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Analyses of observational studies and randomised trials to increase understanding of the occurrence and role of drug resistance in HIV infection

THESIS

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ZOE VALERIE FOX

Department of Primary Care and Population Sciences

Royal Free and University College Medical School

University College London

2008

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration

I, Zoe Valerie Fox, confirm that the work presented in this thesis is my own. Where information is derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

This thesis explores the relationship between the use of antiretroviral therapy (ARVs) and drug resistance emergence in HIV-1 infected individuals. It also describes the combined impact of treatment use and resistance mutations on virological and immunological response in individuals who are under follow-up in one of four trials: MaxCmin1, MaxCmin2, COLATE or SMART; and three observational studies: EuroSIDA, the UK CHIC study and the UK drug resistance database.

The emergence of resistance to an ARV that an individual is receiving may influence viral replication rates, which could increase the risk of $CD4^+$ T cell count deterioration, clinical progression and death, unless changes are made to the treatment regimen. Individuals may exhaust all treatment options if large amounts of resistance mutations are detected in their viral sub-populations. In the current era, new ARVs are still arriving on the market, but resistance to these ARVs is only partially understood. Understanding what mutations emerge in individuals who are failing treatment and the impact of specific mutations and combinations of mutations on the likelihood of responding to future regimens is still essential for being able to administer long-term therapy successfully.

Research for this thesis started just after the introduction of ritonavir boosted protease inhibitors (PI/rs). Drug resistance emergence among individuals who experienced virological failure on a PI/r containing regimen is described in detail, and the relationship between resistance at baseline and virological response is also quantified. Other aspects of drug resistance are investigated, including: the potential benefit of harbouring the M184I/V mutation, the impact of resistance on immunological response and the relationship between resistance and viral re-suppression rates amongst patients who interrupt an NNRTI containing regimen.

This thesis outlines the benefits of resistance testing and highlights some of the key issues with interpreting resistance data. Resistance tests are generally performed on a selective group of individuals so caution needs to be used when extending the results to all HIV-1 infected individuals. Further, consensus sequences are useful for indicating resistance that is present in the predominant virus; however, more minor, archived, species are not usually identified through this method and these may also be an important determinant of therapy response.

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CONTENTS

TABLES

FIGURES

LIST OF ABBREVIATIONS

3TC:	Lamivudine
AAUCMB:	Average-area-under-the-curve-minus-baseline
ABC:	Abacavir
AIDS:	Acquired Immune Deficiency Syndrome
ANN:	Artificial Neural Network
APV:	Amprenavir (including fos-amprenavir)
ARVs:	Anti-retroviral Drugs
ATV:	Atazanavir
AZT:	Zidovudine (also known as ZDV)
B.i.d:	Bi-daily
BHIVA:	British HIV Association
cART:	Combination Anti-Retroviral Therapy
CI:	Confidence Interval
CHIP:	The Copenhagen HIV Programme
d4T:	Stavudine
ddC:	Zalcitabine
ddI:	Didanosine
DHHS:	U.S. Department of Health and Human Services
DLV:	Delavirdine
DRV:	Darunavir (referred to as TMC-114 prior to approval)
DSMB:	Data and Safety Monitoring Board
EFV:	Efavirenz
FDA:	Food and Drug Administration
FTC:	Emtricitabine
GEEs:	Generalised Estimation Equations
GIS:	Genotypic Interpretation System
GRO	The General Registrar Office for Scotland
GSS:	Genotypic Sensitivity Score
HIV:	Human Immunodeficiency Virus
IDV:	Indinavir
IQR:	Inter-Quartile Range
LPV:	Lopinavir
NASBA:	Nucleic Acid Sequence-Based Amplification
NFV:	Nelfinavir
NRTI:	Nucleoside Reverse Transcriptase Inhibitor
NtRTI:	Nucleotide Reverse Transcriptase Inhibitor
NNRTI:	Non-Nucleoside Reverse Transcriptase Inhibitor

NVP:	Nevirapine
ONS:	Office of National Statistics
PBMC:	Peripheral Blood Mononuclear Cell
PCR:	Polymerase Chain Reaction
PHI:	Primary HIV Infection
PI:	Protease Inhibitor
PI/r	Ritonavir-boosted Protease Inhibitor
PK-levels:	Pharmacokinetic drug concentrations
PR:	The Protease section of the *pol* gene
RCT:	Randomised Controlled Trial
RNA:	Ribo-Nucleic Acid (HIV RNA refers to the amount of circulating virus)
RT:	The Reverse Transcriptase section of the *pol* gene
RTV:	Ritonavir
SQV:	Saquinavir
TB:	Tuberculosis
TDF:	Tenofovir
TPV:	Tipranavir
T-20:	Enfuvirtide
WHO:	World Health Organisation

CHAPTER 1: GENERAL INTRODUCTION

1.1. The natural history of HIV-1

The first recognised cases of Acquired Immune Deficiency Syndrome (AIDS) were reported in five, previously-healthy, homosexual men in Los Angeles on 5th June 1981[1]. All of these men had *pneumocystis carinii pneumonia* (PCP), a rare lung infection that was primarily seen in patients who were receiving immunosuppressive therapy or among chronically malnourished patients before the advent of AIDS. Human Immunodeficiency Virus (HIV), the virus that causes AIDS, was discovered soon after[2-5].

AIDS has killed more than 25 million people since it was first diagnosed and it is estimated that, as of November 2007, approximately 33.2 (range: 30.6, 36.1) million individuals worldwide were living with HIV, of which 2.5 million (7.5%) were infected in 2007[6]. Currently there are 15.4 million (46%) women and 2.5 million (7.5%) children under the age of 15 years who are living with the virus[6]. The number of people infected with HIV has doubled over the last ten years and since prevalence rates are increasing, effective measures to deal with this growing population of HIV infected individuals need to be found[7].

HIV is a lentivirus which, in turn, is part of a larger group of viruses known as retroviruses. HIV takes a long time to produce adverse effects in the body (NB: lenti- translates to slow in Latin). HIV can be classed into two strains; HIV-1 and HIV-2. Both strains of the virus are thought to have originated from retroviruses found in non-human primates[8]. HIV-2 is related to *SIVsm*, a strain of Simian Immunodeficiency Virus found in sooty mangabeys. The more virulent strain, HIV-1, descends from *SIVcpz*[9], a retrovirus originally found in the chimpanzee subspecies of pan troglodytes troglodytes in Southern Cameroon. In this thesis I will focus on HIV-1 because it is more infectious and pathogenic than HIV-2, it progresses at a faster rate and because there are many more patients infected with HIV-1, it is a much larger clinical problem.

1.1.1. The immune system

When HIV initially enters the body it starts to infect and cause depletion of $CD4^+$ T cells; in particular those located in the gastrointestinal wall. $CD4^+$ T cells are T helper cells which communicate the presence of invading pathogens to macrophages and dendritic cells, both of which are needed for the body to destroy any invading material (in this case HIV), or direct other immune cells to do so. If these cells are not activated, the body will not be able to fight infection and HIV will continue to replicate unhindered and thus able to deplete the immune system further.

Although the mechanisms of destruction are not fully understood, it is thought that HIV leads to a depletion of $CD4^+$ T cells through the following three methods:

- The presence of HIV leads to increased rates of apoptosis (i.e. cell suicide)
- The CD8 killer cells will recognise and kill the HIV-infected $CD4^+$ T cells
- HIV will also directly kill these cells

Individuals usually develop minor symptoms (e.g. fevers, sweats, malaise, lethargy, nausea, myalgia, arthralgia, headaches, sore throat, diarrhoea, generalized lymphadenopathy or thrombocytopenia)[10] following infection with HIV. Some severe symptoms such as gastrointestinal haemorrhage, encephalopathy, pneumonitis, or rhabdomyolysis associated with acute renal failure may also occur during this so-called acute or primary infection phase, which occurs in the first few weeks following infection. In the majority of patients, the more serious, persistent symptoms only appear a median of ten years after HIV first enters the body, but it can take more than twenty years for some patients to develop major symptoms[11-13]. This period of asymptomatic infection varies according to the individual, from less than two years to more than 25 years. The asymptomatic period is explained in more detail in section 1.1.3.2.

1.1.2. HIV replication

Once HIV has entered the body, its surface glycoprotein, gp120, binds to the $CD4^+$ receptors of host cells where this protein is expressed. In addition, gp120 engages a co-receptor on the surface of target cells – the CCR5 co-receptor on T cells and macrophages and the CXCR4 co-receptor on the surface of T cells. The binding triggers a conformational change of gp120 that allows fusion of the viral envelope with the cell membrane, a process mediated by the viral glycoprotein gp41.

HIV is composed of two copies of positive single-stranded RNA that code for the virus's nine genes[13-15]. When fusion has occurred, the viral RNA (ribonucleic acid) is released into the cell cytoplasm where it undergoes reverse transcription into double-stranded DNA (deoxyribonucleic acid). This process is catalyzed by an HIV enzyme called reverse transcriptase[16-18]. The resulting DNA enters the nucleus of the host cell where it integrates into the cell genetic material using a viral integrase emzyme[17;19;20]. Once viral DNA has integrated into the cell it can remain dormant (i.e. the host cell may be in a resting state and not producing any new virions or exhibiting any signs of infection - it is latently infected). Since HIV can remain in latently infected cells it may go undetected by the immune system for many years[21-23].

The host cell will eventually be activated, resulting in the transcription of viral DNA to messenger RNA (mRNA) which is then translated into viral proteins and genomic RNA. The viral proteins include polypeptides and the enzyme protease, a protein required to cleave other HIV proteins into functional forms. These viral proteins assemble with the genomic RNA to form new HIV virions at the cell surface, which are then released into the body to infect another cells[17;24].

1.1.3. The pathogenesis of infection with HIV

The number of $CD4^+$ T cells and the amount of circulating HIV RNA in plasma (i.e. the "viral load") are the most powerful markers that predict the risk of HIV progression[25-28]. Normal $CD4^+$ T cell counts vary between 500 and 1200 cells/μL in uninfected individuals[29]. In HIV-infected individuals, $CD4^+$ T cell counts fall by approximately 50 to 80 cells/μL per year[30-32]. When $CD4^+$ T cell counts fall to below 200 cells/μL the risk of developing an AIDS related illness is greatly increased[33]. AIDS is defined as the occurrence of one of a number of different opportunistic infections (Table 1.1.3.1)[34-36].

Table 1.1.3.1 AIDS defining illnesses[37]

Candidiasis of bronchi, trachea, or lungs
Candidiasis oesophageal
Cervical cancer (invasive)
Coccidioidomycosis, disseminated or extrapulmonary
Cryptococcosis, extrapulmonary
Cryptosporidiosis, chronic intestinal for longer than 1 month
Cytomegalovirus disease (other than liver, spleen or lymph nodes)
Encephalopathy (HIV-related)
Herpes simplex: chronic ulcer(s) (for more than 1 month); or bronchitis, pneumonitis or oesophagitis
Histoplasmosis, disseminated or extrapulmonary
Isosporiasis, chronic intestinal (for more than 1 month)
Kaposi's sarcoma
Lymphoma Burkitt's, immunoblastic or primary brain
Mycobacterium avium complex
Mycobacterium, other species, disseminated or extrapulmonary
Pneumocystis jiroveci (formerly Pneumocystis carinii)
Pneumonia (recurrent)
Progressive multifocal leukoencephalopathy
Salmonella septicemia (recurrent)
Toxoplasmosis of the brain
Tuberculosis
Wasting syndrome due to HIV

In the 1990s new technologies that were able to measure the amount of plasma HIV RNA in HIV infected individuals were introduced. These include the polymerase chain reaction (PCR) assay[29], the branched DNA (bDNA) assay[38] and the nucleic acid sequence-based amplification (NASBA) assay[39]. Initially, the lower limit of quantification for these assays ranged between 200 and 500 HIV RNA copies/mL, with an upper limit of quantification of between 100,000 and 1,000,000 copies/mL[38;40]. More sensitive assays are now available, with a quantification threshold of between 20 and 50 copies/mL[41-44]. Below this level, HIV RNA still cannot be reliably quantified.

When HIV RNA testing entered clinical practice, it became possible to describe the natural course of HIV by examining HIV RNA levels and $CD4^+$ T cell counts from the time of seroconversion to the time of AIDS diagnosis or death[45-48]. HIV mainly uses $CD4^+$ T cells to replicate and, as a result, these cells die or are removed. In contrast, the amount of plasma HIV RNA gradually increases with time after seroconversion. For simplicity, it is sometimes suggested that three different patterns of disease progression exist: rapid, intermediate and late progression, as depicted in the cartoon in Figure 1.1.3.1. This is broadly the case, but disease progression should really be considered to be a continuous spectrum rather than three distinct categories.

Figure 1.1.3.1: Generalized Time Course of HIV Infection and Disease
adapted from the Centers for Disease Control and Prevention, i) Rapid progressors, ii) Intermediate progressors and iii) Late progressors[49]

1.1.3.1. Primary HIV infection

The first stage of HIV infection is known as primary or acute HIV infection (PHI). In the first few weeks following infection the virus starts to multiply by infecting and killing the T lymphocyte cells, resulting in sharp rises in plasma HIV RNA[13;50]. This results in a decline in the number of $CD4^+$ T cells, which are crucial for maintaining an immune response (section 1.1.1)[51;52]. In the gut lymphoid tissue, where most $CD4^+$ T cells live, the numbers of $CD4^+$ T cells decrease by > 50% in the initial days following infection[53-55].

In PHI, HIV RNA levels can increase to as much as 10^7 copies/mL before declining to a 'set point'[29;49;56-58]. Since HIV RNA levels are high in this period, and the adaptive immune response has still to fully develop, individuals are very infectious, yet not all

patients display clinical symptoms[23;59]. Although HIV RNA appears to stabilise at a lower set-point, replication still continues. The HIV RNA plateau in Figure 1.1.3.1 is not related to the absence of virus replication, instead, it demonstrates that the number of HIV virions being produced (i.e. the number of newly infected cells) in a given period of time is the same as the number of HIV virions being cleared by the immune system. This turnover rate will not remain constant indefinitely and HIV RNA levels will start to increase again as time progresses. The precise reasons for this are still not known. However, it could relate to dimunition of the immune response or to evolution of a virus that is better suited to replicate in a wide range of body compartments[47;48;60].

During PHI there is a decline in $CD4^+$ T cell counts and our bodies start to produce antibodies in order to fight the virus. The time that HIV-antibodies are initially produced is referred to as seroconversion[23;61]. The production of antibodies corresponds to a decline in the HIV RNA levels, possibly due to an immune response (i.e. there are transient increases in $CD4^+$ T cell counts which results in cellular as well as antibody responses) or exhaustion of other susceptible cells for HIV to infect.

1.1.3.2. Asymptomatic HIV infection

HIV infected individuals may remain asymptomatic for many years, however, most (and perhaps all) patients who are left untreated will eventually experience a progressive breakdown of the immune system as a result of their infection[12;57]. When $CD4^+$ T cell counts fall too low the body becomes defenceless and vulnerable to infection, so many individuals progress to AIDS or death. Although $CD4^+$ T cell counts decline gradually over the course of the infection, contrary to expectations, the rate of decline may be lower at more advanced stages of the disease rather than more rapid[62]. The median time to development of AIDS after seroconversion is between 10 and 11 years[11-13;63]. The period following seroconversion is referred to as the asymptomatic period because patients do not present with significant, HIV-related, clinical symptoms.

1.1.3.3. Symptomatic HIV infection

Currently there are a number of different antiretroviral drugs available to treat HIV infection (section 1.2). If HIV infected individuals remain untreated patients may start to develop symptoms. Early symptoms include fever, headache, diarrhoea, skin rashes, fatigue, weight loss, sweats and oral candidiasis[64-68]. As the immune system is destroyed further, more severe opportunistic infections develop. Once one of the conditions in Table 1.1.3.1 has developed the individual is classified as having progressed to AIDS. The definition of AIDS was first put forward in 1985; it was next revised in 1987 and again in 1993. The 1993 version is the version we currently refer

to, although there has been a push for this definition to be updated as more information has become available on some malignancies, such as anal cancers, that could be considered as an AIDS defining event[37].

1.1.4. Routes of HIV transmission

HIV can be transmitted through blood, semen, cervico-vaginal secretions and breast milk. The most common mode of transmission worldwide is sexual intercourse, even though the probability of infection per contact can be as low as one in a thousand[69]. The most efficient mode of transmission is through the transfer of blood. Prior to 1985, patients requiring organ or tissue replacement and haemophiliacs requiring Factor VIII, a blood coagulant, became infected through receiving blood from infected donors[70;71]. Now regular screening is used to remove HIV infected blood from donations.

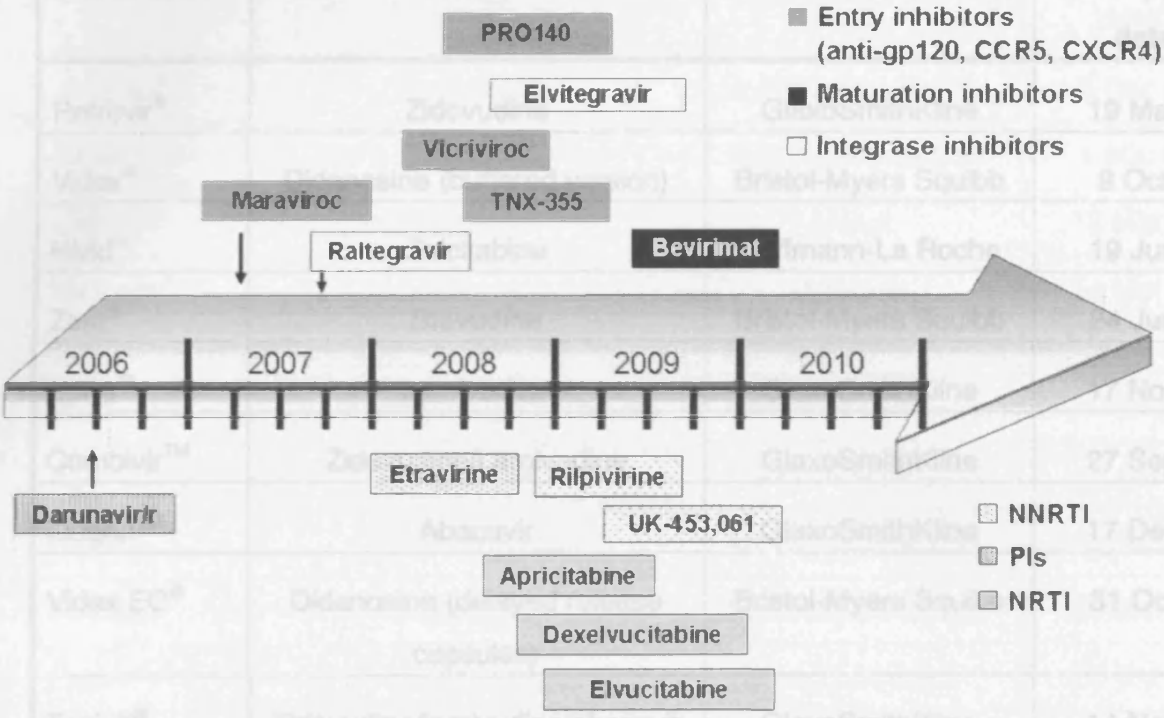
HIV can also be passed from mother to child (vertical transmission) either *in utero*, at birth or through breastfeeding. If the mother does not breastfeed, takes anti-HIV therapy during pregnancy and opts for a caesarean section delivery, the risk of transmission can be reduced to less than 1%[72].

1.2. Antiretroviral therapy

After combination antiretroviral therapy (cART) was introduced between 1995 and 1996, the natural course of HIV changed dramatically in developed countries[73-77]. For many years, there were only three main classes of drugs that were approved by both the FDA and the European Medicines Evaluation Agency to treat HIV; where each drug class was classified by its mechanism of preventing viral replication. Anti-HIV drugs are grouped into nucleoside (and nucleotide) reverse transcriptase inhibitors (NRTIs or NtRTIs), protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs)[33].

Since 2006, other drugs such as entry inhibitors (including fusion inhibitors, small molecule CCR5 inhibitors and CXCR4 inhibitors), integrase inhibitors and maturation inhibitors have arrived/or are arriving on the market, and are now more commonly used in clinical practice[33;78]. The introduction of new drugs from existing classes, combined with the arrival of new drugs from novel treatment classes, means that it will be more possible to personalise treatment for each individual. The time of the arrival of these new antiretrovirals (ARVs), and the estimated timeline for the availability of all new ARVs, is listed in Figure 1.2.1 for each drug class. Since these drugs have recently arrived on the market or are yet to be approved, they have not been used widely by the patients who are included in this thesis and so are not documented in detail.

Figure 1.2.1: Estimated timeline for the availability of new ARVs in all classes



1.2.1. Nucleoside (and nucleotide) reverse transcriptase inhibitors

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first class of drug used as anti-HIV therapy. Zidovudine (AZT) was authorised for treating HIV-infected individuals in the US in 1987[79] and since then, six NRTIs have been approved: didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC) and emtricitabine (FTC), although ddC is no longer commonly used. One nucleotide reverse transcriptase inhibitor (NtRTI), tenofovir (TDF) was also approved for treating HIV infected individuals[80;81]. Table 1.2.1.1 lists all of the NRTIs and NtRTIs licensed for use in the US and Europe. NRTIs and NtRTIs tend to be used in a combination with another drug from the same class in addition to a third drug from a different class.

NRTIs work by inhibiting the enzyme reverse transcriptase during reverse transcription. Following phosphorylation to three-phosphate derivatives, the NRTI binds to the active site on the HIV reverse transcriptase enzyme and is incorporated into the HIV proviral DNA chain, resulting in chain-termination. The remaining viral RNA is subsequently destroyed by other cellular enzymes[82;83]. NtRTIs work in the same way, but, because they already carry a phosphate group, they require only two phosphorolations to be activated intracellulalry rather than the three that are required by NRTIs [84;85].

Table 1.2.1.1: Licensed nucleoside and nucleotide reverse transcriptase inhibitors

Brand name	Generic name	Manufacturer name	FDA approval date[81]
Retrovir®	Zidovudine	GlaxoSmithKline	19 Mar 87
Videx®	Didanosine (buffered version)	Bristol-Myers Squibb	9 Oct 91
Hivid®	Zalcitabine	Hoffmann-La Roche	19 Jun 92
Zerit®	Stavudine	Bristol-Myers Squibb	24 Jun 94
Epivir®	Lamivudine	GlaxoSmithKline	17 Nov 95
Combivir™	Zidovudine/Lamivudine	GlaxoSmithKline	27 Sep 97
Ziagen™	Abacavir	GlaxoSmithKline	17 Dec 98
Videx EC®	Didanosine (delayed release capsules)	Bristol-Myers Squibb	31 Oct 00
Trizivir®	Zidovudine/lamivudine/abacavir	GlaxoSmithKline	14 Nov 00
Emtriva™	Emtricitabine	Gilead Sciences	2 Jul 03
Epzicom™	Abacavir/Lamivudine	GlaxoSmithKline	2 Aug 04
Truvada™	Tenofovir/Lamivudine	Gilead Sciences	2 Aug 04
Viread®	Tenofovir	Gilead Sciences	26 Oct 01

1.2.2. Protease inhibitors

The introduction of protease inhibitors (PIs) coincided with the use of regimens containing three or more ARVs and has consequently changed the course of HIV[86]. PIs act on the virus during the last stage of its reproduction cycle. PIs resemble pieces of the protein chain that the viral enzyme protease cleaves. They prevent the enzyme from processing the HIV proteins, gag and gag-pol, into the smaller segments required by HIV in order to reproduce. The resulting virions are immature and cannot infect other cells or replicate[87].

Currently there are nine approved PIs: saquinavir (hard and soft gel formulations) (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), (fos)amprenavir (APV), lopinavir (LPV), atazanavir (ATV), tipranavir (TPV) and darunavir (DRV)[80;81]. Table 1.2.2.1 displays all of the currently licensed PIs and the dates of their approval.

Table 1.2.2.1: Licensed protease inhibitors

Brand name	Generic name	Manufacturer name	FDA approval date[81]
Invirase®	Saquinavir	Hoffmann-La Roche	6 Dec 95
Norvir®	Ritonavir	Abbott Laboratories	1 Mar 96
Crixivan®	Indinavir	Merck	13 Mar 96
Viracept®	Nelfinavir	Agouron Pharmaceuticals	14 Mar 97
Fortovase®	Saquinavir (soft gel capsules)	Hoffmann-La Roche	7 Nov 97
Agenerase®	Amprenavir	GlaxoSmithKline	15 Apr 99
Kaletra®	Lopinavir/ritonavir	Abbott Laboratories	15 Sep 00
Reyataz®	Atazanavir	Bristol-Myers Squibb	20 Jun 03
Lexiva®	Fosamprenavir	GlaxoSmithKline	20 Oct 03
Aptivus®	Tipranavir	Boehringer Ingelheim	22 Jun 05
Prezista®	Darunavir	Tibotec	23 Jun 06

Since around 2000, small doses of ritonavir have been used in combination with other PIs because ritonavir inhibits the CYP3A4 liver enzyme that metabolises away these other PIs[88]. As a result it provides a pharmacologic boosting effect by raising the trough levels of the other PIs and by extending their half-lives[88]. The improved potency of the regimen is associated with a reduced risk of resistance emergence (i.e. the emergence of mutations that correspond to a decrease in the antiretroviral activity of an ARV – discussed in detail in section 1.6) and generally no substantial increase in toxicity[89-91], although indinavir boosted with ritonavir has been associated with more adverse events than indinavir alone[92]. The inhibition of the CYP3A4 enzyme can also affect (and be affected by) the drug levels of concurrent medication through pharmacologic interaction. Therefore, levels of concurrent medication need to be adjusted accordingly in order to avoid unnecessary toxicities. This can also affect the efficacy and toxicity of the PIs.

Ritonavir-boosted regimens tend to be more potent and simpler, and also avoid the food restrictions that are associated with single PIs, so they are now commonly used in clinical practice. The first study investigating the use of ritonavir boosting was a Danish PI study comparing HIV RNA reductions in patients who received two NRTIs and either

IDV, RTV or RTV and SQV. They found superior HIV RNA reductions in the RTV/SQV arm compared to either the IDV or RTV arm. Although, in this study, RTV was used at a 400mg dose instead of the current 100mg dose, so it is likely that it also had some activity against HIV and was not just used to boost the level of SQV[93].

1.2.3. Non-nucleoside reverse transcriptase inhibitors

Although non-nucleoside reverse transcriptase inhibitors (NNRTIs) work at the same stage of HIV replication as NRTIs, they bind to a different site of the reverse transcriptase enzyme, causing its allosteric inhibition to prevent conversion of RNA to proviral DNA[85]. The NNRTIs currently approved by the FDA are nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV)[80;81]. Table 1.2.3.1 lists all licensed NNRTIs and their approval dates. Another NNRTI called loviride was used in clinical practice before results from a trial showed its inefficacy in combination with other ARVs[94]. Similarly, trials have shown that delavirdine has no superior efficacy compared to other NNRTIs, so the European pharmaceutical licensing authority's scientific advisory board rejected licensing applications for this drug in December 1999. Second generation NNRTIs, etravirine (TMC125) and rilpivirine (TMC278), are currently in advanced clinical development.

Table 1.2.3.1: Licensed non-nucleoside reverse transcriptase inhibitors

Brand name	Generic name	Manufacturer name	FDA approval date[81]
Viramune®	Nevirapine	Boehringer Ingelheim	21 Jun 96
Rescriptor®	Delavirdine	Pfizer	4 Apr 97
Sustiva™	Efavirenz	Bristol Myers-Squibb	17 Sep 98

1.2.4. Entry inhibitors and fusion inhibitors

Entry inhibitors and fusion inhibitors work differently to other classes of drugs because they work outside the cell to prevent HIV from entering the host cell in the first place. The first fusion inhibitor, enfuvirtide (T-20), is injected. Since it is more complicated to use and its complex structure makes it more expensive than other ARVs, it is only used in patients with very limited treatment options available. Maraviroc is another entry inhibitor that has been approved for treatment in the US and Europe in 2007 (Table 1.2.4.1)[80;81].

As we saw in section 1.1.2 the HIV envelope fuses to the membrane of $CD4^+$ T cells and allows its genetic material to be absorbed into the cell. HIV uses the gp120 glycoprotein on its outer surface to bind to the target cell, allowing the gp41 viral protein

to embed itself in the cell plasma membrane and mediate fusion. Since entry inhibitors competitively bind to the gp41 viral protein, they prevent the creation of an entry pore and stop the virus from entering the cell in the first place[95;96].

Table 1.2.4.1: Licensed entry inhibitors and fusion inhibitors

Brand name	Generic name	Manufacturer name	FDA approval date[81]
Fuzeon®	Enfuvirtide	Hoffmann-La Roche and Trimeris	13 Mar 03
Selzentry or Celsentri	Maraviroc	Pfizer	24 Apr 07

1.3. Treatment strategies

1.3.1. Monotherapy (1987-1992)

For the first four years of treating HIV the only available drug was an NRTI called azidothymidine (AZT), now referred to as zidovudine[79;97]. The FDA approved AZT in 1987 after the results of a clinical trial comparing the 4 months survival rate of AZT with placebo were published[98]. This trial showed a decrease in mortality and a reduction in the incidence of opportunistic infections in patients receiving AZT monotherapy. Further studies supported a benefit of the drug; therefore, it was approved for use in symptomatic patients as early as March 1987[79;99;100].

It soon became clear that the benefits of this drug were only short-lasting. The largest study of AZT monotherapy in asymptomatic individuals, the Concorde study, published data showing that AZT monotherapy did not increase the chances of AIDS-free survival over the long-term[101]. Overall, NRTI monotherapy has been shown to reduce the viral load by around 0.5 $\log_{10}$ copies/mL for six months and by 0.3 $\log_{10}$ copies/mL for a further six months, but after that any further reduction is negligible, due to the emergence of resistance (see section 1.6)[102].

1.3.2. Dual therapy (1992-1996)

Although NRTI dual therapy was used from 1992, it was not until 1995 that clear evidence became available indicating that patients who received dual therapy had a slower progression to AIDS and death compared to patients who received monotherapy[103;104]. In contrast, another study examining the benefit of AZT/ddI against AZT monotherapy found the dual combinations to only be effective in patients without previous exposure to AZT, reflecting the effects of drug resistance and cross-resistance (section 1.6.2.1)[105].

1.3.3. Combination antiretroviral therapy (cART) (1996-current)

Highly active antiretroviral therapy (HAART) or combination antiretroviral therapy (cART) was commonly used after 1996, although a few patients were on triple combination regimens prior to this date[86;106-108]. Currently, state-of-the-art cART is composed of a combination of at least three ARVs from at least two classes. The reason why two drugs are better than one and three are better than two is that drug resistant mutants pre-exist within the viral quasispecies (i.e. strains of HIV that contain drug resistance mutations – discussed in more detail in section 1.6). Mutants that are resistant to a single drug are fairly common, those resistant to two drugs less common and those simultaneously resistant to three drugs are very rare. Therefore, by using three drugs to target two different phases of replication, a virus that is resistant to one of the three drugs will still be suppressed by the other two drugs. Patients starting cART usually initiate a combination of two NRTIs and one NNRTI or one PI[109-111].

The era of cART started with the approval of saquinavir, the first PI to enter the market. Compared with a dual NRTI containing regimen, a combination of two NRTIs plus saquinavir was found to reduce the risk of AIDS and death in both ARV-naïve and ARV-experienced patients[106;112]. The same patterns were also seen in patients who received combinations of NRTIs with all of the new PIs that entered the market[113-121]. It then became common practice to treat patients using at least three ARVs including a PI. These regimens are highly potent, but many patients experienced tolerability problems, especially to the PI-component of the regimen[115;116;122;123].

An alternative triple class regimen for patients who struggle to deal with the toxicities of PIs includes the use of an NNRTI instead of a PI[124;125]. NNRTIs are also useful for patients who experience virological problems on their PI and have PI resistance mutations, as well mutations to the NRTI components of the regimen, in their virus population. Due to the presence of resistance, these patients may have limited treatment options available within the classes of ARVs they have already been exposed to, so an ARV from a different class may be required instead (section 1.6)[126;127].

NNRTIs were introduced into cART regimens after it was shown that, when used in combination with NRTIs, they were generally more tolerable than PIs and were equally as effective at suppressing viral replication. However, this trend was primarily seen in ARV-naïve patients. When NNRTIs were used in patients who shwoed large amounts of resistance mutations to their nucleoside backbone, they continued to experience rapid rises in their HIV RNA levels instead of the expected declines. This reflects the

rapid selection of NNRTI resistant mutants when the NNRTI is not well supported by active NRTIs. It was therefore decided to use NNRTIs as first-line therapy instead of PIs in order to avoid compromising the use of NNRTIs. As we will see in section 1.6, NNRTIs are more affected by resistance emergence than PIs, so if they are used in sub-optimal regimens it is likely that resistance mutations will rapidly emerge to the NNRTI and any future use of them will be inhibited[128;129].

As time progressed, more ARVs were developed in each drug-class, new formulations of existing ARVs came out and more novel treatment options were investigated, including triple NRTI-containing regimens[130-134]. This was done to reduce the pill burden and increase adherence so that the drug levels remained high and viral replication was suppressed[135-137]. In addition, NRTIs have less of a drug interaction with anti-tuberculosis drugs compared to the other classes of ARVs, and since tuberculosis is the leading cause of death among HIV infected individuals, maximising HIV treatment and TB treatment simultaneously is an important consideration[138].

Treatment guidelines exist on when to start therapy, what drugs to start with, when to change and what to switch to in the case of therapy failure (see section 1.4.2)[33]. Current guidelines in the UK recommend that patients should start an NNRTI-containing regimen with a dual NRTI backbone when they become symptomatic or reach a $CD4^+$ T cell count between 200 to 350 cells/μL[33;109].

1.4. Risk factors for HIV progression

As illustrated in Figure 1.1.3.1 the natural course of HIV varies according to the individual. The rate of HIV progression is dependent on certain factors including the age at onset of HIV, and gender[139;140]. In addition to these demographic variables, host genetic factors, the level of immune function, co-infections, viral genetics and the presence of symptomatic PHI have also been shown to affect the rate of clinical progression[141-145].

Host genetic variability is associated with susceptibility to HIV infection and progression to AIDS[146;147]. HIV enters the cell through an interaction with both the $CD4^+$ T cell and a chemokine receptor: CCR5 and CXCR4 are the major chemokine receptors used by HIV[147]. CCR5 is present on a broad range of cells that can also be infected by HIV, but CXCR4 is only found on $CD4^+$ T cells. CCR5-strains of HIV target macrophages in addition to $CD4^+$ T cells because the envelope proteins of macrophages are configured in such a way that they work best using CCR5 as their co-receptors. These strains of HIV are called macrophage-tropic and are not able to induce syncytia, hence are also

termed non-syncytium inducing (NSI) strains. The levels of CCR5 expression are up-regulated in chronically HIV infected individuals therefore monitoring levels of CCR5 could potentially be used in the future as an indicator of disease progression. CXCR4 containing strains on the other hand are syncytium inducing (SI) and can only target $CD4^+$ T cells. Patients infected with CXCR4 strains experience faster declines in $CD4^+$ T cells and progress to AIDS and death more rapidly (3- to 5- fold increase) than patients infected with CCR5 strains[147].

A small fraction of the population carry a mutation on their CCR5 gene called the delta 32 mutation, which indicates that there is a deletion of a 32 base pair segment. In patients who are CCR5Δ32 homozygous (i.e. they harbour two copies of this deletion (+/Δ32)) there is very strong protection against HIV entry because variants of CCR5 containing the delta 32 mutation are non-functional[146;148;149]. Patients who are CCR5Δ32 heterozygous (i.e. they harbour one copy of this deletion (+/Δ32) and hence have lower levels of expression of CCR5) also receive some protection against infection with HIV compared to patients who have a CCR5 gene that does not contain the CCR5Δ32 mutation. Patients who are CCR5Δ32 heterozygous can still become infected, but they will show slower disease progression.

In most individuals, CCR5 strains are predominant early on in the infection. As the disease progresses, the more aggressive CXCR4 strains become prevalent[150]. However, 50% of individuals who die of AIDS still have CCR5 strains present. It is still unclear why there is a switch from CCR5 strains to CXCR4 strains in the dominant plasma population. It could potentially be because CXCR4 containing strains deplete $CD4^+$ T cell counts and so results in more rapid disease progression, or alternatively, CXCR4 strains may only emerge once the immune deterioration has become significant. Anti-HIV drugs are also less effective at treating CXCR4 containing strains which may explain why they become more prevalent in later stages of the disease[147].

1.4.1. Modifiable markers of HIV progression

Although some factors associated with HIV progression are not modifiable, we can alter others by using treatment. The most recent $CD4^+$ T cell count (i.e. the absolute number of circulating $CD4^+$ T cells), the $CD4^+$ T cell nadir and the slope of $CD4^+$ T cell decline have been shown to be powerful predictors of the risk of AIDS and death[151-154]. Since there is large measurement variation of $CD4^+$ T cells, a single determination may not accurately reflect the long term risks of the disease and so multiple measurements are required before making any changes to the treatment regimen[155].

HIV RNA levels have also been shown to be powerful predictors of disease progression[26;28]. Currently, the routine measurement of both HIV RNA and $CD4^+$ T cells is employed in the clinical care of HIV-infected individuals. Both markers are used in combination with each other to determine when individuals should initiate antiretroviral treatment and are also used to assess the short-term and long-term risks of disease progression in treated and untreated individuals[156].

1.4.2. Virological failure

It is possible to keep the risk of progression to AIDS and death at a minimum by changing a patient's treatment regimen if they do not experience a virological response on therapy or if they experience a decline in their $CD4^+$ T cell counts. Historically, the primary endpoints of studies were based on clinical events such as AIDS and death, but since the introduction of cART, the rates of disease progression have substantially fallen, so it is impractical to continue to use these endpoints any longer. $CD4^+$ T cell counts and HIV RNA levels are now used as alternative endpoints instead.

There is no set method for assessing virological response over time, but since the main aim of therapy is to suppress HIV RNA levels below the level of quantification of the assay, virological failure definitions usually encapsulate patients who do not have a virological response to treatment initially (i.e. their HIV RNA levels remain above the level of quantification of the assay) and patients who initially respond to treatment but later experience a virological rebound (i.e. their HIV RNA level falls below the level of quantification of the assay and then rebounds above it). If a patient experiences virological failure they may wish to switch therapy to a more effective regimen. In early virological failure the primary aim of therapy is to achieve re-suppression of HIV RNA, usually by substituting one ARV for another ARV[33;109].

The prognostic value of $CD4^+$ T cell counts for predicting clinical events has been shown to be higher than that of HIV RNA levels in patients receiving therapy, but since HIV RNA levels are a better reflection of an early treatment response they are often used as the primary outcomes in trials[157]. In some studies, patients who are receiving treatment have a lower risk of clinical progression for a given $CD4^+$ T cell count and HIV RNA level compared to patients who are off treatment[158;159]. This indicates that potent antiretroviral therapy may protect against clinical progression beyond the protection acquired through lowering HIV RNA levels and increasing $CD4^+$ T cell counts. This could be a result of improving overall immunodysfunction, beyond what is measured by $CD4^+$ T cell counts, or possibly through the presence of resistance mutations (see section 1.6)[160;161].

1.5. Genetic sequencing

The HIV genome is 9749 nucleotides long and the order in which the nucleotides appear defines the genetic sequence[162]. As with all RNA, HIV RNA is a polymer containing four different nucleotide bases: two purine bases, adenine (A) and guanine (G); and two pyrimidine bases, cytosine (C) and uracil (U). The A, C and G bases are the same as those found in DNA, however uracil is different. Instead thymine (T), a pyrimidine similar to uracil, is the complementary base to adenine in DNA. The use of a single symbol to represent a variety of possible nucleotides at a single position has become widespread over the last few years. The symbols R and Y are commonly used to designate purines (A or G) and pyrimidines (C or T), respectively.

Genes that code for proteins are composed of nucleotide triplets called codons where each protein-coding gene/codon can be translated into one of twenty existing amino acids (Table 1.5.1). Each section of the RNA gene encodes for a particular protein that is necessary for the life cycle of HIV to continue. The complete set of genes is called the genome[163].

Table 1.5.1: List of standard amino-acids

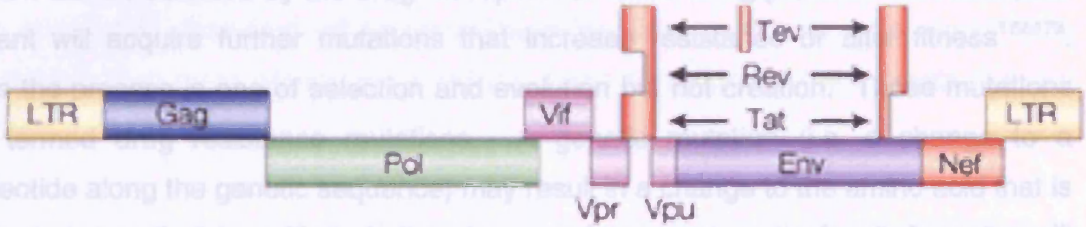
Amino Acid	3-Letter	1-Letter	Nucleotide triplets
alanine	Ala	A	GCU, GCC, GCA, GCG
arginine	Arg	R	CGU, CGC, CGA, CGG, AGA, AGG
asparagine	Asn	N	AAU, AAC
aspartic acid	Asp	D	GAU, GAC
cysteine	Cys	C	UGU, UGC
glutamic acid	Glu	E	GAA, GAG
glutamine	Gln	Q	CAA, CAG
glycine	Gly	G	GGU, GGC, GGA, GGG
histidine	His	H	CAU, CAC
isoleucine	Ile	I	AUU, AUC, AUA
leucine	Leu	L	UUA, UUG, CUU, CUC, CUA, CUG
lysine	Lys	K	AAA, AAG
methionine	Met	M	AUG
phenylalanine	Phe	F	UUU, UUC
proline	Pro	P	CCU, CCC, CCA, CCG
serine	Ser	S	UCU, UCC, UCA, UCG, AGU, AGC
threonine	Thr	T	ACU, ACC, ACA, ACG
tryptophan	Trp	W	UGG
tyrosine	Tyr	Y	UAU, UAC
valine	Val	V	GUU, GUC, GUA, GUG

1.5.1. The HIV genome

There is huge diversity among HIV genomes, even within an individual[164]. It is therefore stipulated that HIV is a quasi-species of a virus[165]. Viral quasi-species are related but non-identical mutant and recombinant viral genomes that result from genetic variation, competition, and selection. The dynamics and structure of RNA allows several viral populations to live and replicate in their hosts simultaneously.

The HIV genome contains several genes: some code for structural proteins and others are accessory genes that have no structural function[166]. The three main functional regions in HIV are the *gag*, *pol* and *env* (Figure 1.5.1.1). The *gag* (group-specific antigen) and *pol* (polymerase) genes together are translated into large poly-proteins which are cleaved by a virus-encoding protein – the protease enzyme – that is contained within *pol*. The *gag* gene encodes the structural proteins of the virus (i.e. it provides the physical infrastructure of the virus and contains the genetic code for producing the following four proteins: matrix, capsid, nucleocapsid and p6), the *pol* gene provides the mechanism by which the virus replicates (i.e. it contains the genetic code for producing HIV reverse transcriptase, integrase and protease) and the *env* (envelope) gene encodes the glycoproteins, which interact with host cell surface receptors and are needed to allow the virus to enter the cell[167].

Figure 1.5.1.1: Diagram of the HIV genome



1.6. Antiretroviral drug resistance

A major barrier to the success of cART is the emergence of drug resistance mutations that inhibit the activity of antiretroviral drugs. Since the start of the pandemic, the HIV-infected patient population has been exposed to over twenty different ARVs, which has led to the emergence of a wide range of drug resistance mutations (i.e. nucleotide changes along the genetic sequence that impact on the antiretroviral activity of ARVs). Although the mechanism of mutation development differs by drug class and, to some extent, by individual drugs, there are some mutations that result in a virus that is better able to replicate in the presence of an ARV.

Mutations that impact on the ability of the virus to replicate in the presence of an ARV tend to occur at, or near, the active sites of the enzymes targeted by antiretroviral drugs. These are generally along the reverse transcriptase (RT) or protease (PR) sections of the *pol* gene. Some viruses have developed large numbers of ARV-specific mutations and consequently patients with these viruses in their viral sub-species have limited or no antiretrovirals that are active (or partially active) against viral replication. Understanding resistance emergence and how it impacts on the virological profiles for patients is crucial for being able to administer successful treatment. It will be the main focus of this thesis.

1.6.1. The emergence of resistance

It is estimated that HIV replication *in vivo* involves the production and clearance of approximately one to ten billion viral particles daily, infecting over ten million host cells every day if a patient does not receive treatment[168;169]. As already described in section 1.5.1, the viral population of an HIV infected person is predominantly heterogeneous (i.e. the majority of virions differ) and since the transcription of RNA into viral DNA is an error prone process that does not contain an inbuilt error checking system to repair any errors that arise, genetic mutations will occur during replication[13;170]. A genetic mutation is defined as an alteration or mistake in the genetic code[171].

In the presence of an ARV and ongoing virus replication, a pre-existing resistant mutant can be selected by the drug. If replication under drug pressure continues, the mutant will acquire further mutations that increase resistance or alter fitness[172;173]. Thus the process is one of selection and evolution but not creation. These mutations are termed drug resistance mutations. A genetic mutation (i.e. a change to a nucleotide along the genetic sequence) may result in a change to the amino acid that is present at a particular position. In the absence of therapy a particular viral species will dominate which is generally referred to as the 'wild-type' virus. Throughout this thesis I will use the term 'wild-type' to refer to viruses that do not contain any drug resistant mutations. These viruses are usually present following infection, although the transmission of drug-resistant strains has become an increasing problem in recent years[174-176].

Mutants with resistance to drugs usually have a reduced fitness (i.e. the ability to replicate) compared to wild-type viruses. However, mutations that compensate for this reduction in fitness often arise in order to allow the virus to replicate at a comparable rate to that of wild-type viruses. These mutations are termed compensatory mutations and play an important role in resistance development and persistence.

Patients who are on a failing cART regimen, and have high levels of viral replication, have the highest risk of experiencing resistance emergence in their dominant virus population. High replication rates mean there is a greater chance of an error occurring, and if these patients are also receiving an ARV it is likely that mutations will emerge that allow the virus to thrive in the presence of that ARV and be preserved[177;178]. In patients who are receiving ARVs, viruses with drug resistant mutations tend to be fitter than viruses without those mutations so these mutated viruses will out-grow the less fit, wild-type viruses and become dominant.

Patients who are receiving sub-optimal therapy tend to experience rapid increases in their HIV RNA levels and resistance emergence. Resistance to a particular ARV tends to emerge when both the potency of the ARV and the genetic barrier (i.e. the number of mutations that the virus requires to become resistant to that ARV) of the ARV is low. The genetic barrier is determined by a number of factors, including the number of resistance mutations that are required for loss of activity of an ARV, the level of pre-existing resistance and the replication rate of the pre-existing resistant viral strains. Defining the genetic barrier involves determining the effect of a single mutation and/or combination of mutations on the susceptibility of HIV to the ARVs in the regimen.

Monotherapy is a treatment strategy where resistance has been shown to emerge easily and rapidly to the ARV received. Monotherapy inhibits certain viruses from replicating, however once mutations emerge that are resistant to the 'monotherapy' drug in question, that drug will no longer be effective at suppressing the virus. As a result replication rates will increase, leading to an elevation in viral mutants that are resistant to the drug.

Dual therapy increases the genetic barrier of the regimen because, for replication to occur effectively, the virus has to have resistance mutations to both drugs in the regimen. Since more mutations are generally needed to develop resistance to two drugs compared to one, viral load increases are not seen as quickly in these patients as in those receiving monotherapy[103]. Now triple combinations of drugs are used to maximise antiretroviral activity and inhibit viral replication; however, adherence rates need to be good in order to keep the potency of the regimen high and avoid resistance.

1.6.1.1. Adherence and risk of resistance

Adherence to antiretroviral treatment is one of the most important determinants of resistance emergence and therapy success[179]. Patients who start cART and have directly observed therapy (DOT) experience virological suppression rates of up to

100%[180;181]. Although it is hard to measure adherence in an individual, physicians continue to promote the optimal use of drugs by trying to enhance the levels of adherence in their patients.

Various different measures of adherence exist (e.g. Medication Event Monitoring Systems (MEMS), DOT, physician perception, plasma pharmacokinetic drug levels, self-reported and self-administered questionnaires amongst others), yet none of them have proved to be wholly reliable[182].

If adherence rates are suboptimal the emergence of resistance becomes a major issue because there is insufficient drug pressure to prevent replication from occurring[183]. In fact, the relationship between adherence and mutation accumulation has an inverse-u shape where low rates of mutation accumulation are seen in patients with low adherence levels, due to minimal selection pressure, and low rates of accumulation are also seen in patients with high adherence levels, due to suppression of viral replication (Figure 1.6.1.1.1 (i)). The rate of mutation accumulation and the magnitude of the relationship between adherence and mutation accumulation has been shown to vary by drug class (Figure 1.6.1.1.1 (ii))[184], however, the same inverse-u shaped relationship between adherence levels and resistance accumulation is observed within all classes.

Figure 1.6.1.1.1: Resistance–adherence curves[183;185]
i) A cartoon demonstrating the relationship between adherence and resistance accumulation

ii) The relationship between adherence and resistance accumulation for patients receiving a nelfinavir or lopinavir/r containing regimen (which also contains lamivudine): A) primary PI mutations B) secondary PI mutations and C) lamivudine resistance mutations

1.6.2. Active drugs and switching

Resistance is the underlying cause of most virological failure (see section 1.4.2) in patients who have moderate to high adherence levels and optimal drug pharmacokinetic levels. Most patients who initiate a first-line of antiretroviral therapy in the absence of transmitted drug resistance have no drug resistant mutations present in their quasispecies at a prevalence equal to or above approximately 0.1%, so they should have a virus that is susceptible to all of the drugs they are receiving. Patients who experience virological rebound on a first-line regimen may have resistance mutations in their predominant virus population, to some, or all of the drugs in their regimen. It is important to identify these mutations and switch the ARVs that are no longer effective to ARVs that are (i.e. active drugs) so that the patient remains on a fully suppressive regimen. Since first-line regimens usually include two NRTIs (referred to here as background drugs) and either a PI or an NNRTI (referred to here as the anchor drug), use of the other anchor drug will not be compromised. Depending on the class of ARVs that has been compromised these patients can initiate two different NRTIs, because cross-resistance (discussed overleaf) among NRTIs is fairly low, and/or an anchor ARV from a class of ARVs that has not been used before. This is termed second-line therapy.

1.6.2.1. Cross-resistance

Drug resistant mutations are not all specific to a particular antiretroviral drug. Mutations may emerge that indicate reduced susceptibility to more than one ARV from a particular class, including an ARV that a patient has never been exposed to before. Although mutations along the RT gene indicate resistance to both NRTIs and NNRTIs there is only one mutation that has been identified so far that indicates reduced susceptibility to both NRTIs and NNRTIs simultaneously; probably because these classes of drugs have different sites of action. The only mutation that is currently known to indicate resistance to both NRTIs and NNRTIs is the little studied N348I mutation[186]. Cross-resistance usually occurs within a particular class of drugs and not across classes.

Reduced susceptibility to more than one PI is most likely to be associated with an amino-acid substitution at one of six different positions along the protease section of the *pol* gene: 10, 46, 54, 82, 84 and 90. Other PI mutations (i.e. D30N, G48V, I50V or I50L – see section 1.6.3.1) are relatively specific for particular PIs and are less likely to produce cross-resistance[187].

Within-class cross-resistance is a major issue because, even though over twenty different ARVs are available for treating HIV, the emergence of a mutation to a particular ARV could inhibit the use of other ARVs from within the same class. Cross-resistance is more likely to happen for NNRTIs than for either NRTIs or PIs. Although there is some overlap among mutations for ARVs from within these latter classes, the latter classes tend to require several mutations for susceptibility to a drug to be compromised. So, the emergence of a single mutation may result in the reduced activity of another drug from the same class but it should not compromise the use of any other ARV entirely. For example, resistance to 3TC is associated with the reverse transcriptase mutation M184V that causes complete cross-resistance to FTC, but has no significant resistance effects on other NRTIs and, in fact, increases susceptibility to NRTIs such as TDF, ZDV or d4T.

The genetic barrier of first-generation NNRTIs is lower than that of both PIs and NRTIs, and, as a result, the emergence of a particular NNRTI mutation may dramatically affect the response to other ARVs from within the same class. There are two main pathways for developing resistance to NNRTIs, one in which the K103N mutation is present, resulting in cross-class resistance to all NNRTIs, and another in which K103N is not present, resulting in a variable degrees of cross-class resistance.

1.6.2.2. Lines of therapy

Although antiretroviral therapy is highly potent and is known to suppress HIV RNA replication effectively, there are still some patients who receive treatment but do not experience virological suppression. This could relate to the presence of drug resistance mutations in their viral populations[188].

The emergence of resistance has been shown to limit the long-term duration of virological suppression[188]. If a patient experiences HIV RNA rebound on their first-line therapy the chances of achieving virological suppression on a second-line or further line regimen are reduced because mutations to some of the drugs in the regimen may already be present in the viral sub-species[189]. It is preferable to use a combination of at least three drugs without any overlapping resistance mutations, in any line of treatment, to minimize the risk of resistance emergence as much as possible[109].

Patients with long-term exposure to a number of different ARVs may have a plethora of resistance mutations in their viral sub-species, some of which may not be present in the dominant viral species, but present in plasma as low frequency mutants and hidden in archived reservoirs of HIV proviral DNA. These mutations could still compromise the use of treatment because viruses containing these mutations are likely to become dominant under selective pressure from antiretroviral drugs.

In the absence of therapy, wild-type viruses have the highest replication capacity (i.e. ability to replicate), so these strains are likely to become dominant if therapy is stopped[172]. Patients with limited treatment options may wish to undergo a treatment interruption in order to allow the wild-type strains of HIV to become dominant again so that they can temporarily restore susceptibility to some ARVs.

If patients who have predominantly wild-type viral strains resume therapy, they will experience noticeable decreases in their HIV RNA levels as the wild-type strains of HIV decline, but strains of HIV harbouring resistance mutations will become dominant again if they contain a mutation to an ARV that the patient is receiving (Figure 1.6.2.2.1). As a result the benefits of treatment interruption are short-lived and the strategy is not currently recommended. In the case of repeat virological failure, a patient should attempt to switch to an active regimen that suppresses viral replication in the presence of all of the mutations they have in their viral strains. Mutations that are present in the dominant viral strains should be considered in combination with mutations that are present in archived sub-species. Patients who have exhausted most treatment options because their viral strains contain too many resistance mutations can be referred to as

at a 'salvage' stage of therapy. The degree of susceptibility that the virus has to a treatment regimen is assessed through the use of resistance testing.

Figure 1.6.2.2.1: Resistance and viral load (www.aidsmap.com)

1.6.3. Resistance testing

If a patient experiences virological failure and needs to switch some of the drugs in their regimen they may wish to assess what other drugs they have susceptibility to. This is done through the use of genotypic and/or phenotypic resistance testing[190]. By using genotypic and/or phenotypic resistance testing it is possible to quantify the amount of resistance there is to each ARV individually, and to the regimen as a whole. As a result it enables a patient to switch any ARV that is compromised by the presence of resistance mutations to an ARV that is not. Although physicians use resistance testing to select an active and potent cART regimen for each individual, its use does not always correlate to virological response[191-195].

1.6.3.1. Genotypic resistance testing

Genotypic resistance testing is used to guide therapy selection by producing lists of the type and positioning of each mutation by comparing the genetic code of the sampled virus to that of a consensus sequence[193;196-198]. Consensus sequences vary according to the subtype of HIV that the patient is infected with, which has been shown to have considerable variation across the world[199-202]. This thesis includes patients who are predominantly infected with subtype B infection and the reference strain commonly referred to for subtype B infected patients is HXB2[203].

Genotyping involves sequencing the RT and PR sections of the *pol* gene, the areas of the gene that are targeted by current antiretroviral drugs. It is now also common to sequence the gp41 envelope gene in patients receiving enfuvirtide because this area is associated with resistance to entry inhibitors[204]. Genotypic resistance tests look for mutations associated with a reduced susceptibility to antiretroviral drugs[197;205;206].

Mutations are described by combinations of letters and numbers. The number relates to the position of the codon, the initial letter refers to the amino acid that is expected at that position in the consensus sequence and the final letter refers to the actual amino acid that was found at that position (see Table 1.5.1). For example, the M184V mutation is a common mutation associated with resistance to lamivudine. It occurs at codon position 184, the expected amino acid at codon 184 is methionine (M) but the mutation that is found at that position is valine (V). I will look at the M184V mutation in more detail in chapter 6 in this thesis.

All drug resistance mutations contribute towards resistance to antiretroviral drugs to a different extent. Some mutations cause resistance to a particular ARV but re-sensitise other ARVs[207-210], other mutations improve the fitness of the virus under selective pressure from ARVs[211;212] and some mutations lie along the pathway for developing major resistance mutations[213]. The relationship between mutations and ARVs is complex and has resulted in disagreements between experts on how each mutation affects response to each ARV. Consequently over twenty different rules-based genotypic interpretation systems (GISs) have been proposed[214-222]. Drug resistance is interpreted using one of these systems[223]. Interpretation systems tend to agree on the antiviral response to common resistance mutations, however substantial discordance exists in the ascribed levels of resistance to some ARVs and to the regimen itself[224-230].

1.6.3.1.1. Genotypic interpretation systems

For a given genotype there is debate on how best to estimate the predicted level of activity for each ARV according to the resistance mutations that are identified. A number of different GISs are currently used for determining the amount of drug resistance to each ARV and guiding therapy changes in patients who are starting a new treatment regimen[217;220;221;225;231;232].

The International AIDS Society (IAS-USA) publishes a list of mutations along the RT, PR and envelope genes that are considered to indicate some level of resistance to the existing ARVs (appendix I)[233]. These are split into primary and secondary resistance mutations. Primary resistance mutations are generally those that occur first during

therapy with a non-suppressive regimen and result in a decrease in the sensitivity of the virus to the drug in question. Secondary mutations are those that occur over time if the virus continues to replicate in the presence of a particular drug (i.e. if a failing regimen is not discontinued or switched to an active regimen). All resistance mutations are specified for each ARV in the IAS-USA listings so that it is possible to get an overall view of how susceptible a virus will be to the regimen a patient is receiving.

Since some mutations are more powerful indicators of resistance than others and some mutations indicate hypersusceptibility to certain ARVs but resistance to other ARVs, complicated GISs that take into account combinations of mutations have been proposed[220;221;231]. As an example appendix II demonstrates the algorithm for the REGA interpretation system. Amino-acid sequences are run through the chosen GIS-specific algorithm to produce a score that relates to the degree of resistance a virus has to each ARV in the regimen.

Most GISs categorise resistance into three distinct categories (i.e. resistance, intermediate resistance or sensitive), however some GISs use a five category system instead (i.e. high-level resistance, intermediate resistance, low-level resistance, potential low-level resistance and sensitive). Rules exist for condensing down systems using five categories to systems containing three categories[220]. For GISs containing three levels of resistance, a score of 0 for an ARV demonstrates full-resistance to that ARV, 0.5 indicates intermediate resistance and 1 relates to ARV-sensitivity. A genotypic sensitivity score (GSS) can then be generated for the regimen by summing the level of resistance shown to each ARV in the proposed regimen[218].

Studies have compared how different GISs interpret amino acid substitutions and explored how their corresponding interpretations relate to phenotypic drug susceptibility and to the clinical activity of ARVs in terms of viral load response[228;229]. Some GISs are rules-based algorithms, updated by experts, which are freely accessible over the internet whereas others are costly and the rules are not always specified[225]. Table 1.6.3.1.1.1 outlines the GISs that have been proposed for clinical use and undergone clinical validation. There is a real need for more information on the prognostic value of these tests to determine whether the impact of resistance testing is long-lasting. Variability exists in the mechanisms underlying the interpretations, the nature of the systems, their intended use and how often they are updated[225;234].

Table 1.6.3.1.1.1: Information on the existing genotypic interpretation systems

GIS	Version	Manufacturer	Date of last update	Rules-based	Freely accessible	For clinical use	Undergone clinical validation
ANRS	15	Agence Nationale de Recherches sur le Sida	2007	Yes	Yes	Yes	Yes
Geno2pheno	3.0	German National Reference Center for Retroviruses	2003	No	Yes	No	Yes
HIV Drug Resistance Database (HIVdb)	4.3.1	Stanford University	2007	Yes	Yes	Yes	Yes
Rega Institute	8.0	Rega	Expected Jan 2008	Yes	Yes	Yes	Yes
VircoTYPE/ VirtualPhenotype	-	Tibotec-Virco	2007	No	No	Yes	Yes
HIV-1 GenotypR PLUS	-	Specialty Laboratories	2001*	Yes	No	Yes	No
TRUGENE HIV-1 genotyping test	12.0	Siemens	2007	Yes	No	Yes	Yes
Retrogram	1.6	Virology networks	2002*	Yes	No	Yes	Yes
ViroSeq HIV genotyping system	2	Celera Diagnostics/Abbo tt Laboratories	2000*	Yes	No	Yes	No
GeneSeq	3	Monogram Biosciences	2003	Yes	No	Yes	No
GenoSure	-	LabCorp and Virco	2004*	Yes	No	Yes	No
DMC	3	Detroit Medical Center	2004	Yes	Yes	No	Yes
CHL	5.0	Centre Hospitalier de Luxembourg	2005	Yes	No	No	Yes
Quest-Stanford	-	Quest diagnostics	2005	Yes	No	Yes	Yes
GAV/USP	4	Grupo de Aconselhamento Virologico, Sao Paulo University	2006	Yes	No	No	Yes
Therapy Edge	3.2	Advanced Biological Laboratories	2005	No	No	Yes	Yes
Los Alamos	?	Los Alamos National Laboratory	2005*	No	Yes	No	No
DR_SEQAN	1.1	Menéndez-Arias	2006	Yes	Yes	Yes	Yes
RCG	-	Resistance collaborative group	2000	Yes	Yes	No	Yes
Viradapt	-		1999	Yes	No	No	Yes
AntiRetroScan	1.5.1	University of Siena	2004	No	No	No	No
IAS-USA	-	IAS-USA	2007	Yes	Yes	No	Yes

* These systems are regularly updated, but no information is available on when the updates occur

Mutations associated with resistance to protease inhibitors were initially selected for unboosted PIs for every GIS. Due to the potency of ritonavir-boosted PIs (PI/rs) and because PIs are now more commonly used in their boosted form, these GISs may require alternative or more sensitive interpretations for ritonavir-boosted regimens. Some GISs have already been updated to account for the use of ritonavir-boosting but others still have not. Although the ascribed levels of resistance to a PI/r, calculated using a GIS that has yet to be updated for the use of ritonavir boosting, still correlates with the activity of the PI/r if it is used in its boosted form[198], most discordance between GISs still occurs amongst PIs[230]. Since limited information exists on the prognostic value of GISs, particularly for PI/rs, I investigated concordance between ascribed PI/r resistance levels using four freely available GISs and related the resulting interpretations to virological response using trial data and longitudinal data. These analyses are described in detail in chapter 4 and chapter 5 of this thesis.

1.6.3.2. Phenotypic resistance testing

Phenotypic resistance assays measure the amount of a drug that is needed to inhibit replication of a viral isolate *in vitro* by either 50% or 90% relative to a control virus. This is referred to as the inhibitory concentration (IC) and the measurement of interest is the IC_{50} or IC_{90}, respectively[235]. The phenotypic assays currently in routine use are based on the production of a recombinant virus containing the patient-derived HIV genetic region of interest (e.g., RT and protease) amplified by PCR and inserted into a defective laboratory vector to produce a complete virus particle. The virus is then grown in the presence of escalating concentrations of drugs.

The growth rate of the cultured HIV is compared to that of wild-type virus and the fold change in the amount of drug required to suppress replication is reported. If larger quantities of drug are needed to suppress the virus than what is required for the wild-type virus, reduced susceptibility is present. If the reduction in susceptibility exceeds a threshold value (see below), the viral isolate is called resistant to the drug examined. For example, if the cultured sample grows ten times as much as the wild-type virus under the same amount of drug concentration then the cultured sample is estimated to be ten-fold resistant to that drug. In other words, the susceptibility of the virus to that drug is ten times lower than wild-type virus or ten times the amount of drug is required to prevent replication to the same extent[236]. Although phenotypic resistance testing accurately reflects drug susceptibility *in vitro,* the *in vivo* activity may differ because other biological factors that influence response to therapy are not taken into consideration.

Technical cut-off levels are selected on the basis of the assay's reproducibility when a single wild-type reference virus is tested repeatedly. The output obtained from using these assays is usually reported as a low-, medium- or high-level resistance score. Since resistance levels obtained through this method do not correlate precisely with virological response *in vivo* the clinical relevance of these cut-off levels is questionable. Biological cut-offs are an improvement over technical cut-offs and are used to determine the level of phenotypic resistance that is considered significant for a certain ARV. Biological cut-offs rely on data derived through testing a large number of viral isolates from ARV naïve patients *in vitro,* thus representing the range of natural variation in susceptibility among different virus strains. Clinical cut-offs, on the other hand, are calculated from assessing the level of resistance that impacts on clinical responses *in vivo*. Phenotypic assays use a variety of different cut-off levels to reflect susceptibility, reporting any isolate that exceeds a specific cut-off level as having resistance. Although the latter seem great, they also have important limitations: they reflect the specific characteristics of the population from which they have been derived and are clearly influenced by the activity of other drugs in the regimen.

1.6.3.3. A comparison of genotypic and phenotypic resistance testing

Both phenotypic and genotypic resistance tests have their strengths and weaknesses[237]. Phenotypic resistance tests report levels of drug susceptibility, taking into account all mutations, and are therefore apparently easier to interpret and apply than genotypic tests. However, they also have their limitations: for shifts in phenotypic susceptibility to become evident, a significant proportion of the viral quasispecies must be resistant to the drug in question. Phenotypic assays also have lower sensitivity than genotypic tests as they are unable to detect the effect of mutations that cause small changes in the viral quasi-species, but predict impaired virological responses *in vivo* (e.g., T215 revertants). Other limitations of phenotypic resistance testing are that: interpretation of low levels of phenotypic resistance is difficult, because even small shifts have been associated with reduced responses *in vivo* (e.g., 1.4 fold for TDF); and the decision on what cut-off to use for interpretation is questionable. Further, they cost about £400 per test and it takes over a month to process the results. Although, there is work being done to lower the cost of phenotypic resistance testing and improve the turnaround time[238].

Genotyping, on the other hand, costs between £150 and £300 per test and results from these come back in about two weeks[190;237]. Genotypic tests report the exact mutations that are present in the virus rather than providing a low-, medium- or high-level resistance output, although these genotypic resistance mutations still need to be

translated into a genotypic resistance score in order to help the physician understand the relationship between resistance and potential response to therapy.

Much discussion has focused on the relative strengths and weaknesses of genotypic and phenotypic resistance testing. However, studies to date, have not sufficiently assessed the predictive value and relative merits of both tests[192;195;239], although most available evidence favours genotyping. Genotypic changes can be present several months before phenotypic changes, and because results are technically and economically more accessible, genotyping is generally preferred to phenotyping in detecting transmitted resistance and in early virological failure, whereas phenotypic resistance tests may be helpful in patients with complex resistance patterns[190].

1.6.4. Mechanisms of resistance to NRTIs

There are two distinct mechanisms for developing resistance to NRTIs: the first is through drug discrimination and the second by pyrophosphorolysis. Drug discrimination is most common and arises due to the occurrence of mutations at or near the binding site of the RT enzyme that lead to a decrease in drug binding. For example, viral strains that contain L74V, Q151M or M184V genotypic mutations (all NRTI mutations, see section 1.6.3.1) inhibit drug activity by discriminating against the drug in favour of the natural substrate (i.e. the enzymes that contain these mutations will select for the natural substrate rather than the nucleoside analogue) and so will be able to replicate in the presence of an NRTI[240;241].

Pyrophosphorolysis, on the other hand, involves the actual removal of the incorporated triphosphate NRTI-derivative from the viral DNA chain using adenosine 5'-triphosphate (ATP), or less commonly, pyrophosphate, as an acceptor. Reverse transcription starts with the nucleoside triphosphate, the active drug in the cell, which is incorporated into the viral DNA chain extending and lengthening the chain, liberating a free pyrophosphate so that it can continue to grow. Nucleoside analogues work by incorporating themselves into the chain and prematurely terminating the DNA chain. In pyrophosphorolysis a mutation arises that enables the enzyme to remove the chain-terminating nucleoside (i.e. the nucleoside analogue) and unblock the primer, and as a result the reverse transcriptase is able to resume its elongation activity. Pyrophosphorolysis occurs relatively slowly, but its impact is significant as it results from the occurrence of thymidine analogue mutations (TAMs) which confer cross-resistance to almost all the nucleoside analogues.

TAMs are mutations that occur in the RT region of the *pol* gene, and are typically selected by AZT and d4T[242]. There are two distinct pathways to developing TAMs, either through the TAM1 pathway (including the 41L, 210W or 215Y mutations) or through the TAM2 pathway (including the 67N, 70R, T215F or 219E/Q mutations)[243-246]. The pathway chosen is driven by the NRTI the patient is using, as well as other undetermined factors[247].

1.6.5. The rate of emergence of resistance and temporal trends

Resistance mutations may arise in the dominant virus population of an individual as a result of a viral drift (i.e. a nucleotide change in the predominant virus), or possibly, and more realistically, as a result of a viral shift (i.e. a change in the dominant virus to a virus from a different viral quasi-species). At the first onset of resistance this is assumed to be the same. The time to the detection of a drug resistant mutation, under drug selection pressure, varies according to the individual.

Factors that contribute to the speed of resistance emergence include the mutation frequency at the time of treatment initiation (i.e. mutations that are present in major populations and in the more minor sub-species), the extent of virus replication and the fitness of the mutant (i.e. the ability of the virus to replicate and infect new cells), the viral fidelity (i.e. the probability of new mutations emerging in each replication round) and the magnitude of drug selection pressure.

It can take a matter of weeks for a mutation to become dominant within the quasispecies. For example, the M184I/V mutation can emerge in two weeks if a patient is failing lamivudine monotherapy. Other mutations, such as the K65R mutation along the RT gene, tend to emerge more slowly during treatment with a failing ddI, ABC or TDF containing regimen. Genotypes, phenotypes and the time to the emergence of some mutations are outlined in Table 1.6.5.1.

Different methods have been used to explore the temporal trends in the prevalence of resistance in HIV infected individuals[248]. Data from the UK Collaborative Group on HIV drug resistance suggests that, in ARV treated patients, the prevalence rates of resistance to one, two or three classes of drugs remains constant over time if rates are calculated using one resistance test per patient, and the mutations that are present in that test are investigated as a proportion of all of the tests performed. Resistance to an entire class of drugs is considered to be the emergence of at least one major mutation associated with reduced susceptibility to a drug in the class. Resistance to only one

class of drugs has been high since 1998 (>70%) whereas resistance to all three classes of drugs has stayed reasonably low (<20%) (Figure 1.6.5.1).

Table 1.6.5.1: Time to resistance emergence after monotherapy with specific ARVs[249]

Figure 1.6.5.1: Prevalence of resistance over time: cross sectional approach. Among treated patients undergoing resistance testing[250]

Figure 1.6.5.2: Prevalence of resistance over time: including archived mutations
Cumulative prevalence of resistance: accounting for archived resistance mutations[250]

When the UK Collaborative Group on HIV drug resistance modelled the cumulative prevalence of resistance over time instead, to take into account archived resistance mutations, they found an increasing number of previously treated patients with major resistance mutations to all three classes of drugs. In 1998 less than 4% of patients exhibited resistance to either one, two or three classes of ARVs, and by 2002 17% of patients had resistance to ≥1 class of ARVs and 4% had resistance to all three classes; however these temporal increases appear to have levelled off since 2000 (Figure 1.6.5.2). Mutations were only identified for patients with resistance testing performed. Since the majority of treated patients will have effective suppression of viral replication, they will have an undetectable HIV RNA level and so will not have resistance testing performed, even if a resistance mutation is present in their viral sub-species. As a result these prevalence rates may be under-estimations.

1.6.5.1. Accumulation of new resistance while on therapy

The prevalence of drug resistance to each drug class appears to have plateaued or even declined in ARV-experienced patients since 1999[250;251]. This corresponds to the proportion of patients who have experienced virological failure to each of the three main drug classes over the same time period[252]. The availability of a large number of more convenient NRTIs as well as the wide use of PI/rs could partially explain why prevalence rates of resistance have stopped increasing, although resistance emergence is still a problem and patients who have mutations in their viral sub-species may continue to experience more emergence of resistance. Of note, much resistance

was accumulated at the time of mono- and dual therapy, but since these regimens are no longer recommended, accumulation of resistance due to these treatment strategies should no longer a problem.

It is estimated that around 85% of patients who are failing a cART regimen have evidence of at least one resistance mutation in their viral sub-species[253;254]. Among patients on potent cART with detectable viraemia, in whom resistance mutations were detected by population sequencing, the incidence of acquiring new mutations was shown to be approximately 1.61 (95% CI: 1.36 to 1.90) mutations per person-years follow-up[254]. In all patients who are starting therapy, the rate of aquiring new mutations in regions that are sequenced routinely, is likely to be slow to start off with because the selection of initial mutations may be difficult. Once initial mutations have emerged to the regimen, the virus will be more susceptible to the emergence of new mutations, so these will occur more easily and the rate of accumulating new mutations is likely to increase. However, once the virus has become highly mutated, resistance accumulation rates will slow down again, because only a finite number of mutations can emerge.

In patients who were kept on the same virologically failing regimen for a median of six months in EuroSIDA, there was considerable accumulation of drug resistance mutations, particularly in patients who had low levels of resistance to the failing regimen initially: thymidine analogue mutations emerged in the predominant virus population of 25% of patients, NNRTI mutations emerged in the predominant virus population of 12% of patients and PI mutations emerged in the predominant virus of 46% of patients[253]. The majority of these patients (92%) were on a failing PI-containing regimen at the time of the analysis. Compared to this study, a study by Maggiolo *et al* included more patients who continued on a failing NNRTI-containing regimen and so fewer PI mutations were acquired: new resistance mutations emerged in 37.5% patients overall; primary RT mutations emerged in the predominant virus of 21.9%; and PR mutations emerged in the predominant virus of 14.3%[255].

As already detailed in 1.6.5, resistance mutations arise at different rates depending on other influential factors. Predictors of resistance emergence include the plasma HIV RNA level, the HIV RNA slope and the number and type of mutation detected in the initial resistance test. In patients who were failing a PI containing regimen and have resistance mutations to each class of drugs they were receiving, new major protease inhibitor mutations emerged more rapidly than new TAMs when the M184V mutation was present. There was no significant difference in the emergence of TAMs and major

protease inhibitor mutations if lamivudine was not part of the thymidine analogue regimen (i.e. the M184V mutation was not present), because of the antagonistic effect of M184I/V on the emergence of TAMs[256].

Little is known about the emergence of new resistance mutations to PIs in patients who are receiving a ritonavir-boosted PI containing regimen. Resistance mutations to unboosted PIs generally emerge more quickly than to NRTIs, but slower than to NNRTIs (Table 1.6.5.1). In the current era of ritonavir-boosted regimens it will be interesting to see whether the same types of resistance mutations emerge and whether they emerge at the same rate. This will be discussed in more detail in chapter 3[248;250-261].

1.6.6. Phylogenetic analysis

Phylogenetic analysis was originally developed by biological systematists to reconstruct evolutionary genealogies of species based on genetic similarities[262]. In HIV, phylogeny is used to identify clusters of genetically similar viral strains, where clusters are depicted in a hierarchical branching diagram called a phylogenetic tree[263]. Phylogenetic trees are initially created through multiple-sequence-alignment where each sequence of interest is aligned to other sequences of interest. Pairwise distances (i.e. the number of nucleotide differences) are then calculated between sequences and these are displayed in an un-rooted tree by using neighbour-joining (NJ) distance methods[264;265]. Boot-strapping is then used to determine the level of support for each branch of the tree, based on the data at hand. Boot-strapping generates new trees using the same methods as above, but through comparing sequences where nucleotides have been removed at random positions along the nucleotide sequence. This uses sampling with replacement and is repeated a large number of times (usually 500-1000) so that many permuted alignments are produced. For each alignment a new tree is generated and a consensus tree is subsequently created from the large set of trees. The consensus tree consists of groups that occur most frequently in the set of trees. It is possible to root the tree by comparing all sequences to a consensus sequence (e.g. by including HXB2 in the initial comparisons).

All sequences that are genetically close irradiate from the same node, and the distance from the node of the branch indicates the difference in nucleotide bases between sequences forming a cluster. Each branch of the tree represents an individual RNA sequence and all sequences that are considered to be descendants of a common ancestor group together to form a cluster or, when describing HIV subtypes, a clade[264;265]. Sequences within each clade are genetically similar to every other

sequence in the same clade. Different approaches are used to determine the most likely tree topology and branch lengths[266-269].

Progressive sequence alignment is used to produce a phylogenetic tree by incorporating new sequences into the calculated alignment according to the order of the genetic distance between them. Phylogenetic methods such as parsimony based analysis (i.e. a method of identifying the phylogenetic trees that require the smallest number of evolutionary events to explain the observed sequence data) and maximum likelihood methods (i.e. a substitution model that is used to assess the probability of a particular mutation occurring) are used to estimate the likelihood of each nucleotide substitution occurring and consequently determine the tree topology and branch lengths. In this thesis I will use phylogenetic analysis to compare nucleotide sequences along the reverse transcriptase (RT) and along the protease genes because these are two areas that are targeted by ARVs.

1.6.7. Resistance testing guidelines

The European HIV Drug Resistance Panel has provided updated guidelines on practical issues associated with resistance testing since 2001[190]. Their recommendations include details on when to perform resistance tests, which test to use and how best to implement the results. This was last updated in October 2004 after a push for more hypersusceptibility mutations to be included[190].

The panel recommend resistance testing for the following indications:

1. Antiretroviral-naïve patients with acute or recent HIV infection
2. At therapy failure, including suboptimal treatment response when an ARV change is required
3. Pregnant HIV infected women and paediatric patients with detectable viral load, when treatment initiation or change is considered
4. For genotype source patients who are to receive post-exposure prophylaxis (PEP).

Resistance testing should also be considered for all ARV-naïve patients who are starting therapy. By following guidelines on when to perform resistance testing in patients who are experiencing virological failure it is possible to switch some ARVs from a failing treatment regimen to other active ARVs with the intention of suppressing viral replication.

1.6.8. Benefits associated with the emergence of resistance

Contrary to expectations, the emergence of mutations may have less of a negative effect than anticipated, at least over the short term, although compensation can still occur over time. Some mutations that indicate resistance to one ARV have been associated with hypersensitivity to other ARVs within the same class[270]. Other mutations may affect the fitness of the virus and impact on the viral replication capacity. For example, evidence from *in vitro* experiments suggests that viruses containing the M184V mutation have a lower fitness (i.e. the ability of the virus to replicate and infect new cells) and increased fidelity (i.e. a lower probability of the emergence of new mutations per replication round) compared to wild-type viruses[271-278].

In the E-184V study, patients with limited treatment options performed better if the M184V mutation was continuously detected through the use of population sequencing during a structured treatment interruption (STI – see section 1.6.9.2)[279]. However, it is still unknown whether, in the era of cART, it is beneficial to preserve this mutation. By lowering the viral replication capacity and increasing the fidelity of the dominant virus, other mutations may emerge at a slower rate and so the activity of other drugs in the regimen may be preserved for longer[280]. In the E-184V study, 3TC preserved the M184V mutation as well as other pre-existing mutations, probably due to linkage on the same genome with M184V. Thus a virus with reduced replication capacity was maintained in this study. Under drug selection pressure from other drugs in a regimen, this may not be the case. I will investigate the benefit of preserving the M184V mutation in the dominant virus population of patients who are failing a 3TC containing regimen, in chapter 6, using data from a clinical trial.

1.6.8.1. Fitness and replication capacity

The presence of resistance mutations may cause a virus to have a reduced capacity for inducing $CD4^+$ T cell count declines because it may not be able to infect the T lymphocyte cells as efficiently[281]. A reduction in viral fitness associated with viruses containing resistance mutations has been shown previously[281-286], but this reduced fitness may act entirely through a reduction in the viral load and not impact on $CD4^+$ T cell counts[287]. In both patients on ARVs and patients off ARVs there is a trend for more favourable (or less unfavourable) $CD4^+$ T cell count changes in patients with lower viral loads.

In chapter 7 I use data from patients who are followed up longitudinally throughout Europe and have an ongoing viral load > 500 cps/mL in order to assess whether the level of resistance present (i.e. specific mutations and classes of mutations) is

associated with $CD4^+$ T cell count changes, independent of the viral load level and the use of ARVs.

1.6.9. Consequences of resistance emergence: salvage treatment

Although over twenty ARVs are currently approved by the FDA, most of them come from just three drug classes and consequently have overlapping resistance profiles[80;81]. Patients can exhaust all treatment options if too many resistance mutations emerge in their viral quasispecies, because ARVs from any one class, or in combination with each other, will no longer be effective[254;261;288]. New strategies aimed at patients in need of salvage therapy have been investigated, yet it is still a problem with 4% of patients in the UK showing resistance to all three major classes of ARVs (Figure 1.6.5.2)[250].

Patients with high levels of resistance can still benefit from the continued use of a failing drug. This is because not every strain of HIV will be resistant to the drug, so the drug may still suppress replication to a certain extent. If no active treatment options are available the patient may benefit from recycling or continuing to use old drugs. Alternatively they could undergo a structured treatment interruption, initiate mega-HAART or enrol into an expanded access program for new drugs[33;110]. However, the discontinuation of all drugs is increasingly discouraged as it may result in rapid immunologic and clinical progression[172;173]. These issues are discussed below.

1.6.9.1. Continued use or recycling of old drugs

Patients with limited treatment options available may continue to receive their current cART regimen even if they experience persistently detectable viraemia. By remaining on the same regimen it may be possible to partially control viral replication and remain immunologically stable[289]. Some ARVs continue to exert antiviral activity against viruses containing drug resistance mutations; this is true for some PIs due to their high genetic barrier and also for some NRTIs[279;290-292]. Data have also shown, *in vitro* and to a less extent *in vivo,* that drug resistance mutations may reduce the ability of HIV to replicate efficiently (i.e. it has a reduced replication capacity or fitness), therefore recycling or rotating ARVs in an attempt to reduce viral fitness could be a treatment strategy. Although this needs to be carried out with caution in order to avoid the emergence of mutations to drugs that still might be partially active.

If the patient remains on a virologically failing regimen there is risk that more mutations will emerge to the existing regimen and this could further compromise future regimens. On the other hand, if the patient switches to another regimen containing only one active drug, the patient will essentially experience functional monotherapy and risks

resistance emerging to the active drug thereby inhibiting its future use. Remaining on a failing regimen is currently considered advisable if the patient does not have the potential of at least two fully active ARVs, the regimen is easy to take and has minimal side-effects. The selection of the regimen must be done with care otherwise mutation development will compromise rather than enhance current and future treatment options.

1.6.9.2. Structured Treatment Interruptions (STI)

Patients may want a break from treatment for several reasons. They may want a break from the undesirable side-effects that many patients experience while on treatment, or they may experience treatment fatigue, regardless of tolerability. One strategy is to undertake an STI as a way to postpone their next regimen until the majority virus has reverted back to wild-type. In the absence of treatment the fittest virus will become dominant and since wild-type viruses are fittest, it was hypothesized that interrupting therapy could expand future treatment options for patients in need of salvage therapy.
In the CPCR064 study that was performed on 270 patients infected with multidrug-resistant HIV, STIs were associated with greater progression of disease and did not confer immunologic or virological benefits or improve the overall quality of life. In addition, the SMART study showed that STIs can actually be harmful in the long term, with patients who discontinued treatment having a 2.6 (1.9 to 3.7) higher hazard of progressing to AIDS or death compared to patients who remained on treatment throughout the period[293]. After the results of SMART entered the public domain more patients who needed to discontinue therapy for any reason started reducing treatment down to either monotherapy or simple uncomplicated regimens, rather than terminating treatment altogether[293]. Although STIs did not confer progression of disease or virological benefits in patients with multiple failures and multi-drug resistance in SMART, they had a prolonged negative impact on $CD4^+$ T cell count recovery[294].

NNRTIs have a prolonged half-life compared to ARVs from other classes, so there is a concern that an interruption from a suppressive NNRTI regimen may result in prolonged exposure to an unprotected NNRTI, leading to a risk of viraemia and resistance as a consequence. Since NNRTIs have longer half-lives than most ARVs from other classes and a low genetic barrier (i.e. only one mutation is required in order to confer high-level drug resistance), they need to be stopped earlier than the other ARVs in the regimen to avoid the potential for NNRTI monotherapy[295].

Limited data are available on the best way to stop an NNRTI-containing regimen in order to avoid effective monotherapy and the potential emergence of drug resistance. I

investigate the emergence of drug resistance according to one of three methods used to interrupt an NNRTI-containing regimen for patients randomised to the discontinuation arm of the SMART study, a large trial that assessed the impact of treatment interruptions, in chapter 8. I summarize the impact the three interruption strategies had on the likelihood of re-suppressing HIV RNA in the eight months following the re-start of treatment, and explore the risk of resistance emergence two months after the treatment interruption, in patients who were virologically suppressed on an NNRTI-containing regimen and interrupted it as part of SMART.

1.6.9.3. Mega-HAART and ARV-switching in patients on salvage therapy

Drug combinations that use five or more ARVs are also used in patients on salvage therapy. This is termed mega-HAART and can consist of as many as up to nine drugs, but is often associated with intolerable side-effects and the potential for significant drug interactions[296]. Patients receiving mega-HAART should have therapeutic drug monitoring (TDM) to ensure their drug levels are optimal because safety is a big concern. This strategy has led to undetectable viral loads over the long-term in highly selected patients on salvage therapy[296-298].

Patients who continue to experience rising viral loads on mega-HAART or patients who are unable to participate in clinical trials because they have no treatment options available may consider using new drugs by enrolling into expanded access programmes. Ideally new drugs should be used in combination with other active drugs for the best results, but some patients may not have the capacity to wait for more drugs to become available because their risk of disease progression or death is too high.

1.7. Project objectives and layout of the thesis

The main aim of this thesis is to investigate the clinical impact of resistance testing in four randomised controlled trials (RCTs), two cohort studies and a resistance database. I will initially examine different aspects of resistance emergence using trial data and then extend these analyses to patients from the cohort studies.

In particular, the aims of this project are:

i) To explore the emergence of resistance among patients experiencing virological failure on a ritonavir-boosted PI

ii) To examine the prognostic value of four freely available genotypic interpretation systems for predicting viral load outcome

iii) To use phylogenetic analysis to examine the relationship between nucleotide distances, treatment use and virological response for patients in whom the M184V mutation is present in the dominant viral population.

iv) To explore the association between the presence of resistance mutations and $CD4^+$ T cell count change in patients who are receiving antiretrovirals and have ongoing viraemia.

v) To assess the impact of different strategies for interrupting an NNRTI on the likelihood of viral re-suppression in the eight months after therapy is re-started

vi) To investigate the emergence of drug resistance mutations over two months of follow-up, according to the method of interrupting an NNRTI for patients randomised to a large clinical trial

All of the analyses are based on data from four clinical trials (SMART, MaxCmin1, MaxCmin2 and COLATE) and two large clinical observational databases (EuroSIDA and the UK CHIC study with its links to the UK HIV Drug Resistance Database that is co-ordinated by the Collaborative Group on HIV Drug Resistance). Since resistance testing is dependent on viral load levels (i.e. they are usually performed in patients with an HIV RNA > 1000 copies/mL) and is conducted on a selective group of individuals, the randomised nature of the trials will not ensure balances between the groups that will be compared throughout this thesis. However, patients in the trials are followed up frequently and as a result will have a more data available than patients in the observational studies. In the observational studies, patients will generally be representative of those seen in routine clinical practice. Since the patients who come from the observational studies have more irregular follow-up compared to the trial patients, data will be more sparse and resistance testing more infrequent. The availability of samples for resistance testing varies according to the study. This is described in more detail in Chapter 2.

CHAPTER 2: INTRODUCTION TO THE DATA

2. Introduction

This thesis includes data from four clinical trials (MaxCmin1, MaxCmin2, COLATE and SMART) and three observational databases. The observational data come from two large multicentre clinical cohort studies: EuroSIDA and the UK CHIC study, where the clinical data in the UK CHIC study can be linked to resistance data from the UK HIV Drug Resistance Database. Since this thesis explores the relationship between clinical variables and different aspects of resistance emergence I have only included patients who contributed data to both the UK CHIC study and the UK HIV Drug Resistance Database and not patients who were recruited to either of these cohorts alone. In this chapter I will describe all of the seven studies that contribute patients to this thesis, with special consideration given to how patients were recruited and how data were collected.

2.1. Randomised Controlled Trials

2.1.1. The MaxCmin trials

The MaxCmin trials were the first two open-labelled, multicentre phase IV trials to perform head-to-head safety and efficacy comparisons between different ritonavir boosted PI containing regimens. MaxCmin1 compared the safety and efficacy of ritonavir-boosted saquinavir (SQV/r) against ritonavir-boosted indinavir (IDV/r) and MaxCmin2 compared the safety and efficacy of SQV/r against ritonavir-boosted lopinavir (LPV/r) over 48 weeks follow-up in HIV-infected adults[299;300].

2.1.1.1. Patients and methods

Between September 2000 and April 2001 sites in Argentina, Austria, Belgium, Denmark, France, Germany, Greece, Italy, Norway, Portugal, Switzerland, Spain, Sweden, the Netherlands, the UK and the USA were invited to enrol patients into MaxCmin1. Patients were randomised 1:1 to IDV/r (800/100 mg bid) or to SQV/r (1000/100 mg bid). MaxCmin2 opened enrolment in May 2001 and randomised patients 1:1 to LPV/r (400/100 mg bid) or to SQV/r (1000/100 mg bid) until recruitment ended in December 2001. Both trials were financially supported by Roche Pharmaceutical Ltd.

2.1.1.2. Randomisation

Randomisation was carried out centrally; treatment was open label and patients were informed of their randomised treatment at or prior to their baseline visit. Randomisation was stratified by region and according to whether a patient was PI-naïve or PI-experienced prior to study entry.

2.1.1.3. Sample size and analysis

The MaxCmin trials were powered to show equivalence between study arms with an 80% chance that the 95% confidence interval for the difference in treatment failure rates would exclude a difference greater than 15% in either direction over 48 weeks follow-up. Treatment failure was considered to be observed virological failure (section 2.1.1.5.1), withdrawal of consent for participation in the study, loss to follow-up or death. This was based on a sample size of 150 per arm and an underlying failure rate of 20% in both arms.

Per protocol, the primary population for analysis was the intention-to-treat/exposed (ITT/e) population including all randomised patients who had taken at least one dose of their assigned treatment[301]. In this analysis, any patient who stopped a component of their treatment regimen was not considered to be a failure at the time of stopping, even if they stopped the randomised PI/r. Instead they were considered to be a success until they experienced the event of interest (i.e. treatment failure) or they were censored at 48 weeks if they did not. The protocol also stipulated an ITT/e/stopping = failure analysis where discontinuation of the assigned PI/r component was considered to constitute failure. In both analyses, patients who withdrew consent to participate in the study, were lost to follow-up or died were also defined as having experienced failure. Exploratory on treatment (OT) efficacy and safety analyses were performed in accordance with CPMP guidelines regarding analysis of equivalence trials[302].

2.1.1.4. Protocol design

The protocols for both trials had the following identical inclusion and exclusion criteria:

Inclusion criteria:

1. Male or female, > 18 years of age.
2. HIV-1 infected as documented by a licensed HIV-1 antibody ELISA.
3. Women of childbearing potential with a negative serum pregnancy test (beta-HCG) within 28 days of trial baseline.
4. Ability to understand and provide written informed consent to participate in the trial.
5. Clinical laboratory values considered not clinically significant - for the potential response to the planned new regimen - in the opinion of the investigator.
6. Fulfilment of at least one of the following five criteria, provided that either of the boosted PI-regimens studied in the trial were judged to be of benefit to the person (see criteria # 7):
    (i) Being protease inhibitor (PI)-naïve
    (ii) Being PI-experienced with a viral load ≥ 400 copies/mL

Being PI-experienced with a viral load < 400 copies/mL **and**:

(iii) Experiencing adherence problems either before or currently on an ongoing unboosted PI-containing regimen (irrespective of type and dosing schedule of the PI) **and/or**

(iv) Experiencing current toxicity to the PI-component of an unboosted PI-containing regimen (other than to any of the study PIs) **and/or**

(v) Experiencing typical ritonavir adverse events (i.e. loose stool or peripheral dysastesia) on a ritonavir (dose no less than 300 mg bid) boosted double PI-containing regimen (regardless of type and dosing schedule of other PI)

7. For all five sub-criteria listed under inclusion criterion #6, the *a priori* probability of responding to each study PI, as judged by the investigator, had to be equal. The judgment had to take into account the factors mentioned below, all of which would preclude enrolment:
    i) Prior dose-limiting toxicity to any of the study PIs (irrespective of dosing)
    ii) Prior switch away from a regimen that included one but not the other study PI because of virological failure, except if resistance testing at time of failure did not show evidence of selective resistance development (not applicable for 6(i))

Exclusion Criteria

A patient was not eligible for inclusion in the MaxCmin trials if any of the following criteria applied:

1. Patients whom in the investigator's opinion were unlikely to complete the 48-week trial period.
2. Patients with current alcohol or illicit drug use which, in the opinion of the investigator, could interfere with the patients' ability to comply with the dosing schedule and protocol evaluations.
3. Patients on concomitant medications which, in the opinion of the investigator and according to drug product labelling, would result in clinically significant interactions with any of the PI/rs assessed in this trial
4. Patients who were pregnant or breast feeding
5. Patients who had renal failure requiring dialysis
6. Patients suffering from a serious medical condition, including one or more AIDS defining events, which in the opinion of the investigator, could compromise the safety of the patient.

2.1.1.5. Background therapy

Patients had to be given a potent background regimen containing at least two non-PIs in addition to their PI/r component. The choice of the non-PI background therapy had to be made by the treating physician at each of the trial sites prior to the time of randomisation. The aim of treatment was to reduce HIV RNA to undetectable levels without prematurely terminating treatment for toxicity or non-adherence. Only locally approved ARVs could be used in combination with the randomised PI/r component. Patients were not permitted to receive other PIs than those assigned by randomisation.

Patients were allowed to discontinue the PI/r component if they experienced treatment limiting adverse events or virological failure (section 2.1.1.5.1). Where appropriate (e.g. exposure related toxicity or failure potentially due to low exposure) investigators were permitted to adjust the dose or the formulation of the randomised PI so that patients could continue on trial treatment. The baseline drug regimen was to be continued for the duration of the trial period (i.e. 48 weeks). Where needed, other ARVs could be discontinued and replaced in case of treatment limiting adverse events, virological failure, immunologic failure or clinical failure (i.e. the development of a new AIDS defining event (Table 1.1.3.1) or a relapse of a previously successfully treated AIDS-defining event) due to the other ARVs, within the first 24 weeks of the study. From Week 24 onwards other ARVs could be changed at the discretion of the investigator.

2.1.1.5.1. Virological failure

In MaxCmin1, virological failure was considered to be:

- For patients entering the study with a viral load <200 copies/mL, an HIV RNA value (confirmed) ≥200 copies/mL
- For patients entering the study with a viral load ≥200 copies/mL:
    1. any rise in HIV RNA ≥ 0.5 logs (confirmed) and / or
    2. a viral load (confirmed) of:

        ≥ 50,000 more than 5 weeks after baseline

        ≥ 5000 " 14 "

        ≥ 200 " 27 "

In MaxCmin2 virological failure was considered to be:

- For patients entering the study with a viral load <200 copies/mL, an HIV RNA value (confirmed) ≥200 copies/mL
- For patients entering the study with a viral load ≥200 copies/mL:
    1. any rise in HIV RNA of ≥ 0.5 logs (confirmed) and / or

2. a viral load (confirmed) of:
   at Week 4: < 0.5 log reduction from baseline if > 200 at Week 4
   at Week 12: < 1.0 log reduction from baseline if > 200 at Week 12
   at Week 24: an HIV RNA ≥ 200 copies/mL

2.1.1.5.2. Immunological failure

In both of the MaxCmin studies immunological failure was considered to be:

- Compared with baseline, a decrease in $CD4^+$ T cell counts of > 50% on two consecutive occasions at least one week apart, providing that the baseline $CD4^+$ T cell count was > 150 cells/μL.
- For patients with baseline $CD4^+$ T cell count of 100 to 150 cells/μL , a follow-up $CD4^+$ T cell count < 50 cells/μL
- For patients with baseline $CD4^+$ T cell count < 100 cells/μL a follow-up $CD4^+$ T cell count < 25 cells/μL

2.1.1.6. Follow-up

Consenting patients were screened in the 28 days prior to the first dose of the study drug. Following randomisation, eligible patients were informed of their treatment assignment and re-attended the clinic for the baseline assessments (Day 1). Baseline data was collected on medical history, demographics and clinical and laboratory parameters (appendix III). Data on clinical evaluation, safety, HIV RNA levels and $CD4^+$ T cell counts were collected at follow-up visits which occurred after 4, 12, 24, 36 and 48 weeks (Table 2.1.1.6.1).

Table 2.1.1.6.1: Clinical Evaluations in the MaxCmin Trials

Evaluation	**Day**		**Week**				
	Screening	Baseline	4	12	24	36	48
Informed consent	X						
Entry criteria	X						
Demography		X					
CDC classification		X					
Height		X					
Weight		X	X	X	X	X	X
Concurrent medications		X	X	X	X	X	X
HIV-associated conditions		X	X	X	X	X	X
Adverse and serious adverse events		X	X	X	X	X	X

2.1.1.7. Data collection

Since all of the PI/r regimens were b.i.d, plasma was collected for pharmacokinetic (PK) drug concentration analysis at weeks 4 and 48, as close to 12 hours (range: 11 to 13 hours) post-dose as possible to obtain the C_{min} (i.e. the minimum plasma drug level in a patient). If the drug was taken less than one hour before the PK measurement was recorded, it was presumed that the most recent dose had yet to "kick in" and that the PK measurement actually referred to the dose that was taken prior to it, so 12 hours was added to the time period (e.g. blood taken at 0.5 hrs after the last dose was recorded as 12.5 hours since the "real" last dose). Samples collected one to six hours post-dosing, and samples collected more than 13 hours post dosing, were considered unusable and not included in any analysis due to the large concentration variability in this time interval. Using regression analysis, it was assumed that there was a stable log-linear period of decline in the PK levels for samples collected between six and 11 hours post-dose. Separate models were created for each PI/r to estimate the C_{min} at 12 hours post-dose for each individual. These were adjusted for the period (i.e. week 4 or week 48), and the amount of time between the dose and the PK measurement.

At baseline and at each of the follow-up visits, four 1 mL vials of plasma were collected and stored locally at minus 70° C for resistance testing. Samples were then shipped to a central repository at the Copenhagen HIV Programme (CHIP) where each sample was uniquely identified in a freezer log. Patients underwent genotypic resistance testing if they had a baseline HIV RNA >500 copies/mL or they experienced virological failure (section 2.1.1.5.1) and had HIV RNA >500 copies/mL. Patients underwent phenotypic resistance testing if they were PI experienced at baseline, had an HIV RNA >1000 copies/mL and had baseline genotypic resistance data available. Phenotypic resistance data was only performed on samples at baseline. Patients were selected for both genotypic and phenotypic resistance testing retrospectively.

Samples of interest were shipped on dry ice, in batches, to a central testing facility for protease gene sequencing. For MaxCmin1 the testing facility was the International Clinical Virology Centre (ICVC, UK), and for MaxCmin2 it was Advanced Biological Laboratories (ABL, Luxembourg). ABL has developed a strong focus on HIV drug resistance, mainly on algorithm-based genotype interpretation systems, the management of sequence and complex integrated clinico-virological databases, and on more advanced genetic analysis tools such as virus subtype characterization.

The MaxCmin resistance data that was obtained at baseline and during follow-up was combined with baseline clinical and demographic data (Table 2.1.1.7.1) in order to

explore resistance emergence in detail, and to assess the impact of mutations on virological response.

Table 2.1.1.7.1: Data included in this thesis from the MaxCmin studies

Baseline data:	
Demographic:	Gender Race Date of birth Mode of HIV infection Region of origin Body Mass Index (weight and height information is collected separately)
Clinical:	Date of HIV diagnosis Stage of HIV infection according to CDC classification Date and diagnosis of any HIV-related diseases (CDC category B or C)
Laboratory markers:	Dates and values of current $CD4^+$ lymphocyte counts and any values recorded in the year proceeding baseline Date and value of $CD4^+$ nadir Date and value of current and past plasma viral load measurements
Treatment information:	Type and duration of all prior ARVs and dates of starting new medication: **NRTIs:** AZT, ddI, 3TC, ddC, d4T, ABC, combivir and trizivir; **NNRTIs:** NVP, DLV, EFV; **PIs:** SQV (hard gel formulation), SQV (soft gel formulation), IDV, RTV (<400 mg/dose), RTV (≥400 mg/dose), NFV, APV, LPV
Other data:	C_{min} levels at week 4 and week 48 (i.e. drug concentrations in each patient 12 hours after taking the PI/r)
Resistance data:	Date of baseline resistance test and nucleotide sequence obtained Date of resistance test at the time of failure and nucleotide sequence obtained Genotypic data: mutations in the dominant viral species, including data on the gene location (i.e. RT or PR), the codon position, the amino acid present at a particular position, and information on whether the mutation is present in a mixture or on its own. Flags were used to highlight any major mutations (using the most up-to-date IAS-USA listings) and compensatory mutations. Phenotypic data: the fold change in ARV required to suppress replication compared to wild-type, for all available ARVs

2.1.2. The COLATE Study

COLATE (COntinuation of LAmivudine Treatment in Europe) was a randomised trial to address whether the continued use of 3TC in patients experiencing virological failure on a 3TC-containing cART regimen could be virologically beneficial. As described in section 1.6.8 it is postulated that viruses containing the M184V mutation have a lower fitness and increased fidelity compared to wild-type viruses. Since use of 3TC preserves the M184V mutation it was hypothesised that continued use of lamivudine could lead to suppressed rates of viral replication in patients who were failing treatment. Patients in COLATE were randomised (1:1) to discontinue or continue 3TC in addition to receiving the most effective cART regimen available (appendix V). COLATE was a phase IV, open-label, multi-centre clinical trial conducted at twenty sites in twelve countries in accordance with the Helsinki II Declaration, the *Good Clinical Practice* guidelines (ICH-GCP Guideline (CPMP/ICH/135/95))[280]. Local independent Ethics Committees approved the protocol. The first patient was enrolled on 28th May 1999 and, after recommendation from the DSMB, recruitment was stopped prematurely on 15th May 2002 because there was slow recruitment to the trial. Recruitment was almost complete at the time that the study was stopped and every attempt was made to follow all enrolled patients for the entire 48 weeks.

2.1.2.1. Patients and methods

HIV positive patients were eligible at screening if they were receiving a failing 3TC containing regimen, had plasma viral load >1000 copies/mL in addition to a documented history of viral load values <500 copies/mL for at least one month whilst taking 3TC, were ≥18 years old, were not pregnant or breastfeeding and did not have a serious medical condition. In addition, all laboratory values had to be without potential adverse clinical significance as per the treating physician's judgement.

2.1.2.2. Randomisation

At screening, patients were divided into two strata: stratum A contained patients who experienced virological failure on their first-line 3TC-containing cART regimen; and stratum B contained patients who were failing a later cART regimen that contained 3TC. At screening the treating physician planned a new three-drug cART regimen, which did not include 3TC, but to which 3TC could be added depending on the outcome of randomisation. After the new cART regimen was planned, patients were randomised (1:1) to either: switch to the planned new regimen and discontinue 3TC or switch to the planned new regimen and continue 3TC (150mg twice daily). Randomisation was performed centrally and stratified for strata, country and planned ddI use in the new regimen. Patients could not receive ABC or ddC in their new

regimen because the M184V mutation is known to confer low level cross-resistance to both of these drugs[303].

Patients were allowed to switch one or more of the antiretroviral drugs in their regimen, including the 3TC component, in the case of treatment-limiting adverse events. All ARVs other than 3TC could be switched in the case of virological failure (section 2.1.2.2.1), immunological failure (using the same definition as the MaxCmin trials – section 2.1.1.5.2) or clinical failure (i.e. the development of a new AIDS defining event or relapse of a previously successfully treated AIDS-defining event).

2.1.2.2.1. Virological failure

In the COLATE study, virological failure was considered to be:

- From Week 4 and thereafter, a confirmed decrease in HIV RNA of < 0.5 $\log_{10}$ compared to baseline HIV RNA

OR

- A confirmed increase in HIV RNA of > 1 $\log_{10}$ compared with nadir HIV RNA,

In both cases the HIV RNA that constituted virological failure had to be >1000 copies/mL. The assessment of virological failure had to be based on two consecutive measurements performed at least one week apart, and could not be taken during an intercurrent illness or in the four weeks following an immunisation.

2.1.2.3. Sample size

The initial sample size calculations were performed to investigate whether continued use of 3TC was superior at reducing HIV RNA compared to discontinuation of 3TC. The trial was powered to detect a difference in HIV RNA reduction of at least 0.5 $\log_{10}$ copies/mL between treatment arms within each stratum using an average-area-under-the-curve-minus-baseline (AAUCMB) approach to estimate HIV RNA declines over 48 weeks of follow-up[280]. It was determined that the trial should randomise (1:1) 160 patients into two treatment arms, assuming 90% power and a significance level of 0.05.

After an unscheduled interim analysis, the DSMB recommended that recruitment should be stopped early because recruitment to the study was slow, but follow-up on patients already recruited should be continued. For this interim analysis the Peto method of repeated significance testing was used to test for treatment difference, with a p-value of <0.001 considered significant. A total of 136 patients were recruited (71 in the discontinuation arm and 65 in the continuation arm).

2.1.2.4. Follow-up

Out of 136 patients who were randomised, we have follow-up data on 131 (96.3%), 129 (94.9%), 124 (91.2%), 125 (91.9%), 120 (88.2%) and 122 (89.7%) patients at baseline, weeks 4, 12, 24, 36 and 48 respectively. During follow-up, the following procedures were performed at each visit: clinical evaluation, blood safety analyses, viral load and $CD4^+$ count (Table 2.1.2.4.1).

Table 2.1.2.4.1: Clinical Evaluations in COLATE

Evaluation	**Day**		**Week**				
	Screening	Baseline	4	12	24	36	48
Informed consent	X						
Entry criteria	X						
Demography		X					
CDC classification		X					
Height		X					
Weight		X	X	X	X	X	X
Concurrent medications		X	X	X	X	X	X
HIV-associated conditions		X	X	X	X	X	X
Adverse and serious adverse events		X	X	X	X	X	X

2.1.2.5. Data collection

In COLATE, genotypic resistance data were available at baseline and at all time points during follow-up if a patient had an HIV RNA >500 copies/mL. Plasma was collected and stored at the specific time point so that it could be used for retrospective evaluation of genotypic and phenotypic resistance patterns. Plasma was also used for quality assurance of the HIV RNA measurements that were performed, real-time, at the sites. All follow-up resistance tests were paired with resistance data that were recorded at baseline and these were matched to baseline demographic data (Table 2.1.2.5.1) in order to investigate factors contributing to resistance evolution.

Table 2.1.2.5.1: Data included in this thesis from the COLATE study

Baseline data:	
Demographic:	Gender Race Date of birth Mode of HIV infection Region of origin Body Mass Index (weight and height information is collected separately)
Clinical:	Date of HIV diagnosis Stage of HIV infection according to CDC classification Date and diagnosis of any HIV-related diseases (CDC category B or C)
Laboratory markers:	Dates and values of current $CD4^+$ T cell counts and any values recorded in the year proceeding baseline Date and value of $CD4^+$ nadir Date and value of current and past plasma viral load measurements
Treatment information:	Type and duration of all prior ARVs and dates of starting new medication: **NRTIs:** AZT, ddI, 3TC, ddC, d4T, ABC, combivir and trizivir **NNRTIs:** NVP, DLV, EFV **PIs:** SQV, IDV, RTV (<400 mg/dose), RTV (≥400 mg/dose), NFV, APV, LPV
Other data:	Strata
Resistance data:	Date of baseline resistance test and nucleotide sequence obtained Date of follow-up resistance test and nucleotide sequence obtained Genotypic data: mutations that are present in the dominant viral species, including data on the gene location (i.e. RT or PR), the codon position, the amino acid present at a particular position, and information on whether the mutation is present in a mixture or on its own. Flags were used to highlight any major mutations (using the most up-to-date IAS-USA listings) and compensatory mutations.

2.1.3. The SMART Study

Although the implementation of antiretroviral treatment guidelines has resulted in substantial declines in morbidity and mortality over the years[76], the effectiveness of cART has been shown to wane over time due to the emergence of HIV drug resistance and because of toxicities associated with ARVs and their impact on adherence[304]. Many asymptomatic patients are not at an immediate risk of developing serious opportunistic diseases, especially if they have suppressed rates of viral replication, so they may wish to undergo a treatment interruption. Other reasons why patients may wish to undergo a treatment interruption are discussed in section 1.6.9.2. This motivated a comparison of two strategies: one which conserved treatment options by interrupting their use while the risk of opportunistic disease was low; and one which aimed to consistently sustain virological suppression (i.e. an HIV RNA < 50 copies/mL) without a break from therapy.

The SMART trial (Strategies for Management of AntiRetroviral Therapy) was a large international study designed to examine the impact of intermittent cART treatment in HIV-infected individuals. SMART was supposed to run for over nine years, but it was stopped in January 2006 after only two years of follow-up in some patients. This was decided after an elevated risk of clinical progression was seen in patients who interrupted treatment.

2.1.3.1. Patients and methods

HIV infected patients were eligible for recruitment into SMART if they were ≥13 years old, were not pregnant or breastfeeding and did not plan to become pregnant over the course of follow-up, had a $CD4^+$ T cell count >350 cells/μL, were receiving ARVs and were willing to initiate, modify or stop ARVs depending on the outcome of randomisation. Patients who were not receiving ARVs at randomisation were required to use them immediately if they were randomised to the VS arm of SMART, and were required to postpone using them until after their $CD4^+$ T cell count fell below 250 cells/μL if they were randomised to the DC arm of SMART.

The study was opened for enrolment in the US and Australia in January 2002 and in April 2004 additional sites in Europe, Asia, South and North America began. The purpose of the SMART study was to compare the long-term clinical consequences of two strategies of antiretroviral management: drug conservation (DC) vs. viral suppression (VS). Enrolment was stopped prematurely on 11th January 2006 because patients in the DC arm had twice the risk of disease progression (i.e. the development

of clinical AIDS or death) compared to patients in the VS arm. When enrolment was stopped, 5,472 patients had been enrolled from 318 sites on six continents.

2.1.3.2. Randomisation

In SMART, patients were randomised (1:1) to either the drug conservation (DC) group or the viral suppression (VS) group. Randomisation was performed centrally and stratified for clinical site. Randomisation schedules were generated to ensure that there were an approximately equal number of individuals in each treatment arm at each site. The DC strategy aimed to conserve drugs through episodic use of ARVs for the minimum time to maintain $CD4^+$ T cell counts >250 cells/μL. Patients in the DC arm were required to re-initiate therapy once their $CD4^+$ T cell count fell below 250 cells/μL. The VS strategy was aimed at suppressing HIV RNA levels as much as possible, immediately following randomization and throughout follow-up, irrespective of the $CD4^+$ T cell count. Patients in the VS arm were expected to undergo a treatment change whenever appropriate in order to keep the HIV RNA below the limit of detection. The primary endpoint of the SMART study was clinical HIV-related disease progression or death.

2.1.3.3. Sample size

It was determined that the trial should randomise (1:1) 6000 patients into two treatment arms, assuming 80% power, a significance level of 0.05 and an annual loss-to-follow-up rate of 2%. The trial was powered to detect a 20% difference in the risk of HIV progression or death between treatment arms, assuming that disease progression would account for 70% of the end-points and death the other 30%. The sample size of 6000 was calculated after varying the assumptions for the expected cumulative rate of disease progression in the control arm (i.e. the viral suppression arm) – see Table 2.1.3.3.1.

Table 2.1.3.3.1: Sample size considerations for the SMART study

Sample Size Required for the SMART Study:

Influence of Hypothesized Treatment Difference
(expressed as a hazard ratio) and Control Group Event Rate*
(expressed as cumulative percent after 5 years)

	Cumulative 5-Year Event Rates		
Hazard Ratio	7.5%	10%	12.5%
0.85	7,600	5,800	4,700
0.80	5,400	4,100	3,300
0.75	3,400	2,600	2,100

* assumes increasing hazard after year 2; 3-year enrollment; 6 years minimum follow-up; 2% loss-to-follow-up per year

2.1.3.4. Follow-up

Patients were to be seen at 1, 2, 4, 6, 8, 10 and 12 months following randomisation and every 4 months thereafter for follow-up data collection visits. During follow-up the following procedures were to be performed at each visit: clinical evaluation, blood safety analyses, viral load and $CD4^+$ count (Table 2.1.3.4.1).

Table 2.1.3.4.1: Clinical Evaluations in SMART

Evaluation	**Screening visit***	**Follow-up visits to year 1 (months)**							**Follow-up visits, years 2 to 9 (every 4 months)**		
		1	**2**	**4**	**6**	**8**	**10**	**12**	**4**	**8**	**12**
Informed consent	X										
Entry criteria	X										
Demography	X										
CDC classification	X										
Any supplemental diagnoses	X	X	X	X	X	X	X	X	X	X	X
Height	X	X	X	X	X	X	X	X	X	X	X
Weight	X	X	X	X	X	X	X	X	X	X	X
Concurrent medications	X	X	X	X	X	X	X	X	X	X	X
HIV-associated conditions	X	X	X	X	X	X	X	X	X	X	X
Adverse and serious adverse events	X	X	X	X	X	X	X	X	X	X	X

* Within 45 days before randomisation

2.1.3.5. Data collection

To evaluate whether resistance emergence was reduced in the DC arm compared to the VS arm in the SMART trial, plasma samples were collected at baseline and at various time points during follow-up when a patient had HIV RNA >1000 copies/mL or when the site chose to perform a resistance test (Table 2.1.3.5.1). Resistance testing was recommended for all patients who had to undergo a treatment change. Plasma was collected and stored at the corresponding time points so that it could be used for retrospective evaluation of genotypic resistance patterns. Resistance testing was performed on site, and repeated centrally where necessary.

If antiretroviral therapy is discontinued for a prolonged period of time the selective pressure for the emergence of resistant strains associated with the treatment regimen will be removed. In addition, any individual who defers therapy will not be at risk of mutations emerging because there will be a lack of selective pressure. As a result we would expect to see fewer mutations emerging in patients randomised to the DC arm of SMART compared to the VS arm.

Table 2.1.3.5.1: Data included in this thesis from the SMART study

Baseline data:	
Demographic:	Gender Race Date of birth Mode of HIV infection Body Mass Index
Clinical:	Date and diagnosis of any HIV-related diseases (CDC category B or C)
Laboratory markers:	Date and value of $CD4^+$ lymphocyte counts Date and value of $CD4^+$ nadir Date and value of plasma viral load
Treatment information:	Type and duration of all prior medications: **NRTIs:** AZT, ddI, 3TC, FTC, ddC, d4T, ABC, TDF, combivir and trizivir; **NNRTIs:** NVP, DLV, EFV; **PIs:** SQV (hard gel formulation), SQV (soft gel formulation), IDV, RTV (<400 mg/dose), RTV (≥400 mg/dose), NFV, AMP, LPV, ATV, TPV; **Other:** T-20
Resistance data:	Date of baseline resistance test and nucleotide sequence obtained Date of follow-up resistance test and nucleotide sequence obtained Genotypic data: mutations that are present in the dominant viral species, including data on the gene location (i.e. RT or PR), the codon position, the amino acid present at a particular position, and information on whether the mutation is present in a mixture or on its own. Flags were used to highlight any major mutations (using the most up-to-date IAS-USA listings) and compensatory mutations.

2.2. THE COHORT STUDIES

2.2.1. EuroSIDA

Some of the data analysed in this thesis come from the EuroSIDA study, a prospective observational cohort of 14262 patients with HIV infection (January 2008). EuroSIDA is one of the largest international cohort studies, so far spanning 93 centres across 31 European countries, Israel and Argentina.

2.2.1.1. Study design

EuroSIDA was initiated as a prospective observational cohort study as a follow-up study to the AIDS in Europe study, after marked differences in survival across Europe were demonstrated[305]. EuroSIDA began enrolment in May 1994 and includes information on patients in the pre-cART era, the early cART era and now some of the same patients are still under follow-up in the late-cART era. It is guided by a Steering Committee consisting of different regional representatives. EuroSIDA is sponsored by the European Commission BIOMED 1 (CT94-1637), BIOMED 2 (CT97-2713), the 5th Framework (QLK2-2000-00773) and the 6th Framework (LSHP-CT-2006-018632) programs. Current support also includes unrestricted grants by Bristol-Myers Squibb, GlaxoSmithKline, Roche, Gilead, Pfizer, Merck and Co., Tibotec and Boehringer-Ingelheim. The participation of centres from Switzerland was supported by a grant from the Swiss Federal Office for Education and Science. It is co-ordinated by CHIP, Copenhagen, Denmark, receives statistical support from the statistical centre at the Royal Free and University College Medical School, London and receives virological support from two virology laboratory groups, one based in London, UK and the other in Badalona, Spain. Permission to perform any analyses on EuroSIDA data is given by a Scientific Committee containing epidemiologists, clinicians and statisticians.

2.2.1.2. Patients and methods

To date, EuroSIDA has enrolled seven cohorts of consecutive HIV infected patients, aged 16 years or over, with consecutive, pre-booked clinic appointments in one of 93 clinical centres across 31 European countries, Israel and Argentina. Patients are enrolled until a predefined number are attained from each centre. Cohort I recruited 3116 patients from May 1994; Cohort II enrolled 1365 patients from November 1995; Cohort III, 2841 patients from February 1997; Cohort IV, 1225 patients from January 1999; Cohort V, 1223 patients from November 2001; Cohort VI, 2121 from November 2003; and Cohort VII, 2419 from January 2006. For cohorts I-III, eligible patients were those with a $CD4^+$ T cell count <500 cells/μL in the previous four months. The $CD4^+$ T cell count restriction was removed for the later cohorts.

2.2.1.3. Follow-up data collection

Information is collected from patient charts and by patient interview onto a standardized data collection form at the first visit (baseline) and every six months thereafter. At baseline, clinical and laboratory markers such as haemoglobin, platelets, leukocytes, total lymphocyte counts, absolute and percent $CD4^+$ count, plasma HIV RNA and AST and ALT levels are measured and the following demographic variables collected: date of birth, gender, race, country of origin, and transmission category. HIV infection is confirmed by a positive HIV-antibody test result. At each visit, details on all $CD4^+$ T cell counts and HIV RNA levels measured since the last follow-up are collected. The dates of starting and stopping each antiretroviral and each prophylactic drug are recorded. Dates of diagnoses of all AIDS-defining illnesses are recorded, including re-current or new events made subsequent to the initial diagnosis, using the 1993 clinical definition of AIDS from the Centers for Disease Control[37].

The database is updated every six months, unless a patient experiences a clinical event, hospitalisation, treatment initiation or interruption, or death - in which case this information is recorded as soon after the event as possible. Members of the co-ordinating office visit all centres to facilitate correct patient selection and to verify accurate data collection, through regular monitoring according to a set of standard operating procedures.

Plasma is collected and stored at least every six months for resistance testing; patients generally undergo genotypic resistance testing when they initiate a new regimen or prior to initiation of their first cART regimen. The EuroSIDA virology group is coordinated by Dr. Bonaventura Clotet and contains other *ad hoc* virologists from participating sites in the EuroSIDA Study. The intention of the virology group is to investigate the clinical and virological efficacy of different therapeutic strategies and to reduce morbidity and mortality related to HIV infection by using antiretroviral combinations that are able to control plasma viral replication. The group utilises several techniques directed to the evaluation of genotypic and phenotypic resistance, and they also analyse the behaviour and replication capacity of viruses harbouring one or several mutations in the RT and PR genes.

This thesis concentrates on the data items collected up to April 2007 shown in Table 2.2.1.3.1.

Table 2.2.1.3.1: Data included in this thesis from the EuroSIDA study

Baseline data:	
Demographic:	Gender Race Date of birth Mode of HIV infection Region of origin Body Mass Index
Clinical:	Date of HIV diagnosis Stage of HIV infection according to CDC classification Date and diagnosis of any HIV-related diseases (CDC category B or C)
Laboratory markers:	Date and value of $CD4^+$ lymphocyte counts Date and value of $CD4^+$ nadir Date and value of plasma viral load
Treatment information:	Type and duration of all medications used: **NRTIs:** AZT, ddI, 3TC, FTC, ddC, d4T, ABC, TDF, combivir and trizivir **NNRTIs:** NVP, DLV, EFV **PIs:** SQV (hard gel formulation), SQV (soft gel formulation), IDV, RTV, NFV, AMP, LPV, ATV, TPV **Other:** T-20
Resistance data:	Date of baseline resistance test and nucleotide sequence obtained Date of follow-up resistance test and nucleotide sequence obtained Genotypic data: mutations that are present in the dominant viral species, including data on the gene location (i.e. RT or PR), the codon position, the amino acid present at a particular position, and information on whether the mutation is present in a mixture or on its own. Flags were used to highlight any major mutations (using the most up-to-date IAS-USA listings) and compensatory mutations.

2.2.2. The UK CHIC study

The UK Collaborative HIV Cohort study (The UK CHIC study) is a collaboration of ten of the largest HIV clinics in the UK. Eight of these centres are situated in London (Chelsea and Westminster, King's College Hospital, Mortimer Market Centre, St. Mary's Hospital, The Royal Free Hospital, Barts and the London NHS trust, Homerton and North Middlesex), one in Brighton (Brighton and Sussex University Hospital) and one in Edinburgh (Edinburgh hospital). Currently the UK CHIC database contains information on 29,055 HIV-infected patients in the UK.

2.2.2.1. Study design

The study began in 2001 with the aim of establishing a large clinical follow-up study of HIV infected patients in the UK. Routinely collected data on patients attending one of the ten clinical HIV centres after 1 January 1996 were retrospectively and prospectively merged into a single database.

The ongoing objectives of CHIC are to: monitor and describe changes over time in the frequency of AIDS-defining illnesses and survival; describe the uptake of and response to cART and; identify factors associated with virological and immunological response to cART. Since the number of new infections in the cohort reflects new infections in the UK, and there have been an increasing number of women and heterosexuals recruited to the study in recent years, the UK CHIC population is generally representative of all HIV-infected patients seen for care in the UK. Since it is crucial to examine the impact of resistance (both transmitted and that acquired whilst on treatment) on virological response, links between the UK CHIC study and the UK Collaborative Group on HIV Drug Resistance are essential. The UK CHIC study is closely linked to the UK HIV Drug Resistance Database, a database set up in 2001 in order to collect information on all routinely performed resistance tests in the UK (see section 2.3.1).

2.2.2.2. Patients and methods

HIV positive patients, aged 16 years or older, who attended at least one of the participating centres in 1996 or thereafter, were included in the UK CHIC study. Patients may have attended more than one centre for care so could have duplicate records between centres. All patients were matched on the basis of their soundex code, date of birth in addition to other clinical information and all likely duplicates were flagged. These were electronically and manually checked and if a patient was identified as a duplicate, one composite record for this patient was included in the UK CHIC database. Other methods to identify potential duplicates were also employed[306].

2.2.2.3. Data collection

Loss-to-follow-up rates in the UK CHIC study are kept as low as possible over time, nonetheless some patients become lost. Death registers from the Office for National Statistics (ONS) for England and Wales, and the General Registrar Office for Scotland (GRO) are used to ascertain whether patients are reported to have died, and if so, the database is updated with this information and each centre is informed of the death.

Each centre provides electronically formatted data in specified data sets: demographic information; AIDS diagnoses and deaths; laboratory data (absolute and percent $CD4^+$ counts, HIV RNA levels, haemoglobin, platelets etc) and antiretroviral treatment history (start and stop dates as well as reasons for stopping treatment and treatment-limiting toxicities). Clinical data and treatment data are updated annually at all of the centres.

2.3. OTHER DATABASES

2.3.1. The UK Collaborative Group on HIV Drug Resistance

The UK Collaborative Group on HIV Drug Resistance is a collaboration between laboratories performing HIV resistance tests in the UK, clinical sites using resistance testing, academic specialists in HIV drug resistance, the MRC Clinical Trials Unit, UCL Centre for Virology, the UK CHIC study, the Health Protection Agency and the Department of Health. Understanding HIV drug resistance in the clinical setting helps to improve clinical management of HIV-infected patients, and since HIV resistance is a complex field, involving a number of different mutations at a number of different sites all with different prevalence rates, combining resistance data from different sources is important to increase the number of mutations that can be investigated.

There is a high level of overlap between the UK CHIC centres and the centres who contribute data to the UK HIV Drug Resistance Database. Collaborators come from the following centres: the Royal Infirmary of Edinburgh; Gartnavel General Hospital; the Health Protection Agency; Guy's and St. Thomas' NHS Foundation Trust; Brighton and Sussex University Hospital; King's College Hospital; the Royal Free Hospital; Southmead Hospital; Leeds Teaching Hospital; St. Marys Hospital; University of Edinburgh; St. Bartholemews Hospital; Chelsea & Westminster Hospital; Manchester Royal Infirmary; and Mortimer Market Centre. Data collection is co-ordinated by the MRC Clinical Trials Unit.

2.3.1.1. Study design – The HIV Drug Resistance Database

The collaboration began in 2001, alongside the UK CHIC study, with the aim of establishing a large HIV drug resistance database containing sequence data on resistance tests performed as part of routine clinical care in the UK. This database contains the results of over 30,000 resistance tests from approximately 21,000 patients. There are a total of 6195 (21.3%) patients in the UK CHIC study who have resistance data in the UK HIV Resistance Database

The objectives of the collaboration are to use data from the HIV Drug Resistance Database to: estimate the prevalence of drug resistance in recent and untreated infections within the UK and describe changes over time; to describe the pattern of drug resistance in patients failing therapy; and to assess the effect of specific mutations (in the context of specific drug regimens) on virological response. The collaboration is currently funded by the Department of Health to provide surveillance data on the extent of HIV drug resistance in treated and untreated patients.

2.3.1.2. Patients and methods

Approximately 80% of all routine resistance tests performed in the UK are included in the HIV Drug Resistance Database.

2.3.1.3. Data collection

All laboratories provide the results of the resistance tests (as full nucleotide sequences) performed in the previous calendar year for all of their patients. Sequences are processed via programs developed by Stanford University and adapted by the Centre for Virology, UCL. In brief, each input nucleotide sequence is aligned to an amino acid reference sequence (i.e. HXB2 – consensus B subtype). The inferred mutations are then fed into Stanford's HIVDB algorithm, which is a set of rules for determining the relative resistance to all available antiretrovirals[220]. Along with each resistance test result (genotypic result and sequence data where available), data are collected on patient demographics, clinical details, antiretroviral treatment history and laboratory markers at the time of the resistance test.

The clinical information that can be linked to this resistance data is obtained from the UK CHIC study, and from the UK Register of HIV Seroconverters. A collaboration between the UK CHIC study and the UK Collaborative Group on HIV Drug Resistance was set up to monitor the incidence of resistance over time in relation to the treatment received, and to examine the impact of resistance (both transmitted and that acquired whilst on treatment) on virological response. There is substantial overlap between

patients in the two databases, and thus the clinical information is co-ordinated between the two studies. This thesis concentrates on the data items from the UK CHIC study and from the UK Resistance Database shown in Table 2.3.1.3.1.

Table 2.3.1.3.1: Combined data from the UK CHIC and the UK Resistance Database

Baseline data:	
Demographic:	Gender Race Date of birth
Clinical:	Stage of HIV infection according to CDC classification Date and diagnosis of any HIV-related diseases
Laboratory markers:	Date and value of $CD4^+$ lymphocyte counts Date and value of $CD4^+$ nadir Date and value of plasma viral load
Treatment information:	Type and duration of all medications used: **NRTIs:** AZT, ddI, 3TC, FTC, ddC, d4T, ABC, TDF, combivir and trizivir **NNRTIs:** NVP, DLV, EFV **PIs:** SQV, IDV, RTV (<400 mg/dose), RTV (≥400 mg/dose), NFV, AMP, LPV, ATV, TPV **Other:** T-20
Resistance data:	Date of baseline resistance test and nucleotide sequence obtained Date of follow-up resistance test and nucleotide sequence obtained Genotypic data: mutations that are present in the dominant viral species, including data on the gene location (i.e. RT or PR), the codon position, the amino acid present at a particular position, and information on whether the mutation is present in a mixture or on its own. Flags were used to highlight any major mutations (using the most up-to-date IAS-USA listings) and compensatory mutations.

CHAPTER 3: ACCUMULATION OF NEW PI-SPECIFIC MUTATIONS IN PATIENTS ON PI/rs

3. Introduction

Even though resistance mutations for unboosted PIs were widely documented at the time of performing the analysis described in this chapter, there was little information on resistance among patients receiving a ritonavir-boosted protease inhibitor (PI/r)[307-309]. Resistance mutations may evolve at different rates in patients treated with boosted PIs compared to those treated with unboosted PIs, so the rates of occurrence of protease mutations and their impact on virological response may need to be updated for patients receiving a PI/r. Updating the interpretation systems to account for the use of ritonavir-boosting may be an important consideration for the older PIs, such as indinavir and saquinavir, both of which were initially used in their unboosted form but are now more commonly boosted with ritonavir. However, the main PIs that are currently prescribed include lopinavir and darunavir – both of which are, and have always been, combined with low-dose ritonavir. Therefore, updating the systems to account for the use of ritonavir boosting is less of an issue in the current era. Since many of the boosted PIs share the same characteristics as each other, much of the information we can obtain from exploring these older PI/rs may still be relevant for the newer PI/rs.

In general, patients receiving a PI/r experience less resistance evolution in their viral populations to those receiving an NNRTI because of the higher genetic barrier of PI/rs (section 1.6.2)[310]. In a study by Phillips *et al*, the risk of a PI mutation emerging in the viral populations of patients starting a PI/r containing regimen was significantly lower than the risk of an NNRTI mutation emerging in the viral populations of those starting an NNRTI-containing regimen (relative hazard 0.31, 95% CI: 0.15 to 0.61)[311]. However, new protease mutations were shown to emerge more rapidly than TAMs in adherent patients who remained on stable treatment with a thymidine analogue and/or protease inhibitor after the onset of virologic failure – which may also relate to the genetic barrier of these classes of drugs[256].

3.1. Aims of the chapter

In this chapter I will identify predictors of the risk of virological failure in patients receiving a PI/r containing regimen. I will then examine the presence of primary PI mutations in the predominant virus of patients at the time of virological failure, and explore the emergence of PI-mutations in the predominant virus of patients who experience virological failure whilst on a PI/r. To do this, I focus on new mutations that occur along the protease (PR) section of the *pol* gene in the predominant viral

population of patients enrolled into three 48-week, international, multi-centre, clinical trials (MaxCmin1, MaxCmin2 and COLATE).

3.2. Literature review

As described in section 1.6.5.1 the rate of acquiring new drug resistance mutations varies according to the individual (i.e. age, adherence levels, history of exposure to ARVs, HIV RNA levels and the number of resistance mutations already present in the virus are contributing factors). Some factors, such as adherence rates, can be modified in order to reduce the amount of resistance, but drug resistance mutations can still emerge over time even if a patient has perfect adherence[312]. Although it is unlikely that resistance mutations will emerge in the virus population of patients who start cART with ≥3 active drugs, accumulation of resistance can occur in the case of sub-optimal therapy.

The emergence of resistance to HIV drugs essentially depends on:

i) The extent of viral replication (i.e. the replication capacity of the virus[58;170;313;314], the viral fidelity and HIV RNA levels[58;315])

ii) The drug levels of the ARVs in the regimen (including adherence rates[179] and pharmacological factors that determine drug absorption rates[314]) and

iii) The genetic barrier (including the number of mutations that are already present in the virus and the number of mutations that are required in order for the virus to become more resistant to an ARV or to a regimen).

The rate of acquiring new mutations increases with the number of mutations that are already present in the virus[183;316]. However, a ceiling effect exists because only a finite number of mutations can emerge. Once all possible mutations have emerged no new mutations can arise because the resistant genotype is already present in the virus population. If a patient changes an ARV in their regimen, some mutations may disappear from the predominant virus (i.e. they are archived in more minor sub-species), and other mutations may appear because there is a change in the dominant virus due to the drug selection pressure. Not all changes that occur to the virus will necessarily be to a more resistant virus[179].

Patients with ongoing viraemia, who are on a stable antiretroviral regimen, are likely to have drug resistance mutations in their predominant virus populations. In these patients the incidence rate of acquiring new drug resistant mutations has been shown to vary between 0.5 to 3.5 new mutations per person-years of follow-up[254;288;312;317]. In a recent analysis conducted on patients from the Swiss HIV cohort study who were

virologically failing a cART regimen, resistance mutations were found in the viral populations of 84% (95% CI: 75% to 92%) of patients failing a PI, 30% (95% CI: 12% to 54%) of patients failing a PI/r, and 66% (95% CI: 49% to 80%) of patients failing an NNRTI, demonstrating differences in the rate of accumulating new mutations between regimens[318]. Low levels of resistance have been identified in the virus populations of patients who are failing the newer PI/rs (tipranavir or darunavir), and also among those failing the older PIs (saquinavir/r, indinavir/r and lopinavir/r) when used in their boosted form[319-323].

In a study looking at resistance among patients who were failing a saquinavir/r containing regimen, two out of eight patients had a virus in which a new PR mutation emerged over 4.8 person-years of follow-up (i.e. 0.4 new mutations per person-years of follow-up)[319]. Overall, in the viral populations of patients receiving one of the three PI/rs investigated in this chapter (i.e. saquinavir/r, indinavir/r or lopinavir/r) the rate of accumulating new PR mutations has been estimated to vary from 0.0 to 1.4 new PR mutations per person-years of follow-up. The most common PR resistance mutations to occur in patients who failed a PI/r containing regimen were at codon positions 10, 36 or 84 for saquinavir/r; 46 or 84 for indinavir/r; and 32, 33, 35, 46, 63 or 82 for lopinavir/r[258;310;319;320;324].

3.3. Methods

3.3.1. Chapter description

The analysis in this chapter investigates the presence of mutations at virological failure and mutations that emerge along the PR section of the HIV *pol* gene, because this is the area targeted by PI/rs. The mutations of interest are primary PI mutations, as listed in the IAS-USA 2006 drug resistance guidelines[233], as well as all other PR mutations. In PI-experienced patients, the PR section of the *pol* gene was sequenced at baseline (i.e. the time the PI/r was started) and at the time of failure to identify new mutations that emerged during follow-up, possibly due to the failing PI/r.

PI-naïve patients have a virus that exhibits a higher genetic barrier to PIs than PI-experienced patients, because they will generally have fewer mutations in their virus population at the start of the PI (i.e. usually no primary PI mutations) and hence more PR mutations will be required in order to confer resistance to any particular PI. In PI-naïve patients without a baseline resistance test I assumed there were no drug resistant mutations present in their virus at baseline and that all PR mutations observed at failure arose during follow-up. Since the risk of transmitting PI-resistance is low, this is a reasonable assumption to make[176;325;326].

The approach used to detect mutations in the virus populations of these patients does not capture all of the PR mutations in the more minor sub-species, and it does not account for shifts in the circulating virus when a new cART regimen is initiated (i.e. a change in the dominant viral sub-species after a new regimen is started). However, it can still be used to examine resistance emergence in the dominant viral species to some extent. Since the emergence of new mutations can influence the subsequent choice of therapy and consequently have an effect on clinical progression in patients who are failing therapy, it is necessary to examine all mutational changes that occur to the virus[190;327].

3.3.2. The MaxCmin trials

This analysis investigates resistance emergence in MaxCmin1, MaxCmin2 and COLATE combined. Combination of the two MaxCmin trial populations is justified because the studies had identical inclusion/exclusion criteria, the same enrolment procedures, the same data collection instruments and similar analytical methods[299;300]. Similarly, data collection and follow-up visits occurred at the same time points in the MaxCmin and COLATE trials, so patients from COLATE were included in these comparisons to increase numbers. The definitions of virological failure vary between the three trials and are outlined in 2.1.1.5.1 and 2.1.2.2.1.

To enable us to explore patients who experienced virological failure using a consistent definition of failure among the MaxCmin trials I re-ran the MaxCmin2 definition of failure on the combined trials population.

In MaxCmin2, virological failure was considered to be:

- For patients entering the study with a viral load <200 copies/mL, an HIV RNA value (confirmed) ≥200 copies/mL
- For patients entering the study with a viral load ≥200 copies/mL:
    1. any rise in HIV RNA of ≥ 0.5 logs (confirmed) and / or
    2. a viral load (confirmed) of:
       at Week 4: < 0.5 log reduction from baseline if > 200 at Week 4
       at Week 12: < 1.0 log reduction from baseline if > 200 at Week 12
       at Week 24: an HIV RNA ≥ 200 copies/mL

MaxCmin1 and MaxCmin2 together represent a case series of 630 patients taking cART. More than 300 patients received a bi-daily (b.i.d) SQV/r regimen and the remaining MaxCmin patients received either IDV/r or LPV/r.

3.3.3. The COLATE trial

Patients in COLATE who started a PI/r that was used in either of the MaxCmin studies (i.e. SQV/r, IDV/r or LPV/r) were included in these comparisons. Even though COLATE patients had different entry criteria to MaxCmin patients[280], including additional patients in this chapter increases the power of our analysis. Furthermore, there was already a large amount of heterogeneity among patients in the MaxCmin trials, so combining all three trial populations provides us with more power to identify factors associated with more rapid resistance accumulation in a more heterogeneous sample.

Patients in COLATE are followed up as frequently as MaxCmin patients (i.e. at baseline, and after 4, 12, 24, 36 and 48 weeks of follow-up) but they have more resistance data available than patients in the MaxCmin trials. In COLATE, resistance tests were performed whenever a patient had an HIV RNA > 500 copies/mL whereas in the MaxCmin studies, resistance tests were only performed at baseline and at the time of virological failure (if HIV RNA > 500 copies/mL). I re-ran the MaxCmin2 definition of failure on the COLATE patients and considered the resistance test taken closest to the time of failure to reflect the actual resistance profile at the time of failure. All three trials included patients who were ARV-experienced, PI-experienced as well as ARV-naïve.

3.3.4. Treatment failure

Protocol-defined treatment failure was the primary outcome of the MaxCmin trials. This definition included observed virological failure, withdrawing consent, loss to follow-up and death. In this chapter I focus solely on patients who experienced observed virological failure because most of these patients will have paired resistance tests – at baseline and at the time of failure (see 3.3.2). So a caveat of interpretation is that this chapter only investigates accumulation of resistance in the viral populations of patients with detectable viral load at baseline and at some point in follow-up (i.e. at failure) and not in all patients who started a PI/r.

3.3.5. Mutation emergence:

Mutation emergence is considered to be the emergence of one or more new mutations in the PR gene between baseline and the time of virological failure (defined in one of the below three ways). Mutations that were categorised as being part of a mixture originally, that emerged as a full mutation or were observed as being part of another mixture at the time of failure, were not considered as evolution.

Primary PI mutations are defined by the IAS-USA Drug Resistance Mutations Group (September 2006)[233] as one or more of: D30N, V32I, L33F, M46I/L, I47A/V, G48V, I50L/V, I54L/M, L76V, V82A/F/LT/S, I84V, N88S and L90M.

PI-specific mutations – mutations in bold reflect primary mutations to the PI/r in question, those not in bold reflect secondary PI mutations.

IDV/r mutations are defined by the IAS-USA Drug Resistance Mutations Group (September 2006)[233] as one or more of: L10I/R/V, K20M/R, L24I, V32I, M36I, **M46I/L**, I54V, A71V/T, G73S/A, V77I, **V82A/F/T**, **I84V**, and L90M.

SQV/r mutations are defined by the IAS-USA Drug Resistance Mutations Group (September 2006)[233] as one or more of: L10I/R/V, L24I, **G48V**, I54V/L, I62V, A71V/T, G73S, V77I, V82A/F/T/S, I84V, and **L90M**.

LPV/r mutations are defined by the IAS-USA Drug Resistance Mutations Group (September 2006)[233] as one or more of: L10F/I/R/V, K20M/R, L24I, **V32I**, L33F, M46I/L, **I47V/A**, I50V, F53L, I54V/L/A/M/T/S, L63P, A71V/T, G73S, **V82A/F/T/S**, I84V, and L90M.

Any PR mutation

The emergence of a PR mutation is defined to be the emergence of a new amino-acid at any of the entire list of PR codons sequenced, compared to the amino-acids seen in the baseline sequence (i.e. any amino-acid change from HXB2 that was not seen at baseline). The HIV protease is a 99 amino-acid peptide, so if a patient had a virus that contained the same amino-acids, aligned in the same way as HXB2 at baseline, that patient's virus could undergo a maximum of 99 mutational changes between baseline and virological failure, although not all changes would result in a functional virus.

3.3.6. Laboratory methods

For this analysis, samples were extracted from the central plasma repository based on the following criteria. Baseline samples were sequenced if the viral load was detectable above >500 copies/mL. Samples at the time of virological failure were taken as close as possible to the time of virological failure in the MaxCmin studies (if HIV RNA >500 copies/mL), although the original definitions of virological failure did differ between trials. In COLATE, samples were extracted for resistance testing whenever HIV RNA was >500 copies/mL. Samples from patients fulfilling one or both of these criteria were selected for further analysis.

These samples were shipped on dry ice, in batches, to a central testing facility for protease gene sequencing. For MaxCmin1 and COLATE the testing facility was the International Clinical Virology Centre (ICVC, UK), and for MaxCmin2 it was Advanced Biological Laboratories (ABL, Luxembourg). Nucleotide sequences in each database were collected, quality assured and translated to the corresponding amino acid sequence.

3.3.7. Statistical analysis

The primary objective of this analysis was to summarise the new primary PI mutations that emerged at the time of virological failure and to investigate factors associated with resistance emergence. I included all patients who started a PI/r in any of the three trials and investigated factors associated with virological failure using logistic regression analysis. The subgroup of patients who experienced virological failure and had resistance tests available was then identified in order to investigate resistance emergence in the predominant virus of patients who were failing a PI/r containing regimen.

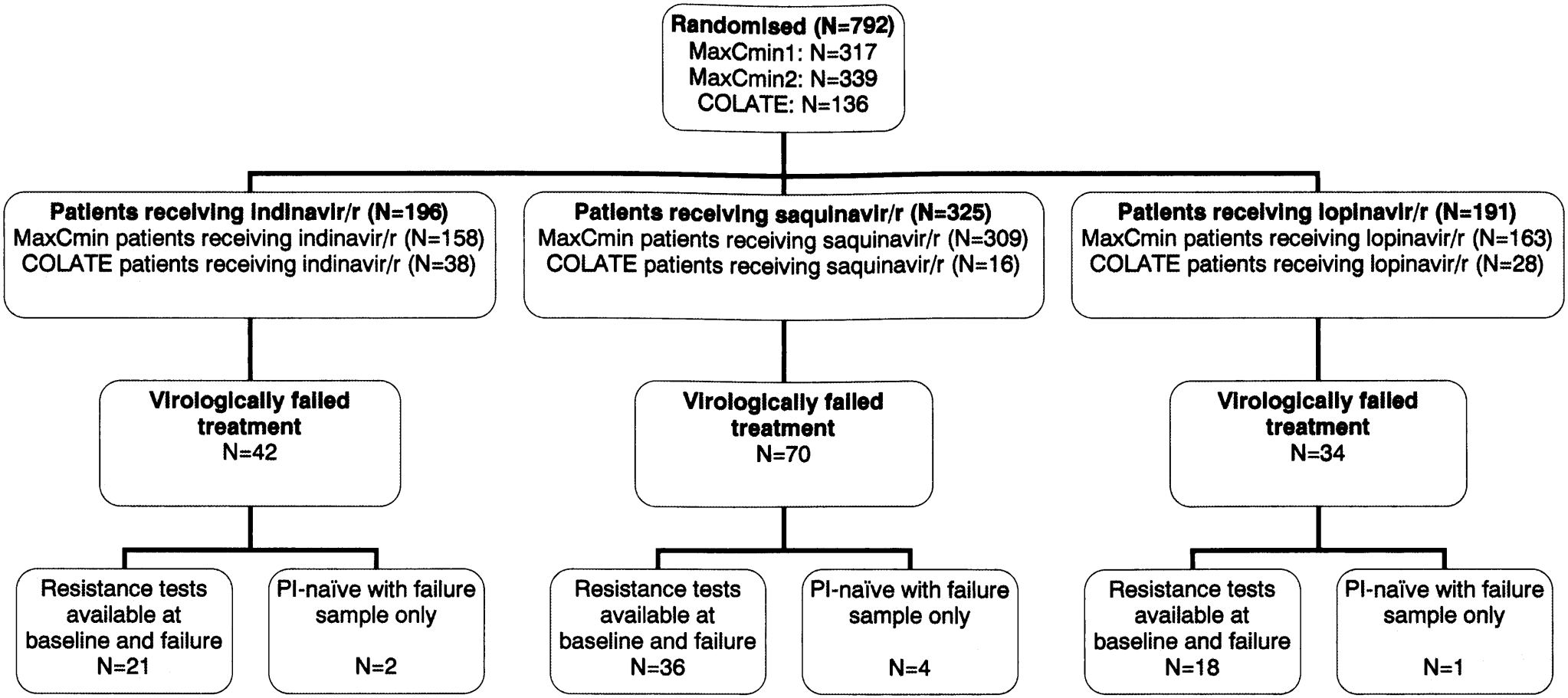
Among patients with resistance data, chi-squared and Fisher's exact tests were used to compare categorical variables at baseline between patients receiving each PI/r. Continuous variables were analysed using analysis of variance analysis (ANOVA) or Kruskal-Wallis tests depending on the distribution. Logistic regression analysis (unadjusted and multivariable) was performed to investigate predictors of virological failure and predictors of resistance emergence (appendix IV). All non-co-linear variables that were significant at the 20% level in the unadjusted analysis were retained for inclusion in the final multivariable models. All statistical analyses were performed using STATA (StataCorp. 2001. Stata Statistical Software: Version 9.2, College Station, Texas, USA).

3.4. Results

3.4.1. Patient flow and disposition

There were 799 patients who were enrolled into the three trials (324 in MaxCmin1, 339 in MaxCmin2 and 136 in COLATE). A total of 663 patients were enrolled into the MaxCmin trials and of these 630 initiated a PI/r. The other 33 patients did not initiate their randomised PI/r and were not included in the comparisons. In COLATE, there were 38 patients who received IDV/r, 16 who received SQV/r and 28 who received LPV/r. Overall, a total of 712 patients initiated a PI/r in the three trials: 196 (27.5%) patients initiated IDV/r, 325 (45.6%) received SQV/r and 191 (26.8%) received LPV/r (Figure 3.4.1.1).

Figure 3.4.1.1: Disposition of patients in this chapter



Of the 712 patients who started a PI/r in any of the trials: 562 (78.9%) were male, 349 (49.0%) were infected through homosexual contact and 246 (34.6%) through heterosexual contact, 564 (79.2%) were white and 220 (30.9%) had a CDC stage C disease at baseline. The majority of patients were ARV-experienced at baseline (N=530, 74.4%), but fewer were PI-experienced (N=420, 59.0%). PI-experienced patients had been exposed to a median (IQR) of one (one to two) PIs prior to entry, for a median (IQR) duration of 3.2 (1.8 to 4.3) years. At baseline the median (IQR) HIV RNA was 4.9 (4.3 to 5.5 $\log_{10}$ cps/mL) for PI-naïve patients and 3.2 (1.7 to 4.5 $\log_{10}$ cps/mL) for PI-experienced patients. Overall, the median (IQR) $CD4^+$ T cell count was 268 (125 to 431 cells/μL).

A total of 146 patients experienced virological failure in the combined trials using the MaxCmin2 definition of failure (54 in MaxCmin1, 70 in MaxCmin2 and 22 in COLATE). In unadjusted logistic regression analysis, the specific PI/r used was not associated with virological failure. After adjustment, in a multivariable logistic regression analysis, there was still no association with the PI/r, but there was a strong association between the use of ARVs prior to study entry and the odds of virological failure (odds ratio: 4.73 for patients with previous ARV exposure compared to those who are ARV-naïve; 95% CI: 2.73 to 8.20, $p<0.0001$) and between the baseline HIV RNA level and the odds of virological failure (OR: 1.69 per $\log_{10}$ cps/mL higher; 95% CI: 1.41 to 2.03, $p<0.0001$). Other predictors of virological failure are outlined in Table 3.4.1.1.

It was not possible to include all patients who failed their PI/r in the analyses investigating the emergence of resistance mutations. Ten (6.8%) patients had an HIV RNA < 500 copies/mL at baseline so did not have a baseline resistance test available. In 23 of the remaining 136 (16.9%) patients, a lack of ability to amplify and sequence the protease gene meant that the baseline resistance test was not available and in 38 of the remaining 113 (33.6%) cases it was not possible to amplify and sequence the protease gene at failure.

So, of the 146 patients who experienced virological failure, 75 (51.3%) had genotypic resistance tests (IDV/r: N=21; SQV/r: N=36; LPV/r: N=18) at both baseline and at the time of failure and an additional seven patients were PI-naïve at baseline and had a failure resistance test only (IDV/r: N=2; SQV/r: N=4; LPV/r: N=1, Figure 3.4.1.1). The seven PI-naïve patients who only had a resistance test at the time of failure were assumed to have a virus that did not contain any mutations at baseline. These patients were combined with the 75 patients with paired resistance tests to give a total study population of 82.

Table 3.4.1.1: Virological failure according to baseline characteristics

The odds of experiencing virological failure, for all three trials combined

Baseline parameter	Unadjusted analysis Odds ratio (95% CI)	P-value	Multivariable analysis Odds ratio (95% CI)	P-value
PI/r:		0.55		0.17
IDV/r	Ref		Ref	
LPV/r	0.79 (0.48, 1.31)		0.64 (0.37, 1.11)	
SQV/r	1.01 (0.65, 1.55)		0.99 (0.62, 1.58)	
ARV naïve (experienced vs naïve)	2.22 (1.36, 3.62)	0.0007	4.73 (2.73, 8.20)	<0.0001
PI-naïve (experienced vs naïve)	1.61 (1.09, 2.36)	0.02	-	-
Gender (female vs male)	1.12 (0.72, 1.74)	0.61	-	-
Age (per 5 years older)	0.95 (0.86, 1.05)	0.29	-	-
Race:		0.02		0.08
White	Ref		Ref	
Black	1.95 (1.23, 3.09)		1.69 (0.97, 2.94)	
Other/unknown	1.06 (0.48, 2.36)		0.66 (0.26, 1.64)	
HIV exposure group:		0.03		0.34
Homo-/bisexual	Ref		Ref	
IVDU	2.19 (1.18, 4.06)		1.93 (0.98, 3.80)	
Haemophiliac/transfusion	1.34 (0.43, 4.15)		1.33 (0.40, 4.47)	
Heterosexual	1.69 (1.12, 2.54)		1.28 (0.78, 2.10)	
Other/unknown	2.14 (0.97, 4.70)		1.79 (0.75, 4.27)	
CDC, cat. C (Y vs N)	1.53 (1.04, 2.23)	0.03	1.70 (1.11, 2.60)	0.01
HIV RNA (per $\log_{10}$ cps/mL higher)	1.36 (1.19, 1.56)	<0.0001	1.69 (1.41, 2.03)	<0.0001
$CD4^+$ count (per 100 cells/µL higher)	0.90 (0.82, 0.98)	0.01	1.02 (0.91, 1.14)	0.76
$CD4^+$ nadir (per 100 cells/µL higher)	0.99 (0.86, 1.14)	0.93	-	-
BMI (per 5 kg/m^2 higher)	0.98 (0.78, 1.25)	0.89	-	-

I assumed that PI-naïve patients without a baseline resistance test had a virus without any baseline mutations in the analysis investigating PI-specific mutations and in the analysis investigating primary PI mutations because the prevalence of transmitted PI resistance is low. However, these patients were excluded from the analysis investigating the emergence of any new PR-mutation (i.e. not only IAS-USA mutations) due to a high prevalence of naturally occurring polymorphisms.

3.4.2. Baseline characteristics

Of the 82 patients with a resistance test at virological failure, 14 (17.1%) were ARV naïve at baseline, 16 (19.5%) PI naïve but ARV experienced and 52 (63.4%) were PI-experienced (Table 3.4.2.1). The 52 PI-experienced patients had been exposed to a median (IQR) of two (one to three) PIs prior to entry, for a median (IQR) duration of 3.0 (1.0 to 4.4) years. Evidence of PI failure in the year preceding baseline (i.e. HIV RNA ≥400 cps/mL) was found in 39 (75.0%) of the 52 PI-experienced patients.

Table 3.4.2.1: Baseline characteristics of the patients included in the analysis

Baseline parameter		IDV/r N=23	SQV/r N=40	LPV/r N=19
Antiretroviral naïve	No. (%)	3 (13.0%)	10 (25.0%)	1 (5.3%)
PI-naïve		5 (21.7%)	8 (20.0%)	3 (15.8%)
PI-experienced with				
Viral load ≥400 copies/mL		15 (65.2%)	20 (50.0%)	15 (79.0%)
Viral load <400 copies/mL		0 (0.0%)	2 (5.0%)	0 (0.0%)
Of PI-experience patients, the no. (%) with PI-failure in the year before entry		9 (60.0%)	19 (86.4%)	11 (73.3%)
CDC, cat. C	No. (%)	10 (43.5%)	14 (35.0%)	9 (47.4%)
HIV RNA (c/mL $\log_{10}$)	Median (IQR)	4.3 (3.5, 5.1)	4.7 (4.0, 5.2)	4.6 (4.0, 5.3)
CD4+ (cells/μL)*	Median (IQR)	260 (115, 379)	236 (104, 334)	233 (32, 359)
CD4+ nadir (cells/μL)	Median (IQR)	107 (43, 170)	99 (31, 227)	47 (20, 180)
Age	Median (IQR)	40 (30, 42)	39 (32, 49)	41 (38, 45)
Gender	No. Male (%)	16 (69.6%)	32 (80.0%)	17 (89.5%)
HIV exposure group	No. (%)			
	Homo-/bisexual	9 (39.1%)	18 (45.0%)	5 (26.3%)
	IVDU	2 (8.7%)	3 (7.5%)	3 (15.8%)
	Haemophilia/transfusion	1 (4.4%)	0 (0.0%)	1 (5.3%)
	Heterosexual	9 (39.1%)	18 (45.0%)	10 (52.6%)
	Other/unknown	2 (8.7%)	1 (2.5%)	0 (0.0%)
Race	No. (%)			
	White	18 (78.3%)	26 (65.0%)	11 (57.9%)
	Black	4 (17.4%)	11 (27.5%)	8 (42.1%)
	Other/unknown	1 (4.4%)	3 (7.5%)	0 (0.0%)
BMI*	Median (IQR)	23.9 (20.8, 26.5)	23.4 (20.6, 26.8)	23.8 (22.3, 25.1)
PI C_{min} level at week 4	Median ng/mL (IQR)	1924 (579, 3482)	1161 (424, 1831)	4684 (891, 7296)
Ritonavir C_{min} at week 4*	Median ng/mL (IQR)	1356 (365, 1719)	348 (142, 955)	217 (39, 301)

* Variables followed by a star indicate missing measurements.

Baseline resistance profiles are only summarised for the 75 patients who have baseline resistance data available. At baseline, 24 (32.0%) patients with a resistance test had a virus containing a primary PI mutation and 73 (97.3%) had a virus containing an amino-acid change from HXB2 (Table 3.4.2.2).

Table 3.4.2.2: Baseline resistance patterns of patients with baseline resistance tests

Baseline resistance:		**IDV/r N=21**	**SQV/r N=36**	**LPV/r N=18**
Primary PI mutations at baseline, (No. (%))	None	14 (66.7%)	26 (72.2%)	11 (61.1%)
	1-2	3 (14.3%)	6 (16.7%)	4 (22.2%)
	>2	4 (19.1%)	4 (11.1%)	3 (16.7%)
Baseline PR differences from HXB2, (No. (%))	None	1 (4.8%)	1 (2.8%)	0 (0.0%)
	1-3	9 (42.9%)	19 (52.8%)	7 (38.9%)
	4-6	5 (23.8%)	8 (22.2%)	4 (22.2%)
	7-9	3 (14.3%)	7 (19.4%)	3 (16.7%)
	>9	3 (14.3%)	1 (2.8%)	4 (22.2%)
Phenotypic sensitivity to indinavir* Median IC_{50} fold↑ (IQR)		1.0 (1.0, 1.5)	-	-
Phenotypic sensitivity to saquinavir* Median IC_{50} fold↑ (IQR)		-	1.0 (1.0, 1.8)	-
Phenotypic sensitivity to lopinavir* Median IC_{50} fold↑ (IQR)		-	-	1.2 (1.0, 2.0)
Phenotypic sensitivity to ritonavir* Median IC_{50} fold↑ (IQR)		1.0 (1.0, 2.0)	1.0 (1.0, 2.4)	1.8 (1.0, 3.0)

*Missing for patients in COLATE and patients in the MaxCmin studies without phenotyping, only available for 57 out of 75 (76.0%) patients.

3.4.3. Follow-up characteristics

Among the 82 patients with paired resistance data at baseline and at virological failure, 59 (71.9%) were still on their baseline PI/r (i.e. their trial PI/r) at the time of failure. There were 63 (76.8%) patients who were receiving a PI/r (not necessarily the trial PI/r) at the time of failure and eight patients (9.8%) who were off antiretrovirals completely (IDV/r: N=4; SQV/r: N=4), yet this was still considered to be virological failure. The remaining 11 patients had switched to a non-PI/r containing regimen before virological failure. The median (IQR) time from baseline to virological failure was 24 (12 to 33) weeks.

Overall, the presence of primary PI mutations was relatively uncommon at the time of virological failure: 23 (28.1%) patients had a primary PI mutation and 80 (97.6%) patients had a PR difference from HXB2 in their predominant virus population at failure (Table 3.4.3.1). Among the 30 patients who were PI-naïve at baseline, two (6.7%) had a virus in which a primary PI mutation was detected and 29 (96.7%) had a virus in which a PR difference from HXB2 was detected, at failure. In contrast, among the 52 patients who were PI-experienced at baseline, 21 (40.4%) had a virus in which a primary PI mutation was detected and 51 (98.1%) had a virus in which a difference from HXB2 was detected at the time of failure.

Table 3.4.3.1: Follow-up characteristics

Follow-up parameter:		IDV/r N=23	SQV/r N=40	LPV/r N=19
Time from baseline to virological failure Med wks (IQR)		25 (13, 46)	24 (12, 30)	23 (11, 25)
On baseline treatment at therapy failure No. (%)		16 (69.6%)	25 (62.5%)	18 (94.7%)
Primary PI mutations at therapy failure: (No. (%))	None	19 (82.6%)	28 (70.0%)	12 (63.2%)
	1-2	0 (0.0%)	8 (20.0%)	4 (21.1%)
	>2	4 (17.4%)	4 (10.0%)	3 (15.8%)
Any PR difference from HXB2: (No. (%))				
	None	1 (4.4%)	1 (2.5%)	0 (0.0%)
	1-3	11 (47.8%)	21 (53.5%)	6 (31.6%)
	4-6	7 (30.4%)	10 (25.0%)	6 (31.6%)
	7-9	1 (4.4%)	4 (10.0%)	2 (10.5%)
	>9	3 (13.0%)	4 (10.0%)	5 (26.3%)

3.4.4. Emergence of primary PI mutations between baseline and failure

Of the 75 individuals who had their virus sequenced at both baseline and virological failure, there were 14 (18.7%) who experienced a change to their primary PI resistance profile. In five cases the change was from a virus, in which a primary PI mutation was detected at baseline, to a virus in which a primary PI mutation could not be detected at the time of failure, using population sequencing. This was regarded as a matter of de-selection. De-selection can occur because most of the patients changed their whole antiretroviral regimen at baseline. So, mutations that occurred due to drug pressure from a previous regimen may no longer be detected because the drug pressure corresponding to the baseline mutations will have been removed. All five of these patients were PI-experienced prior to entry. Three of the five (60.0%) were still on their baseline PI/r at the time of failure, suggesting that de-selection of these mutations due to lack of pressure may not apply in all cases.

From the seven PI-naïve patients with a resistance test at failure only, there was one (14.3%) who had a virus in which a primary PI mutation was detected (assumed not to have been present at baseline). Overall, there were a total of 15 (18.3%) patients who experienced changes to the primary resistance mutations in the dominant virus population and 9 (11.0%) who showed emergence of a new primary PI resistance mutation (Table 3.4.4.1).

Table 3.4.4.1: Individuals who experienced a change to their primary PI mutations

Patient	Primary PI mutations		New mutations gained	Mutations lost	PI-naïve at baseline	On BL-PI/r at failure	On a PI at failure
	Baseline	Failure					
IDV/r							
1500004	M46I; V82L; L90M			M46I; V82L; L90M	No	Yes	Yes
2004112	M46I			M46I	No	No	No
2012105	D30N; L90L/M	D30D/N; I54I/L; L90L/M	I54I/L		No	No	Yes
8000001	M46I; N88S			M46I; N88S	No	Yes	Yes
SQV/r							
1100213	L90M	I84V; L90M;	I84V		No	Yes	Yes
1111203	M46I/M; I84I/V; L90M	I84V; L90M		M46I/M	No	Yes	Yes
1213214		M46I/M; V82A; L90M;	M46I/M; V82A; L90M		No	Yes	Yes
1224202*		L90L/M	L90L/M		Yes	Yes	Yes
1503115	V82A;	V82A; I84I/V;	I84I/V		No	Yes	Yes
1503202	D30D/N; M46I/M; L90L/M;			D30D/N; M46I/M; L90L/M	No	No	No
3003102	D30N; M46I;	D30N; M46I; I84V;	I84V		No	Yes	Yes
5502102		M46I/M;	M46I/M		Yes	No	No
8500102	M46I/M; G48V; V82A/V; L90L/M;	M46I/M; G48V; V82A; I84V; L90L/M;	I84V		No	Yes	Yes
LPV/r							
2500061	M46I/M; V82A/V; L90M	M46I/M; V82A/S; L90M			No	Yes	Yes
2500063	M46I; I84V; L90M	M46I; L76V/L; I84V; L90M	L76V/L		No	Yes	Yes

* PI-naïve at baseline with a failure sample only.

• BI-PI/r refers to the PI/r received at baseline

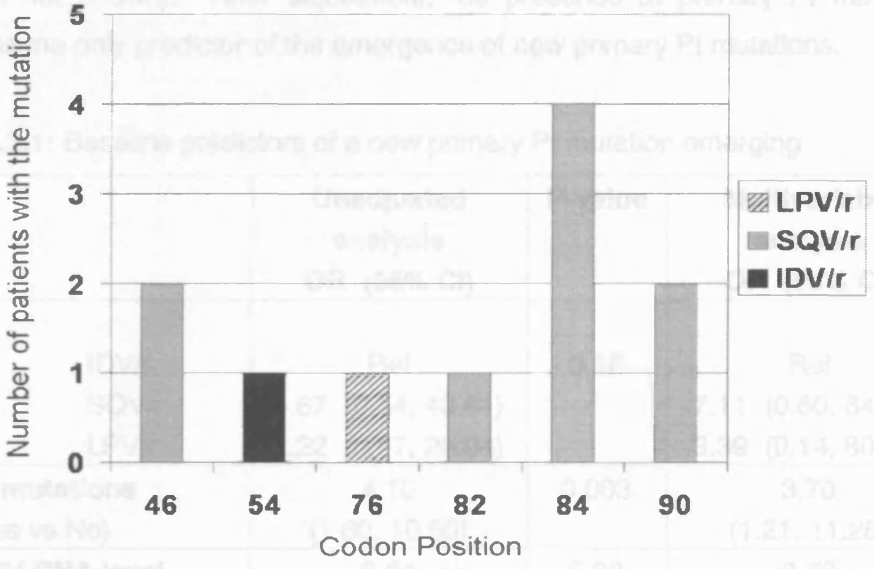
3.4.4.1. New primary PI mutations

Patients included in these analyses have a cumulative follow-up time of 36.9 person years between baseline and the time of virological failure. Over this time period, 11 new primary

PI mutations emerged and ten primary PI mutations disappeared from the dominant virus populations of these patients. This translates to an acquisition rate of 0.30 (0.12 to 0.47) new primary PI mutations per person-years follow-up and a de-selection rate of 0.27 (0.10 to 0.44) primary PI mutations per person-years follow-up. These figures should be interpreted with caution because some mutations may actually be present in the viral subspecies at baseline, but remain undetected because resistance testing methods do not always capture every mutation in the virus populations, especially those that are present in low levels.

Out of the nine patients in whom a new primary PI mutation emerged in their dominant virus population, seven (77.8%) were exposed to PIs prior to baseline and six had primary PI mutations in their predominant virus at baseline (Table 3.4.4.1). Among the new primary PI mutations that emerged at time of failure, emergence at codon 84 was the most frequently observed (Figure 3.4.4.1.1).

Figure 3.4.4.1.1: Emergence of mutations at each codon for patients with a virus that acquired a primary PI mutation



3.4.4.2. Treatment use at virological failure

Seven of the nine (77.8%) patients who experienced emergence of a new primary PI mutation, remained on their PI/r at the time of failure (IDV/r: N=0 (0.0%); SQV/r: N=6 (85.7%); LPV/r: N=1 (100%)). Similarly, 52 of the 73 (71.2%) patients who did not experience primary PI mutation emergence in their viral populations were on their PI/r at the time of failure (IDV/r: N=16 (72.7%); SQV/r: N=19 (57.6%); LPV/r: N=17 (94.4%)). The

proportion of individuals who experienced primary PI mutation emergence in their viral populations, among those receiving their PI/r at time of failure, was 11.9% (7 out of 59).

3.4.4.3. Predictors of primary PI mutation emergence

To identify factors associated with the emergence of a new primary PI mutation a logistic regression model was used. All variables that were associated with the emergence of a new primary PI mutation were identified using the 20% level of significance because of the small numbers. These variables were then entered into a multivariable model and retained in the model if they were still significant ($p<0.20$) after adjustment (Table 3.4.4.3.1). Since logistic regression models are not very stable if there are few events, the results of this model should also be interpreted with caution.

Marginally more patients who received SQV/r or LPV/r acquired a new primary PI-mutation in their predominant virus, compared to patients who received IDV/r; albeit this trend was not significant. Plasma PI levels and plasma ritonavir levels four weeks after PI/r initiation were not significant predictors of the emergence of a new primary PI mutation in unadjusted analysis. PI naivety was not protective against primary PI mutation emergence either (data not shown). After adjustment, the presence of primary PI mutations at baseline was the only predictor of the emergence of new primary PI mutations.

Table 3.4.4.3.1: Baseline predictors of a new primary PI mutation emerging

	Unadjusted analysis OR (95% CI)	**P-value**	**Multivariable analysis OR (95% CI)**	**P-value**
Treatment:				
IDV/r	Ref	0.16	Ref	0.19
SQV/r	4.67 (0.54, 40.61)		7.11 (0.60, 84.88)	
LPV/r	1.22 (0.07, 20.94)		3.39 (0.14, 80.90)	
Primary PI mutations present (Yes vs No)	4.10 (1.60, 10.50)	0.003	3.70 (1.21, 11.28)	0.02
Baseline HIV RNA level (per 1 $\log_{10}$ higher)	0.64 (0.32, 1.27)	0.20	0.79 (0.29, 2.19)	0.65
Baseline CD4$^+$ T cell count (per 100 cell higher)	1.24 (0.93, 1.66)	0.14	1.17 (0.74, 1.86)	0.51
Nadir CD4$^+$ T cell count (per 100 cell higher)	1.19 (0.81, 1.75)	0.37	-	-

3.4.5. Additional analyses

3.4.5.1. Mutations associated with resistance to the baseline PI/r

PI-specific resistance mutations were identified from the IAS-USA (Sept 2006) mutation lists and are defined in section 3.3.5. Of the 82 individuals who were included in these comparisons, 25 (30.5%) had a virus that underwent a change to their PI-specific resistance profile (i.e. either a PI-specific mutation emerged or disappeared from the predominant virus). In 15 (18.3%) of these patients a new PI-specific resistance mutation emerged in the dominant virus population (Table 3.4.5.1.1). All of these patients were still on their baseline PI/r when they experienced virological failure. Since 59 patients overall were on their baseline PI/r at the time of virological failure, the proportion of individuals in whom a PI-specific mutation emerged whilst receiving a PI/r was 25.4% (i.e. 15 out of 59).

Table 3.4.5.1.1: PI specific mutations

Patient	Mutations to the baseline PI/r		New mutations gained	Mutations lost	PI-naïve at BL	On BL-PI/r at failure	On a PI at failure
	Baseline	Failure					
IDV/r							
1500004	**M46I**, A71T, V77I, L90M;	M36I	M36I	**M46I**, A71T, V77I, L90M;	No	Yes	Yes
1501002	L10V, M36I	L10I, M36I			No	Yes	Yes
1501003	L10L/V			L10L/V	No	Yes	Yes
2004112	L10L/V, M36I, **M46I**	M36I		L10L/V, **M46I**	No	No	No
2012105	A71V, V77V/I, L90L/M	V77I, L90L/M		A71V	No	No	Yes
2500059	L10I, I54V, A71V, G73G/S, **I84V**, L90M	A71V, **I84V**, L90M		L10I, I54V, G73G/S,	No	Yes	Yes
8000001	L10V, K20R, M36I, **M46I**	M36I		L10V, K20R, **M46I**	No	Yes	Yes
SQV/r							
1100213	I62I/V, **L90M**	A71V, I84V, **L90M**	A71V, I84V	I62I/V	No	Yes	Yes
1111203	L10F/I/L, I62I/V, A71A/V, V77I/V, I84I/V, **L90L/M**	I62V, A71V, G73S, V77I, I84V, **L90M**	G73S	L10F/I/L	No	Yes	Yes
1213214	I62I/V	L10I, I54V, I62V, A71V, G73S, V77I/V, I82A, **L90M**	L10I, I54V, A71V, G73S, V77I/V, I82A, **L90M**		No	Yes	Yes
1224202	-	**L90L/M**	**L90L/M**		Yes	Yes	Yes
1501103	V77I	L10V, I62V	L10V, I62V	V77I	No	Yes	Yes

Patient	Mutations to the baseline PI/r		New mutations gained	Mutations lost	PI-naïve at BL	On BL-PI/r at failure	On a PI at failure
	Baseline	Failure					
SQV/r							
1503115	L10I, L24I, I54V, I62I/V, A71A/I/V/T, V77I, V82A	L10I, L24I, I54V, I62I/V, A71A/I/V/T, V77I, V82A, I84I/V	I84I/V		No	Yes	Yes
1503202	I62V, **L90L/M**	I62I/V		**L90L/M**	No	No	No
1504102	L10I/L, I54V, **L90M**	I54V, **L90M**		L10I/L	No	Yes	Yes
3003102	L10I/V, A71A/V V77I/V	L10V, A71V, I84V	I84V	V77I/V	No	Yes	Yes
8500102	**G48V**, I54I/V, V77I/V, V82A/V, **L90L/M**	L10I, **G48V**, I54I/V, V77I/V, V82A, I84I/V, **L90L/M**	L10I, I84I/V		No	Yes	Yes
LPV/r							
1101203	L10I, K20K/M, I54V, A71V, **V82A**, L90M	L10I, I54V, A71V, **V82A**, L90M		K20K/M	No	Yes	Yes
1401209	L10I, K20R, M46I, I54V, **V82A**	L10I/V, K20R, L24I/L, M46I, I54V, **V82A**	L24I/L		No	Yes	Yes
2000215		L63P	L63P		Yes	Yes	Yes
2500054	L10I, L24I, M46I, F53F/L, I54V, **V82A**	L10I/F, K20R, L24I, M46I, F53L, I54V, **V82A**	K20R		No	Yes	Yes
2500061	L10F, M46I/M, I54V, L63P, A71A/V, **V82A/V**, L90M	L10F, K20R, M46I/M, I54V, L63P, A71V, **V82A/S**, L90M	K20R		No	Yes	Yes
2500063	L10I, M46I, L63P, A71V, G73S, I84V, L90M	L10I, M46I, I54V, L63P, A71V, G73S, I84V, L90M	I54V		No	Yes	Yes
6500201		L63P	L63P		Yes	Yes	Yes
8500058	L10I/L/V, M46I, F53F/L, I54V, L63P, A71V, **V82A**, L90M	L10I/L, M46I, F53F/L, I54V, L63P, A71V, **V82A**, L90M			No	Yes	Yes

* Mutations in **bold** reflect primary PI-specific mutations (not all primary PI mutations).

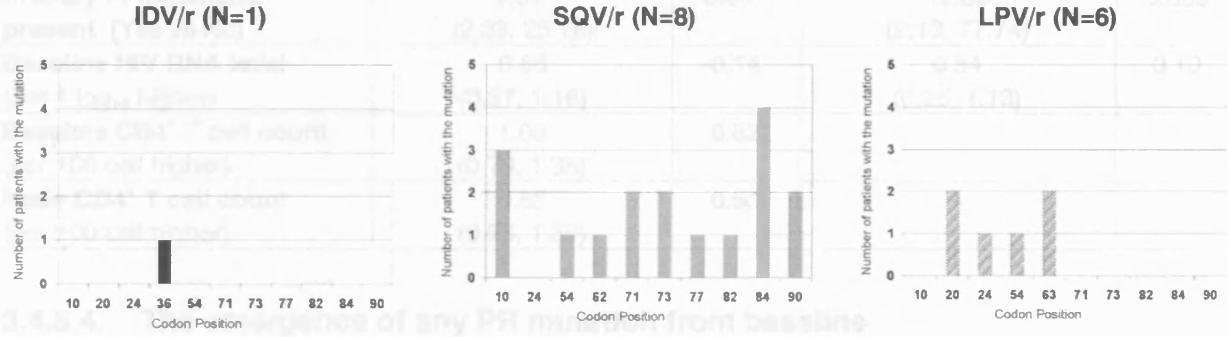
Mutations not in bold refer to secondary PI-specific mutations.

3.4.5.2. The rate of acquisition of new PI-specific mutations (i.e. mutations associated with resistance to the baseline PI/r)

During follow-up, 24 new PI-specific mutations emerged in the dominant virus of 15 patients (1 IDV-specific mutation over 12 person years of follow-up; 17 SQV-specific mutations over 17.7 person years of follow-up; and 6 LPV-specific mutations over 7.2 person years of follow-up). This translates to an overall acquisition rate (95% CI) of 0.65 (0.39 to 0.91) new PI-specific mutations per person-years follow-up. Patients who received SQV/r had the fastest acquisition rate (0.96 (0.50 to 1.41) new SQV-specific mutations per person-years follow-up) and patients who received IDV/r the slowest (0.08 (0.00 to 0.25) new IDV-specific mutations per person-years follow-up). Patients who received LPV/r had an acquisition rate of 0.83 (0.17 to 1.50) new LPV-specific mutations per person-years follow-up.

For the 15 patients in whom a PI-specific mutation emerged, 12 (80.0%) were exposed to PIs prior to baseline and ten (66.7%) had a primary PI mutation in their dominant virus at baseline. The most common site for SQV-specific mutations was at codon 84 (N=4), for LPV-specific mutations it was at codons 20 (N=2) and 63 (N=2) and only one IDV-specific mutation emerged and this was at codon 36 (Figure 3.4.5.2.1).

Figure 3.4.5.2.1: Mutational emergence at each codon, for patients with a virus that acquired a PI-specific mutation



Of note, in three (50.0%) of the six patients who failed an IDV/r containing regimen, who no longer had indinavir-specific mutations in their predominant virus, one of the mutations that was no longer detectable was a primary indinavir-specific mutation (i.e. the M46I mutation in all cases). Only two primary mutations to the PI/r in question emerged in the dominant viral species overall; and both of these were seen in patients treated with SQV/r (i.e. the L90M mutation in both cases). The other 22 mutations that emerged were all secondary PI mutations (for the PI/r the patient was receiving).

3.4.5.3. Predictors of PI-specific mutation emergence

To identify factors associated with the emergence of a new PI-specific mutation, a logistic regression model was used. Since more PI-specific mutations emerged in the dominant virus of these patients than primary PI mutations, results from this logistic regression analysis will be more robust than the model in 3.4.4.3. The odds of a PI-specific mutation emerging in the predominant virus population was lowest for patients who received IDV/r and highest for patients who received LPV/r (Table 3.4.5.3.1). Patients who were PI-experienced prior to entry were at marginally higher odds of experiencing PI-specific mutation emergence in their viral population, but after adjustment for the presence of primary PI mutations at baseline, this trend reversed and was not significant. In this analysis, the presence of primary PI mutations at baseline was the strongest predictor of a PI-specific mutation emerging.

Table 3.4.5.3.1: Baseline predictors of the emergence of a PI-specific mutation

	Unadjusted analysis OR (95% CI)	**P-value**	**Multivariable analysis OR (95% CI)**	**P-value**
Treatment:				
IDV/r	0.18 (0.02, 1.56)	0.05	0.11 (0.01, 1.10)	0.02
SQV/r	Ref		Ref	
LPV/r	1.85 (0.53, 6.38)		2.10 (0.48, 9.16)	
PI-experienced at baseline (Yes vs No)	2.70 (0.70, 10.48)	0.15	0.42 (0.05, 3.22)	0.40
AIDS at baseline (Yes vs No)	1.38 (0.45, 4.26)	0.58	-	-
Primary PI mutations present (Yes vs No)	7.57 (2.23, 25.76)	0.001	12.86 (2.13, 77.74)	0.005
Baseline HIV RNA level (per 1 $\log_{10}$ higher)	0.65 (0.37, 1.16)	0.14	0.54 (0.26, 1.13)	0.10
Baseline $CD4^+$ T cell count (per 100 cell higher)	1.03 (0.79, 1.35)	0.82	-	-
Nadir $CD4^+$ T cell count (per 100 cell higher)	0.85 (0.53, 1.36)	0.50	-	-

3.4.5.4. The emergence of any PR mutation from baseline

This analysis was restricted to patients with both baseline and failure resistance tests and did not include patients with resistance tests at the time of failure only, due to the unknown prevalence of naturally occurring polymorphisms. Of the 75 individuals with baseline and failure genotyping, there were 46 (61.3%) who experienced a change in their predominant virus populations (i.e. any change to an amino-acid in any position); 33 (44.0%) of these patients had a virus in which the change was from a baseline amino-acid that was expected according to HXB2 to a follow-up amino-acid that was not (Table 3.4.5.4.1).

Table 3.4.5.4.1: Emergence of any PR mutation from baseline

Patient	Any PR mutation from baseline		New mutations gained	Mutations lost	PI-naïve at BL	On BL-PI/r at failure	On a PI at failure
	Baseline	Failure					
IDV/r							
1000103	I13V, S37N, I64V, V77I, L90FL	I13V, S37N, I64V, V77I		L90FL	Yes	Yes	Yes
1100102	T12N, S37D, K43R, L63P, L89LM	T12N, S37D, K43R, L63P		L89LM	No	No	No
1500003	V3I, I13V, I15V, M36I, S37DN, K43R, I62V, L63P	V3I, T12ST, I13IV, I15IV, M36I, S37DN, K43R, I62V, L63P	T12ST		No	Yes	Yes
1500004	V3I, T12AT, S37N, M46I, Q58E, L63P, A71T, V77I, V82L, L90M	V3I, I13V, I15V, M36I, S37D, K43R, I62V, L63P	I13V, I15V, M36I, K43R, I62V	T12AT, M46I, Q58E, A71T, V77I, V82L, L90M	No	Yes	Yes
1501002	V3I, L10V, I13V, G16EG, E35D, M36I, S37N, R41K, R57K, L63PS, H69K, L89M	V3I, L10I, I13V, G16E, E35D, M36I, S37N, R41K, R57K, L63S, H69K, L89M			No	Yes	Yes
1501003	V3I, L10LV, T12A, K14KR, S37N, R41K, L63P, I93L	V3I, T12A, S37N, R41K, L63P, I93L		L10LV, K14KR	No	Yes	Yes
1501008	V3I, M36I, S37D, Q61H, H69HQ, I72IV	V3I, I15IV, M36I, S37D, Q61H, I72IMV	I15IV	H69HQ	No	Yes	Yes
1503101	L10IL, K20R, V32I, E35D, M36I, S37D, M46I, I47V, K55R, Q58E, I62IV, L63P, V82A, I93L	L10IL, K20R, V32I, E35D, M36I, S37D, M46I, I47V, K55R, Q58E, L63P, V82A, I93L		I62IV	No	Yes	Yes
2004112	L10LV, I13V, I15V, E35D, M36I, S37N, R41K, M46I, R57K, H69K, L89T	I13V, I15V, E35D, M36I, S37N, R41K, R57K, H69K, L89M		L10LV, M46I	No	No	No
2012105	I13V, K14R, I15V, K20T, D30N, S37N, L63P, I66FI, A71V, I72V, V75IV, V77IV, N88D, L90LM	D30DN, S37N, R41K, I54IL, I62V, L63P, V77I, V82IV, N88D, L90LM, I93L	R41K, I54IL, I62V, V82IV, I93L	I13V, K14R, I15V, K20T, I66FI, A71V, I72V, V75IV	No	No	Yes
2012115	S37N, L63P, I72IV, V77IV, I93L	S37N, L63P, V77IV, I93L		I72IV	Yes	No	No
2500059	V3I, L10I, I13V, L19I, E21DE, L33F, E35D, S37D, I54V, D60E, I62V, L63P, I66F, A71V, I72L, G73GS, I84V, L90M	V3I, L10F, I13V, L19I, E21D, L33F, E35D, S37DE, I54A, D60E, I62V, L63P, I66F, A71V, I72L, I84V, L90M		G73GS	No	Yes	Yes
8000001	V3I, L10V, I13V, K20R, E35D, M36I, S37D, R41K, M46I, R57K, H69K, N88S, L89M	V3I, T12AT, L19I, M36I, S37N, R41K, Q61HQ, L63S, H69K, L89M, I93L	T12AT, L19I, Q61HQ, L63S, I93L	L10V, I13V, K20R, E35D, M46I, R57K, N88S	No	Yes	Yes
8500101	I13V, I15V, K20R, M36I, S37N, R41K, H69K, L89M, I93L	I13V, I15V, K20R, M36I, S37N, R41K, H69K, K70KR, L89M, I93L	K70KR		Yes	Yes	Yes
8500106	S37N, L63P, V77I, I93L	S37N, L63P, I64IM, I72IV, V77I, I93L	I64IM, I72IV		Yes	No	No

Patient	Any PR mutation from baseline		New mutations gained	Mutations lost	PI-naïve at BL	On BL-PI/r at failure	On a PI at failure
	Baseline	Failure					
SQV/r							
1100116	I15V, K20R, M36I, S37D, R41K, K45KR, R57K, Q61N, L63T, I72IT, I93L	I15V, K20R, M36I, S37D, R41K, K45KR, R57K, Q61N, L63T, I93L		I72IT	No	No	No
1100213	V3I, I13V, I15V, K20T, E35D, M36I, S37N, R41K, R57K, D60E, Q61D, I62IV, L90M, I93L	V3I, I13V, I15V, K20T, E35D, M36I, S37N, R41K, R57K, D60E, Q61D, A71V, I84V, L90M, I93L	A71V, I84V	I62IV	No	Yes	Yes
1102208	V3I, L10IL, E35D, M36I, S37N, R41K, K45KR, R57K, Q61N, L63FLPS, I64L, I93L	Q2P, V3I, L10I, E35D M36I, S37N, R41K, K45KR, R57K, Q61N, L63FS, I64L, I93L	Q2P		Yes	No	No
1110212	V3I, L10LV, I15V, E35D, M36I, S37N, P39Q, R41K, R57K, D60E, Q61D, I62IV	V3I, T4NT L10LV, I15V, E35D, M36I, S37N, P39Q, R41K, R57K, D60E, Q61D, I62IV	T4NT		Yes	Yes	Yes
1111203	V3I, L10FIL, K14KR, I15LV, G16AG, K20KT, S37N, M46IM, I62IV, L63P, A71AV, V77IV, I84IV, I85IV, L90LM, I93IL	V3I, L10F, K14KR, I15V, G16A, K20T, S37N, I62V, L63P, A71V, G73S, V77I, I84V, I85V, L90M, I93L	G73S	M46IM	No	Yes	Yes
1111204	E35D, M36I, S37N, R41K, K45R, R57K, Q61S, I93L	V3I, E35D, M36I, S37N, R41K, K45R, R57K, Q61S, I93L	V3I		No	Yes	Yes
1205102	L19Q, D25DN, S37CS, L63P, V77IV	L19Q, S37C, L63P, V77IV		D25DN	No	Yes	Yes
1208204	V3, T4PT, S37N, R41KR, K43KR, L63LP, I64V	V3I, S37N, R41K, K43KR, L63P, I64V		T4PT	Yes	No	No
1213208	V3I, S37N, L63P, H69HQ	V3I, S37N, R41KR, L63P	R41KR	H69HQ	No	No	No
1213214	V3I, S37N, K45R, I62IV, L63P, K70R, I72E	V3I, L10I, I13IV, L19IL, S37N, K45R, M46IM, I54V, Q61KQR, I62V, L63P, K70R, A71V, I72E, G73S, V77IV, V82A, L90M	L10I, I13IV, L19IL, M46IM, I54V, Q61KQR, A71V, G73S, V77IV, V82A, L90M		No	Yes	Yes
1225205	V3I, S37N, L63A, E65DE	V3I, P9PS, I13ILPT, S37N, L63A, E65DE	P9PS, I13ILPT		Yes	Yes	Yes
1501103	S37N, V77I	L10V, K14R, I15V, E35D, S37N, I62V, L63P	L10V, K14R, I15V, E35D, I62V, L63P	V77I	No	Yes	Yes

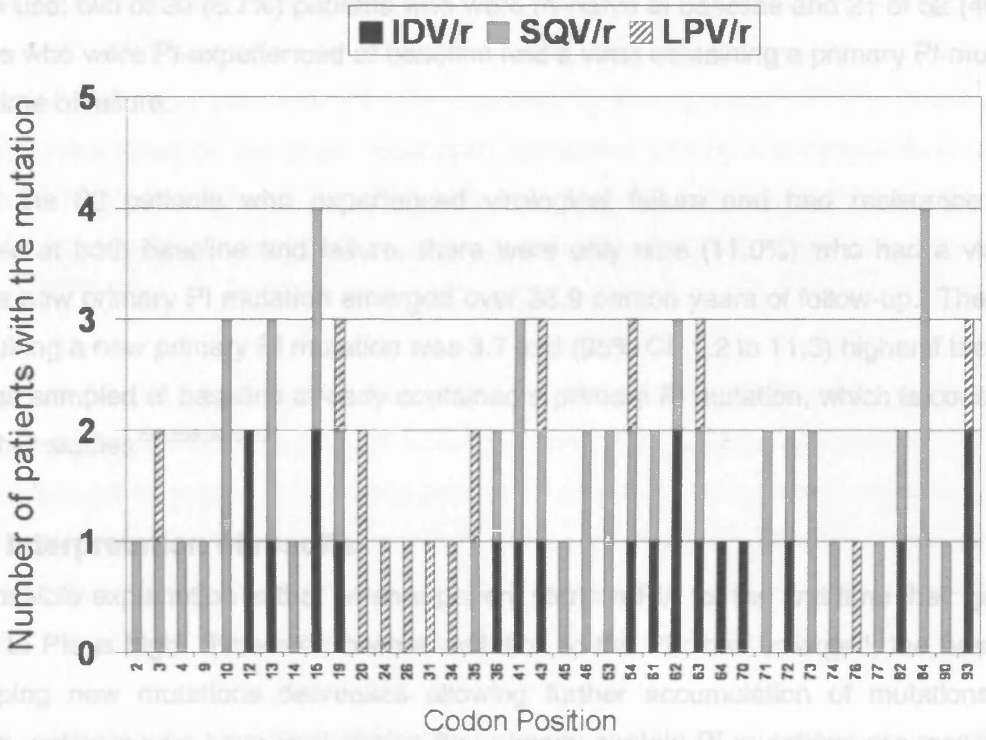
Patient	Any PR mutation from baseline		New mutations gained	Mutations lost	PI-naïve at BL	On BL-PI/r at failure	On a PI at failure
	Baseline	Failure					
SQV/r							
1503002	V3I, T12S, I15V, L19T, M36I, S37N, R41K, Q61E, L63P, H69K, V82I, L89M, I93L	V3I, T12S, I15V, L19T, M36I, S37N, R41K, K45KR, Q61EQ, L63P, H69K, V82I, L89M, I93L	K45KR		No	Yes	Yes
1503115	L10I, L24I, S37T, R41K, I54V, I62IV, L63P, A71AITV, V77I, V82A, I93L	L10I, L24I, S37T, R41K, F53FL, I54V, I62IV, L63P, A71AITV, V77I, V82A, I84IV, I93L	F53FL, I84IV		No	Yes	Yes
1503202	V3I, K20KT, D30DN, E35D, M36I, S37N, R41K, M46IM, I62V, L63P, T74ST, N88DN, L90LM	V3I, E35D, M36I, S37N, R41K, I62IV, L63P, I72IT	I72IT	K20KT, D30DN, M46IM, T74ST, N88DN, L90LM	No	No	No
1504102	L10IL, I15V, S37N, M46I, I54V, K55R, L63P, G73T, V82C, L90M	I15V, S37N, M46I, F53L, I54V, K55R, L63P, G73T, V82C, L90M	F53L	L10IL	No	Yes	Yes
1504104	T12S, I13V, L19I, S37N, L63P, I64V, Q92K	T12ST, I13V, I15IV, L19IM, S37N, R41KR, K43KR, L63P, I64V, Q92K	I15IV, R41KR, K43KR		No	No	No
3003102	L10IV, D30N, S37N, M46I, L63PQ, I64V, E65D, A71AV, V75IV, V77IV, N88D, I93L	L10V, D30N, S37N, M46I, L63Q, I64V, E65D, A71V, T74P, I84V, N88D, I93L	T74P, I84V	V75IV, V77IV	No	Yes	Yes
5502102	L10I, T12RT, I13V, M36I, S37N, R41K, L63T, I64V, E65D	L10I, T12RT, I13V, M36I, S37N, R41K, M46IM, L63T, I64V, E65D	M46IM		Yes	No	No
8500102	S37N, M46IM, G48V, I54IV, I64V, T74AT, V77IV, V82AV, L90LM	L10I, M36IM, S37N, M46IM, G48V, I54IV, I64V, T74AITV, V77IV, V82A, I84IV, L90LM	L10I, M36IM, I84IV		No	Yes	Yes
LPV/r							
1101203	V3I, L10I, K20KM, E35D, M36I, S37N, R41K, I54V, R57K Q61N, L63S, I64L A71V, V82A, L90M, I93L	V3I, L10I, E35D, M36I, S37N, R41K, I54V, R57K, Q61N, L63S, I64L, A71V, V82A, L90M, I93L		K20KM	No	Yes	Yes
1102207	V3I, L10I, I15V K20R E35D, M36I, S37N, R41K, I54V, R57K, Q61N, L63LP, I72T, T74AT, V82A	V3I, L10I, I15V K20R, E35D, M36I, S37N R41K K43KR, I54V, R57K, Q61N, L63P, I72T, T74AT, V82A	K43KR		No	Yes	Yes
1213202	V3I, L10I, I13V, L19I, S37N, L63P, H69Q, A71T	V3I, L10I, I13V, L19I, T26ST, T31AT, S37N, L63P, H69Q A71T	T26ST, T31AT		Yes	Yes	Yes

Patient	Any PR mutation from baseline		New mutations gained	Mutations lost	PI-naïve at BL	On BL-PI/r at failure	On a PI at failure
	Baseline	Failure					
LPV/r							
1213209	R41K, L63P, I64L, V77I	V3I, E35DE, R41K, L63P, I64L, V77I	V3I, E35DE		No	Yes	Yes
1219201	V3I, M36I, S37D, R57K, D60E, L63P, C67CW	V3I, M36I, S37D, R57K, D60E, L63P		C67CW	No	No	No
1401209	V3I, L10I, I13V, K20R, M36I, S37N, R41K, M46I, I54V, I62V, L63T, I64V, E65D, V82A	V3I, L10IV I13IV, K20R, L24IL, M36I, S37N, R41K, M46I, I54V, I62V, L63T, I64V, E65D, V82A, I93L	L24IL, I93L		No	Yes	Yes
1501006	V3I, T12I, E35D, R41K, I62M, L63P, A71V, I93L	V3I, T12AITV, L19IL, E35D, R41K, I62IMV, L63P, A71AV, I93L	L19IL		No	Yes	Yes
2000215	V3I, I13V, E35D, M36I, S37N, R41K, R57K, H69K, L89M	V3I, I13V, E35D, M36I, S37DN, R41K, R57K, L63P, H69K, L89M	L63P		Yes	Yes	Yes
2500054	V3I, L10I, I15V, L24I, E35D, M36V, S37N, K43T, M46I, F53FL, I54V, K55R, I62V, L63A, I64V, V82A	V3I, L10FI, I15V, K20R, L24I, E35D, M36V, S37N, K43T, M46I, F53L, I54V, K55R, I62V, L63A, I64V, V82A	K20R		No	Yes	Yes
2500061	V3I, L10F, L33IL, E35DE, M36IM, S37N, R41K, M46IM, I54V, K55KN, D60E, I62V, L63P, A71AV, V82AV, L90M, I93IL	V3I, L10F, K20R, E35D, M36I, S37N, R41K, M46IM, I54V, K55N, D60E, I62V, L63P, A71V, V82AS, L90M, I93L	K20R	L33IL	No	Yes	Yes
2500063	V3I, L10I, I15V, K20T, M46I, Q58E, I62V, L63P, A71V, I72IL, G73S, V77I, I84V, I85V, L90M, I93L	V3I, L10I, I15V, K20T, E34EK, M46I, I54V, Q58E, I62V, L63P, A71V, I72L, G73S, L76LV, V77I, I84V, I85V, L90M, I93L	E34EK, I54V, L76LV		No	Yes	Yes

Overall, there were a mean (95% CI) 2.19 (1.69 to 2.69) PR amino-acid changes per person-years follow-up between the virus that was sampled at baseline and the virus that was sampled at failure. The viral population of patients who received IDV/r underwent 1.76 (0.99 to 2.53) PR changes per person-years follow-up, in patients who received SQV/r there were 2.53 (1.74 to 3.31) PR changes per person-years follow-up and in patients who received LPV/r there were 2.14 (1.02 to 3.27) new PR changes per person-years follow-up.

For the 33 patients, in whom a PR amino-acid change occurred between baseline and virological failure, the most common sites for these changes were at codons 15 and 84 (Figure 3.4.5.4.1).

Figure 3.4.5.4.1: Mutational emergence at each codon, for patients with a virus in which a PR change occurred from HXB2



3.5. Discussion

3.5.1. Summary of main results

This analysis examines the emergence of mutations along the PR section of the *pol* gene in the predominant viral species of patients enrolled into the MaxCmin or COLATE trials. I explored resistance emergence in the predominant virus of patients who experienced virological failure, regardless of whether they remained on a PI/r or not. On reflection, restricting the analysis to patients who were on a PI/r at the time of failure would have been a better approach to examine resistance emergence to a PI/r containing regimen. However, this would have resulted in a reduction in numbers. So, to retain power, as many patients as possible were included in these comparisons, including those who were on and those who were off therapy at the time of failure. Although not all patients were on a regimen containing a PI/r when they experienced virological failure, the majority of patients were (i.e. 76.8%), so most of the drug mutations that emerged in this analysis are likely to result from the failing PI/r component of the regimen.

Our primary analysis concerns the prevalence and emergence of primary PI resistance mutations at the time of failure, defined according to the September 2006 IAS-USA

resistance tables[233]. There were 23 out of 82 (28.1%) patients who had a virus containing a primary PI mutation at the time of virological failure. Differences existed according to prior PI use: two of 30 (6.7%) patients who were PI-naïve at baseline and 21 of 52 (40.4%) patients who were PI-experienced at baseline had a virus containing a primary PI mutation at the time of failure.

Out of the 82 patients who experienced virological failure and had resistance tests available at both baseline and failure, there were only nine (11.0%) who had a virus in which a new primary PI mutation emerged over 36.9 person years of follow-up. The odds of acquiring a new primary PI mutation was 3.7 fold (95% CI: 1.2 to 11.3) higher if the virus that was sampled at baseline already contained a primary PI mutation, which is consistent with other studies[253;255;309;327].

3.5.2. Interpretation of results

One possible explanation is that when a patient starts a PI/r for the first time their genetic barrier to PIs is high. However, once a mutation to the PI/r has emerged, the barrier to developing new mutations decreases allowing further accumulation of mutations. In addition, patients who have viral strains that already contain PI mutations are more likely to be incompletely adherent patients (i.e. previous low levels of adherence may explain why the mutation appeared in the first place); this may explain further why mutations are more likely to emerge in the predominant virus of these patients compared to those in which no mutations were present in their sampled virus at baseline.

Although new primary PI mutations were seen in the virus populations of some patients at the time of failure – most of whom were still receiving their PI/r at failure – it is not possible to assume these mutations occurred solely due to the selection pressure exerted by the PI/r. However, such a causal link is likely and plausible. Of note, new mutations at codon 84 prevailed in the virus populations of patients who were using SQV/r, and since this mutation is known to be associated with a reduced response to unboosted SQV[220;328] it is also likely to arise and have an association with virological failure in patients who are treated sub-optimally with SQV/r.

I performed several other analyses to capture all of the changes along the PR section of the *pol* gene. Overall, I found an acquisition rate of 0.30 (0.12 to 0.47) new primary PI mutations per person-years follow-up, 0.65 (0.39 to 0.91) new PI-specific mutations per person-years follow-up and 2.19 (1.69 to 2.69) new PR amino-acid changes per person-

years follow-up. Analysis of a subgroup of patients who remained on their PI/r at the time of failure found comparably low rates of new mutations emerging from baseline in all of the analyses. Other studies looking at the accumulation of new PR mutations found similar event rates[254;258;288;317] and similar mutations were identified in the virus populations of patients failing one of the three PI/rs investigated in this analysis[309;329;330]. Most of the published information on genotypic resistance associated with PI/rs is derived from studies using each PI without ritonavir boosting, although recently, more information on virological response amongst patients receiving a ritonavir-boosted regimen has been published[216;331-335].

In 15 out of 82 (18.3%) patients, the virus that was sampled at failure contained a PI-specific mutation that was not seen at baseline: nine (60%) patients had a virus in which a primary PI-mutation emerged (including primary PI mutations other than those to the PI/r in question) and the six remaining patients had a virus in which only secondary PI-mutations emerged. In addition, 33 out of 75 (44.0%) patients had a virus that underwent any change to the PR section of the *pol* gene between baseline and failure.

This analysis provides a heterogeneous picture of mutations that occur as a result of treatment with a PI/r. All of the mutations that occurred as a result of LPV/r containing regimens have previously been associated with resistance to LPV/r. A small proportion of mutations that arose in the viral populations of patients receiving IDV/r or SQV/r are not known to be associated with the corresponding PI/r (i.e. at codons 12, 15, 62 or 93 for IDV/r; and at codons 13, 15, 41 or 53 for SQV/r). These mutations may augment resistance to the PI, or represent compensatory mutations which restore virus fitness.

Longer follow-up of patients who are failing a PI/r containing regimen should indicate whether these mutations are actually associated with virological failure themselves or whether they are on the pathway for developing other resistance mutations. However, this may not be possible because it is advisable to change regimens if a patient is failing therapy in order to stop the emergence of mutations. So, *in vitro* studies may help to elucidate their role in PI resistance instead. Heterogeneity in the amino-acid changes identified along the PR section of the *pol* gene could arise because of the low signal to noise ratio (i.e. a large number of codons were sequenced using various approaches), and because these analyses are lacking in power. Thus, it is not possible to say for certainty that these mutations emerged as a result of the PI/r the patient was receiving.

3.5.3. Limitations of the analysis

This analysis was based on 82 of the 146 patients who had observed virological failure in the three trials combined. The reason for excluding some patients was either lack of available sample or lack of ability to amplify and sequence the protease gene. This possibly introduced selection bias by excluding patients who had low levels of HIV RNA either at baseline and/or failure. However, baseline characteristics such as previous treatment exposure were balanced between patients who were included in these comparisons compared to those excluded (data not shown).

New PI mutations associated with reduced viral efficacy were acquired in patients who received all three PI/rs, but this analysis was substantially under-powered to identify any clinically meaningful differences in the rate of mutation emergence. In order to maximize the sample size to assess the objective of this analysis, I merged data from three independent trials and included patients with genotypic data at time of failure but no baseline samples as long as they had not received a PI prior to baseline. It is important to underline that by doing so, I assumed that in the seven patients without prior exposure there were no PR mutations present in their virus at baseline.

This assumption is reasonable because primary PI mutations are rarely transmitted and are rarely developed spontaneously[248;250;336;337]. However, these patients had never been exposed to PIs before the trial, so would therefore be expected to respond favourably to a PI/r containing regimen. Since they failed treatment, resistance that has not been accounted for in these comparisons may actually be present at baseline. After excluding patients who were PI-naïve from the analysis, the proportion of patients in whom a primary PI mutation emerged was 14% and therefore comparable to the 11% if all patients are studied together.

Since this analysis was performed on combined data from the three trials I re-ran the MaxCmin2 definition of virological failure on patients in MaxCmin1 and COLATE to produce 'new' dates of virological failure. Although there was no difference in the new dates of virological failure for the majority of patients in MaxCmin1 (78.6%) and for the majority of patients in COLATE (81.1%), the new dates did differ in some patients (from 12 weeks before to 14 weeks after in MaxCmin1 and from 6 weeks before to 19 weeks after in COLATE). Of note, four patients in COLATE were considered to have experienced virological failure using the new MaxCmin2 definition of failure when they did not satisfy the original COLATE definition of virological failure.

Resistance data from patients who are failing a PI/r containing regimen allows us to identify mutations that arise under selection pressure from a failing a PI/r. Since clinicians are likely to switch a patient from a failing PI/r regimen to an active regimen as soon as they experience virological failure, these types of data are fairly uncommon. To allow us to investigate resistance emergence in patients on a PI/r I combined data from the three trials because, even though the study methodology differed slightly between studies, combination of the three trials increases the number of patients who could be included in this analysis.

Combining the three trials could theoretically represent a methodological problem because they were set up to answer different questions and are conducted in different ways. However, all three trials were conducted between 1999 and 2002 so treatment approaches were likely to be similar in each trial, especially with regard to using a ritonavir-boosted PI containing regimen because they had just been introduced into clinical practice. Furthermore, all definitions of virological failure that were used in the trials incorporate information on the baseline HIV RNA levels and the expected response to therapy at specific time points over follow-up. These patients should have good suppression over the first 24 weeks because they are recruited on the basis that they will respond favourably to the drug, and since the purpose of this analysis was not to compare the groups but to collect resistance data on patients who experience virological failure, it is assumed that it does not constitute a significant methodological issue to merge these data.

3.5.4. Conclusion

New treatment strategies should be intensively studied and all available resistance data on mutations leading to changes in virological resistance patterns for a particular ARV should be investigated. Combining patients from MaxCmin1, MaxCmin2 and COLATE meant it was possible to examine resistance evolution in the viral populations of patients receiving PI/rs in some detail. The results of the multivariable logistic regression analyses suggest that patients who have a virus that contains no primary resistance mutations at baseline are less likely to experience primary resistance emergence in their virus populations. This supports evidence suggesting that the longer one has been treated with a PI/r the greater the risk of virological resistance emerging[288]. This trend was found to be independent to the drug concentrations in these patients. However, patients with both drug concentration data and resistance data available had drug concentrations that were well above the level required to inhibit HIV replication by more than 90%.

In conclusion, the emergence of new primary PI resistance mutations along the PR section of the *pol* gene was demonstrated in 11% of patients who virologically failed a regimen containing a PI/r. Existence of resistance mutations at the start of the PI/r predicted an increased risk of new mutations emerging.

CHAPTER 4: THE ABILITY OF FOUR GISs TO PREDICT VIRAL LOAD RESPONSE TO RITONAVIR-BOOSTED PIS, USING TRIAL DATA

4. Introduction

In chapter 3 I investigated the emergence of newly detected PR mutations in the dominant virus of patients who were failing a PI/r containing regimen. Although there was a low rate of mutations emerging (i.e. an acquisition rate of 0.30 (95% CI: 0.12 to 0.47) new primary PI mutations per person-years follow-up), primary PI mutations still emerged in the dominant virus of 11.0% of patients. Most of the patients in the trials had a virus with only a few protease mutations at the time of starting a PI/r (i.e. trial baseline); therefore, their virus should have been sensitive to the drugs that were in the regimen, especially the PI/r component. If the virus is sensitive to therapy, these patients are not expected to experience high levels of resistance emergence in their virus populations because viral replication should be kept under control.

The short term response to therapy has been associated with longer term response[317;338] so it is worthwhile identifying if any mutation in the dominant virus at baseline can predict short term viral load changes in order to maximise the overall longer term response. The dominant virus in a patient is unlikely to change much over the first few weeks following treatment initiation, so any viral load changes that occur during this interval are likely to be related to mutations that are present in the virus at baseline. If viral load changes are studied at a later time point it is likely that, under drug selection pressure, changes will have occurred within the viral subspecies. As a result, any changes in HIV RNA levels may be due to the emergence of new resistance mutations rather than mutations that were present in the virus at baseline (Figure 1.6.2.2.1). In this chapter I examine HIV RNA declines at two early time points to identify whether baseline mutations can predict early virological response as an indicator of the potential success of therapy.

4.1. Aims of the chapter

Rules-based genotypic interpretation systems (GISs) take into account a large number of different mutations and assign them all a weight, depending on whether they are detected alongside other mutations. As a result they predict varying degrees of susceptibility the virus has to the regimen a patient is receiving. In this chapter I will explore the differences between four freely available GISs in their ascribed levels of resistance to a PI/r and examine whether these interpretational differences impact on the ability of each system to

predict short term virological response (appendix V)[339]. This analysis was performed using data from the three randomised controlled trials (MaxCmin1, MaxCmin2 and COLATE).

4.2. Literature review

As described in section 1.6.3.1, genotypic resistance testing is used to guide therapy selection by producing lists of the type and positioning of each mutation in the sampled virus[193;197;198]. Studies exploring the relationship between resistance and response to therapy have so far been limited by a low prevalence of mutations or inadequate data on virological response, because extremely large data sets are required in order to produce reliable results. As a result, there are disagreements between experts on how each mutation affects response to each ARV and consequently over twenty rules-based GISs have been proposed[214-221].

Differences between GISs have been illustrated previously[228;229;340;341]. They tend to agree on the level of resistance inferred by the common resistance mutations, especially if the patient is receiving an ARV that is compromised by the emergence of a single mutation in the virus (e.g. 3TC and the M184V mutation). Conversely, high levels of discordance exist between GISs if the virus requires complex mutational patterns for the ARV to be compromised by the presence of resistance, (e.g. zidovudine, didanosine, stavudine and abacavir)[224;225;227-230].

This is also true for protease inhibitors (PIs) where disagreements cannot be attributed to the presence of a single mutation or specific mutational pattern[229]. For example, resistance to darunavir/r is considered to be a problem if at least 4 mutations emerge among: V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, L89V using the ANRS GIS, and cannot be attributed to the emergence of any one mutation specifically.

Ritonavir is currently used in low doses to boost levels of other PIs. Due to the potency of these combinations, ritonavir-boosted PIs (PI/rs) may require alternative or more sensitive interpretations to those for unboosted PIs, although the current systems have also been shown to apply to PI/rs[198]. Some GISs have been updated to account for the use of ritonavir-boosting, but still most discordance between GISs occurs amongst PIs[230].

Individual mutations and resistance to the overall regimen, as illustrated by a genotypic sensitivity score (GSS), have been shown to be associated with HIV RNA declines at various time-points[198;218;340]. Since limited information exists on the prognostic value of

GISs for PI/rs I investigated concordance between ascribed PI/r (IDV/r, LPV/r and SQV/r) resistance levels using four freely available GISs and related the resulting interpretations to virological response after 4 and 12 weeks of follow-up. Although most of the PI/rs studied in this chapter (i.e. SQV/r and IDV/r) are less frequently used nowadays, they were commonly used at the time of performing the analysis described here.

4.3. Methods

Current treatment guidelines identify week 12 as the optimal time point for assessing virological responses to a new combination regimen[342]. Since the week 12 viral load response has been shown to reflect long-term success this was chosen as the time point for this chapter[343]. I also wanted to see whether there were differences in HIV RNA change according to the ascribed PI/r resistance levels at an earlier time point. If differences are noticeable at an earlier stage then changes can be made to the regimen to ensure patients receive the maximum benefit from their treatment without risk of further resistance emergence in their virus population.

Since the trials have viral load data at four weeks follow-up, I also investigated the relationship between GISs and HIV RNA decline after four weeks follow-up. To identify the additional effect of other drugs in the regimen (i.e. other than the PI/r) on viral load response I assigned a GSS to the rest of the regimen and explored the relationship between this GSS and viral load response at both time points.

4.3.1. The trials

Between May 1999 and May 2002, 799 HIV infected patients were enrolled into the three 48-week, multi-centre trials (MaxCmin1 (N=324), MaxCmin2 (N=339) and COLATE (N=136)). Patients in COLATE who were receiving a PI/r (i.e. SQV/r, IDV/r or LPV/r) were combined with MaxCmin patients to maximise numbers for these comparisons.

4.3.2. Genotypic interpretation systems

Genotypes were provided as amino acid substitutions (full or as mixtures) from a reference clade B strain (i.e. HXB2) for both the RT and protease genes. The amount of resistance was quantified by running the amino-acid sequences through the following GISs: ANRS (version 13)[215]; DMC (October 2004)[217]; REGA (version 6.4)[221]; and Stanford (version 4.2.0)[220] to produce a score for each ARV in the regimen relating to whether a patient had a virus showing resistance, intermediate resistance or sensitivity to that drug (see

1.6.3.1.1). These four GISs were chosen because they are rules-based, updated regularly (usually annually), freely available and possible to programme. Rules for these GISs are presented as tables listing mutations that confer possible genotypic resistance to ARVs. All of the GISs were created from data based on genotype-to-phenotype correlations, with a small part based on genotype-to-viral-load-response.

The ANRS algorithm was created by the French ANRS (Agence Nationale de Recherches sur le Sida) AC11 resistance group in 2002 in order to help physicians select the most effective treatment regimen for a patient. The rules of the current version are almost exclusively based on data examining statistically significant correlations between drug resistance mutations and virological outcomes from a large database of patients failing antiretroviral therapy.

The REGA Institute GIS, Leuven, is a rules-based system based on published evidence and expert interpretation, and has a particular focus on the impact of drug resistance on the response to ARVs. They also use data from *in vitro* genotypic and phenotypic studies. The first REGA GIS was created in January 2000, which was clinically validated in a cohort of patients on salvage therapy, where the three month virological response was used as the outcome variable.

The DMC GIS is another rules-based GIS and was created in 2000[344] by the Detroit Medical Centre. All three GISs provide a three-level resistance output (i.e. resistance, intermediate resistance or sensitive) for each ARV according to the mutations that are present in the virus. DMC resistance algorithms were not available for PI/rs so interpretations for the unboosted PI were used instead.

The Stanford GIS is another resistance algorithm that was made available online in September 2000. This GIS usually provides the inferred resistance level as a continuous output which is then translated into a 5-level resistance score: susceptible (score 0 to 9); potential low-level resistance (score 10 to 14); low-level resistance (score 15 to 29); intermediate resistance (score 30 to 59); or high-level resistance (score ≥60). Resistance levels ascribed by Stanford were reduced from a 5-level system to a 3-level system using the recommended method: an overall score of 0 to 14 represents susceptibility, 15 to 59 intermediate resistance and ≥60 high-level resistance.

A GSS was generated for the background regimen by summing the level of resistance to each ARV in the regimen other than the PI/r (see 1.6.3.1.1). Separate GSSs were calculated using each of the four GISs. Resistance to ritonavir was not included in this calculation since it was not administered in therapeutic doses. I programmed each GIS in Stata (version 8.2) and cross-checked the programming with a colleague (Alessandro Cozzi Lepri) in SAS (version 8.2). Final checks were made by running 5% of the sequences through the online Stanford resistance programme[345] and cross-checking the inferred resistance levels with the levels that I obtained using my programmes.

4.3.3. Laboratory methods

Plasma samples were used for sequencing if baseline HIV RNA >500 copies/mL. HIV RNA was quantified using the Roche Ultrasensitive Amplicor HIV-1 Monitor Assay (Roche Molecular Systems, Alameda, California)[41] and the Chiron branched chain (bDNA) assay (Chiron Corp., Emeryville, California)[38].

Different sequencing procedures were used for each study (see section 3.3.6). Virus sequencing was performed centrally (Advanced Biological Laboratories, Luxembourg) in batches using cryopreserved plasma samples in MaxCmin2 and COLATE. Sequence data for the reverse transcriptase (RT) and protease (PR) reading frames (i.e: PR amino acid positions 1 to 99 and RT positions 1 to 335) were obtained by extracting HIV RNA from 500 µl plasma using the NucliSens isolation kit. Through nested PCR amplification, a 1.8-kb amplicon encompassing the protease and the first 1005 nucleotides of the RT gene was generated, spanning a total of 1302 nucleotides. Purified amplicons were sequenced using the ABI Prism BigDye terminator cycle sequencing kit and the resulting nucleotide sequences were translated into amino acids. Sample mix-up or lab-contaminated samples were identified by generating phylogenetic trees.

In MaxCmin1, virological sequencing was performed at the International Clinical Virology Center (High Wycombe, UK) where they used the QIAamp kit for RNA extraction from plasma (Qiagen, Barcelona, Spain) according to the manufacturer's instructions. Sequence data for the RT (positions 1 to 335) and PR (positions 1 to 99) genes were obtained using Trugene HIV-1 Genotyping Kit and OpenGene DNA Sequencing System (Visible Genetics, Barcelona, Spain).

4.3.4. Statistical analysis

All patients who initiated a PI/r, had HIV RNA data available at four and 12 weeks of follow-up and had a genotypic resistance test available at baseline were included in these comparisons. Concordance in the ascribed PI/r resistance levels at baseline was evaluated between GISs using kappa statistics[346]. A kappa >0.8 reflects very good concordance, 0.6<kappa≤0.8 good concordance, 0.4<kappa≤0.6 moderate concordance, 0.2<kappa≤0.4 fair concordance and kappa ≤0.2 poor concordance or no relationship.

Censored linear regression analysis was used to relate PI/r resistance levels to HIV RNA reductions from baseline after 4 and 12 weeks of follow-up. I assumed there was a linear relationship between the change in HIV RNA and sensitivity to the PI/r in question (i.e. the difference in change in HIV RNA between resistant and intermediate viruses is the same as the difference in change in HIV RNA between intermediate and sensitive viruses). This was performed after evaluating the relationship between the decline in HIV RNA levels and the degree of resistance, classified as a three-level categorical variable (i.e. resistant, intermediate resistance and sensitivity). Since the initial analysis indicated a linear relationship across categories, the relationship between the change in HIV RNA and sensitivity to the PI/r was modelled as a linear relationship throughout this chapter.

Patients with missing HIV RNA data at either the 4- or 12- week time point had their HIV RNA level estimated through linear interpolation if they had data available at both the preceding and following visits. The most commonly used HIV RNA assay has a lower limit of quantification of 50 copies/mL therefore it was not possible to estimate exact HIV RNA declines for patients with an HIV RNA <50 copies/mL. Factors associated with HIV RNA change were identified through censored linear regression analysis (appendix IV) to take into account the partial observation of the extent of viral load reduction due to this censoring[347]. All variables associated with HIV RNA decline in unadjusted analysis were entered into separate multivariable models for each GIS. Each multivariable model was adjusted for the GSS to the rest of the regimen calculated using the same GIS. Statistical analyses were performed using STATA.

4.4. Results

4.4.1. Patient characteristics

Seven-hundred-and-twelve of the 799 (89.1%) patients who were enrolled in the trials initiated a PI/r. Overall, 657 of 712 (92.3%) patients had HIV RNA values available at four

and 12 weeks of follow-up and of these 368 (56.0%) had a baseline resistance test in the three weeks prior to baseline. These 368 patients started one of the following single PI/rs (as opposed to double-boosted PI/rs): IDV/r (N=89, 24%); LPV/r (N=126, 34%); and SQV/r (N=153, 42%). The baseline characteristics for these patients are shown in Table 4.4.1.1.

Patients were primarily male (75%), white (73%), were a median age of 39 years (IQR: 34 to 46 years) and 29% had an AIDS defining event prior to entry. There were 201 (55%) patients who were PI-experienced prior to entry. Median $CD4^+$ cell counts were 223 (IQR: 100 to 360) cells/μL and median HIV RNA levels were 4.7 (IQR: 3.9 to 5.2) log_{10} copies/mL.

Table 4.4.1.1: Baseline characteristics for the 368 patients from the trials

Baseline parameter		IDV/r (N=89)	LPV/r (N=126)	SQV/r (N=153)	Total (N=368)
Gender	No. Male, (%)	61 (69)	95 (75)	121 (79)	277 (75)
Age	Med (IQR)	39 (32, 47)	40 (35, 46)	38 (35, 45)	39 (34, 46)
Antiretroviral naïve	N, (%)	8 (9)	40 (32)	38 (25)	86 (23)
PI-naïve	N, (%)	26 (29)	15 (12)	40 (26)	81 (22)
PI-experienced	N, (%)	55 (62)	71 (56)	75 (49)	201 (55)
CDC, cat. C	N, (%)	23 (26)	43 (34)	41 (27)	107 (29)
HIV RNA (cps/mL log_{10})	Med (IQR)	4.4 (3.8, 5.1)	4.8 (4.0, 5.3)	4.7 (4.0, 5.2)	4.7 (3.9, 5.2)
CD4+ (cells/μL)*	Med (IQR)	249 (119, 364)	214 (82, 388)	217 (71, 319)	223 (100, 360)
CD4+ nadir (cells/μL)	Med (IQR)	138 (55, 224)	108 (35, 199)	102 (33, 224)	110 (39, 215)
HIV exposure group	N, (%)				
Homosexual/bisexual		39 (44)	55 (44)	63 (41)	157 (43)
IVDU		5 (6)	9 (7)	18 (12)	32 (9)
Heterosexual		34 (38)	52 (41)	60 (39)	146 (40)
Other/unknown		11 (12)	10 (8)	12 (8)	33 (9)
Race	N, (%)				
White		65 (73)	91 (75)	114 (72)	270 (73)
Black		20 (22)	26 (21)	29 (19)	75 (20)
Other/unknown		4 (5)	9 (4)	10 (9)	23 (7)
Any major NRTI mutation*	N, (%)	55 (62)	65 (52)	69 (45)	189 (51)
Any NNRTI mutation*	N, (%)	17 (19)	35 (28)	41 (27)	93 (25)
Any primary PI mutation*	N, (%)	28 (31)	35 (28)	29 (19)	92 (25)

* Refers to any major mutation as defined in the IAS-USA 2006 tables[233]

Overall 189 (51%), 93 (25%) and 92 (25%) patients had a virus with a major resistance mutation to an NRTI, NNRTI or PI, respectively[233]. Patients started a variable number of ARVs in addition to the PI/r, with the majority (80%) starting two other ARVs.

4.4.2. Concordance of the interpretation of genotypic data by all GISs

At baseline there were 367 patients who received an NRTI, 35 who received an NNRTI and 368 who received a PI/r. Using the IAS-USA (Sept 2006)[233] mutations guidelines to crudely examine the prevalence of mutations to the baseline regimen, 151 (41%) patients were estimated to have a virus containing a reverse transcriptase (RT)-mutation to an NRTI they were receiving at baseline, 4 (11%) patients had a virus containing an RT-mutation to an NNRTI they were receiving at baseline and 41 (11%) patients had a virus containing a primary PI-resistance mutation to a PI/r they were receiving at baseline (N=12 (14%), N=19 (15%) and N=10 (7%) patients on IDV/r, LPV/r and SQV/r, respectively).

Using the REGA GIS (version. 6.4) to estimate the prevalence of baseline resistance, there were 42 (11%) patients with a virus that was deemed to have full or intermediate resistance to the baseline PI/r (N=15 (17%), N=16 (13%) and N=11 (7%) patients who were receiving IDV/r, LPV/r and SQV/r, respectively). These resistance levels are similar to the levels of major IAS-USA PI mutations described above. ANRS (version 13) calculated comparable levels of resistance to REGA but both DMC (October 2004) and Stanford (version 4.2.0) predicted nearly double the number of patients with a virus exhibiting full or intermediate resistance to their baseline PI/r (Table 4.4.2.1).

Table 4.4.2.1: Patients with a virus exhibiting full resistance, intermediate resistance or sensitivity to the PI/r according to each GIS

Interpretation System	**IDV/r (N=89)**	**LPV/r (N=126)**	**SQV/r (N=153)**	**Total (N=368)**
ANRS	18/0/71	3/11/112	6/0/147	27/11/330
No. (%) with resistance to the PI/r (R/I/S)*	(20/0/80)	(2/9/89)	(4/0/96)	(7/3/90)
REGA	9/6/74	3/13/110	6/5/142	18/24/326
No. (%) with resistance to the PI/r (R/I/S)*	(10/7/83)	(3/10/87)	(4/3/93)	(4/7/89)
DMC	11/22/56	10/13/103	12/9/132	33/44/291
No. (%) with resistance to the PI/r (R/I/S)*	(12/25/63)	(8/10/82)	(8/6/86)	(9/12/79)
Stanford	7/11/71	5/24/97	5/14/134	17/49/302
No. (%) with resistance to the PI/r (R/I/S)*	(8/12/80)	(4/19/77)	(3/9/88)	(5/13/82)

* R/I/S – Full resistance/Intermediate resistance/sensitivity to the PI/r

When I examined concordance between interpretations using the REGA and ANRS GISs, complete concordance was identified in the ascribed level of resistance to the PI/r in 349 (94.8%) cases (i.e. 16 were deemed to have full resistance, 11 intermediate resistance and 322 sensitivity using both systems). The interpretations differed for the other 19 (5.2%) isolates using these systems.

Overall concordance between GISs was moderate. There was complete concordance in the predicted level of resistance to IDV/r, LPV/r and SQV/r between all four GISs for 61 (69%), 102 (81%) and 134 (88%) of the isolates, respectively. Kappas between GISs ranged from 0.37 to 0.75 for IDV/r; 0.46 to 0.93 for LPV/r; and 0.38 to 0.68 for SQV/r (Table 4.4.2.2).

Table 4.4.2.2: Kappas values to illustrate concordance between GISs
Bold reflects concordance among the whole group; *italics* reflect concordance among PI-experienced patients only

		REGA	DMC	Stanford
IDV/r	**ANRS**	**0.68**	**0.43**	**0.64**
		0.64	*0.49*	*0.60*
	REGA	-	**0.37**	**0.75**
			0.43	*0.73*
	DMC	-	-	**0.46**
				0.54
LPV/r	**ANRS**	**0.93**	**0.46**	**0.57**
		0.92	*0.40*	*0.52*
	REGA	-	**0.54**	**0.64**
			0.48	*0.59*
	DMC	-	-	**0.66**
				0.60
SQV/r	**ANRS**	**0.51**	**0.42**	**0.38**
		0.52	*0.50*	*0.41*
	REGA	-	**0.56**	**0.68**
			0.65	*0.69*
	DMC	-	-	**0.64**
				0.71

Poor $\kappa \leq 0.2$; Fair $0.21 \leq \kappa \leq 0.40$; Moderate $0.41 \leq \kappa \leq 0.60$; Good $0.61 \leq \kappa \leq 0.80$ and Very Good $\kappa > 0.80$

Concordance between the DMC GIS and the other GISs increased among a sub-group of patients who were PI-experienced at baseline. All of these GISs were originally designed for PI-experienced patients (i.e. those with more PI-mutations in their viral populations) so we may expect to find improved concordance in this subgroup of patients. Overall, similar kappas were identified in PI-experienced patients compared to the combined population of PI-experienced and PI-naïve patients: kappas ranged from 0.43 to 0.73 for IDV/r; 0.40 to 0.92 for LPV/r; and 0.41 to 0.71 for SQV/r (Table 4.4.2.2).

Through detailed examination of the GISs it is possible to identify mutations that are predictors of resistance with certain GISs but not with others. Figure 4.4.2.1 crudely shows the codons that cause discordance between GISs for each drug class. Although ANRS considers 25 codons in the protease gene to be associated with resistance to a PI/r in this analysis (i.e. three more mutations than any other GIS), it estimates that fewer patients have a virus that shows full or intermediate resistance to their PI/r compared to the other GISs. This is because mutations occurring at these codons are either infrequent or need to be considered in combination with other mutations to infer resistance using ANRS.

Figure 4.4.2.1: Mutational discrepancies between GISs, by drug class

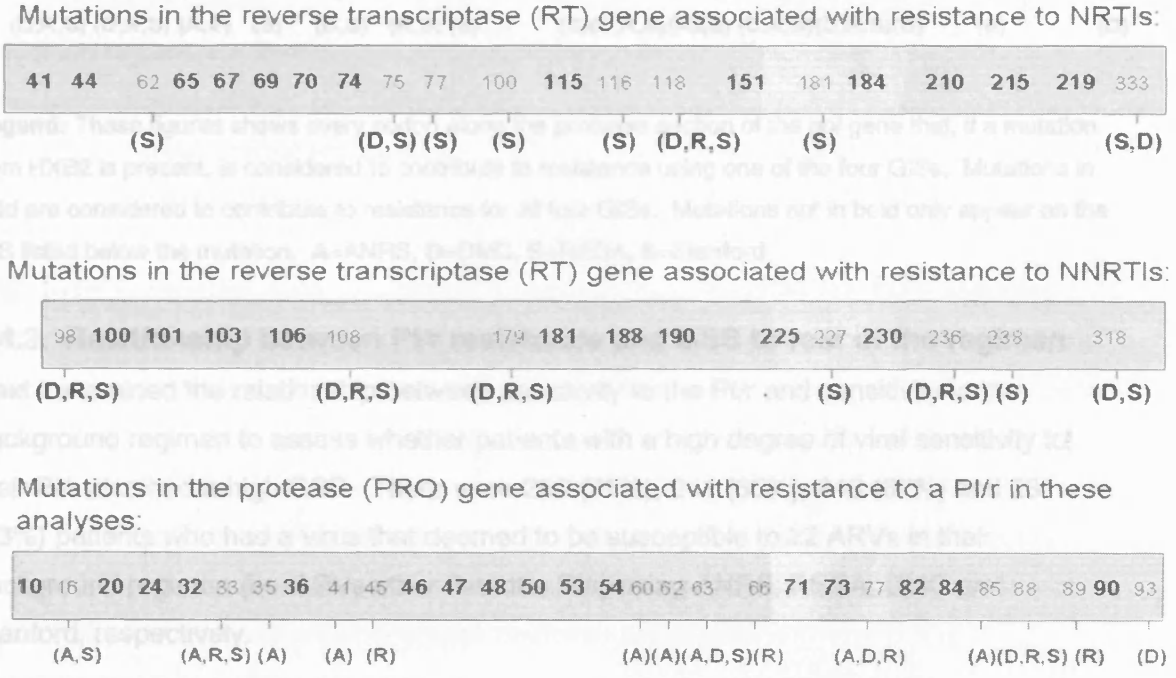
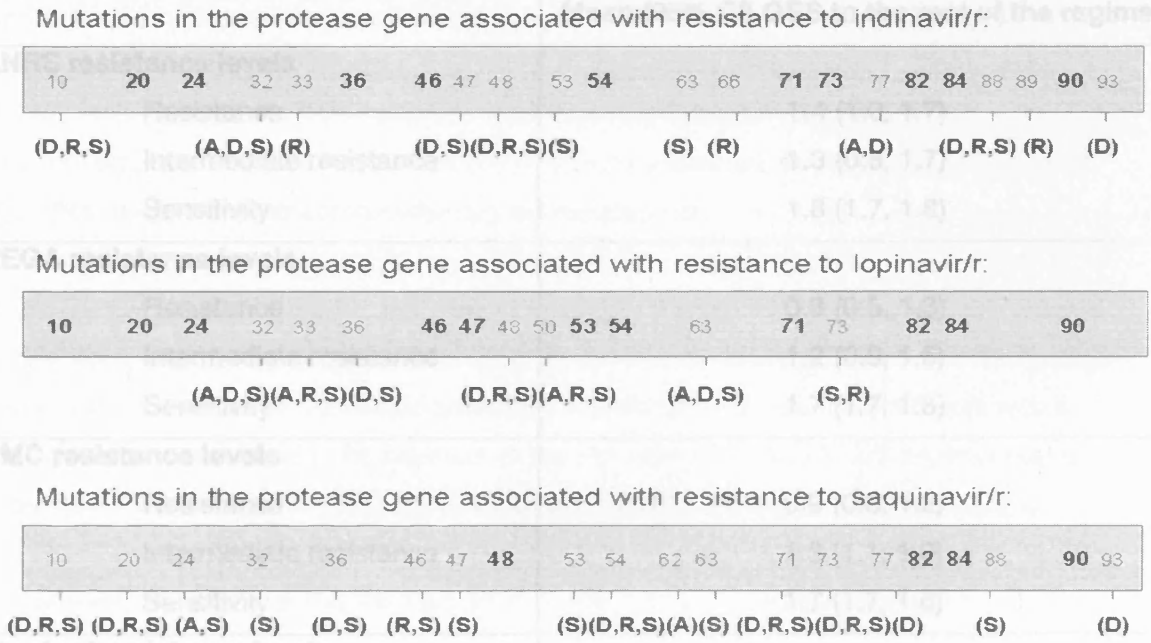


Figure 4.4.2.2 shows there is a high level of discordance when examining interpretations for each PI/r separately. DMC predicts significantly higher levels of resistance to IDV/r and SQV/r than any other GIS. This is primarily driven by the presence of the I93L protease mutation, which occurs in 146 (40%) of our patients but is not considered to have an effect on resistance using any GIS other than DMC.

Figure 4.4.2.2: Mutational discrepancies between GISs, by PI/r



Legend: These figures shows every codon along the protease section of the *pol* gene that, if a mutation from HXB2 is present, is considered to contribute to resistance using one of the four GISs. Mutations in bold are considered to contribute to resistance for all four GISs. Mutations not in bold only appear on the GIS listed below the mutation. **A**=ANRS, **D**=DMC, **R**=REGA, **S**=Stanford

4.4.3. Relationship between PI/r resistance and GSS to rest of the regimen

Next I examined the relationship between sensitivity to the PI/r and sensitivity to the background regimen to assess whether patients with a high degree of viral sensitivity to their PI/r also had a high GSS. There were 260 (71%), 241 (65%), 246 (67%) and 231 (63%) patients who had a virus that deemed to be susceptible to ≥2 ARVs in their background regimen (i.e. ARVs other than the PI/r) using ANRS, REGA, DMC and Stanford, respectively.

For all of the GISs, there was an increasing amount of sensitivity to the ARVs in the background regimen with increasing levels of sensitivity to the PI/r. Using the REGA GIS, the mean (95% CI) GSS to the rest of the regimen was 0.9 (0.5 to 1.3) for patients who had a virus that showed resistance to the PI/r at baseline, 1.2 (0.9 to 1.6) for patients who had a virus that showed intermediate baseline resistance and 1.7 (1.7 to 1.8) for patients who had a virus that showed sensitivity to the PI/r (Table 4.4.3.1).

Table 4.4.3.1: The GSS to the rest of the regimen according to PI/r resistance levels

	Mean (95% CI) GSS to the rest of the regimen
ANRS resistance levels	
Resistance	1.4 (1.0, 1.7)
Intermediate resistance	1.3 (0.8, 1.7)
Sensitivity	1.8 (1.7, 1.8)
REGA resistance levels	
Resistance	0.9 (0.5, 1.3)
Intermediate resistance	1.2 (0.9, 1.6)
Sensitivity	1.7 (1.7, 1.8)
DMC resistance levels	
Resistance	0.9 (0.6, 1.2)
Intermediate resistance	1.3 (1.1, 1.6)
Sensitivity	1.7 (1.7, 1.8)
Stanford resistance levels	
Resistance	0.8 (0.4, 1.2)
Intermediate resistance	1.2 (1.0, 1.4)
Sensitivity	1.7 (1.7, 1.8)

This trend is expected because patients in the trials had varied entry criteria, including patients who were ARV-naïve or PI-naïve prior to entry. Patients with a virus that showed resistance mutations at baseline are likely to be patients with high degrees of exposure to ARVs prior to the start of the trial and, therefore, they may show mutations along both the protease and RT sections of the gene. ARV-naïve patients will only show mutations if they were infected with a strain of HIV carrying resistance mutations and therefore they are likely to show few mutations in their viral sub-species and have sensitivity to both the PI/r and the background components of their regimens at baseline.

4.4.4. Prognostic value of GISs for predicting viral load decline at week 4

The median (IQR) baseline HIV RNA level was 4.7 (3.9 to 5.2) $\log_{10}$ copies/mL (3.5 (3.1 to 4.6) $\log_{10}$ for patients who had a virus that showed resistance to the PI/r at baseline, 4.1 (3.4 to 4.9) $\log_{10}$ for patients who had a virus that showed intermediate resistance at baseline and 4.7 (4.0 to 5.3) $\log_{10}$ for patients who had a virus that showed sensitivity to the PI/r at baseline, according to the REGA GIS).

HIV RNA declined by a median (IQR) 1.8 (1.2 to 2.3) $\log_{10}$ copies/mL by week 4. This was similar for patients receiving each PI/r: 1.7 (0.8 to 2.2) $\log_{10}$ copies/mL (IDV/r), 1.8 (1.3 to 2.3) $\log_{10}$ copies/mL (LPV/r) and 1.8 (1.2 to 2.3) $\log_{10}$ copies/mL (SQV/r). There were 14 (16%), 14 (11%) and 17 (11%) patients who received IDV/r, LPV/r and SQV/r, respectively, who had an HIV RNA recorded as <50 copies/mL in whom the reduction in HIV RNA levels were censored in the regression analysis.

In unadjusted analyses, the level of resistance to the PI/r (all PI/rs combined) was related to HIV RNA reductions using all of the GISs ($P<0.10$ in all cases): all GISs showed greater HIV RNA reductions with increased sensitivity to the PI/r. For example, patients with a virus that was considered to be resistant to the PI/r using the REGA GIS experienced a mean (95% CI) 1.4 (0.9 to 1.8) $\log_{10}$ decline; patients harbouring a virus that showed intermediate resistance had a mean 1.6 (1.2 to 2.0) $\log_{10}$ decline; and patients with a virus that showed sensitivity to the PI/r had a 1.8 (1.7 to 1.9) $\log_{10}$ copies/mL decline.

Due to the strong association between the GSS to the rest of the regimen and PI/r sensitivity, the GSS to the rest of the regimen may confound the relationship between PI/r sensitivity and HIV RNA change. After adjustment for the PI/r used, HIV RNA at baseline and the GSS to the rest of the regimen (using the same GIS that was used to calculate resistance to the PI/r) none of the GISs remained predictive of HIV RNA change to week 4 when looking at the level of resistance to the PI/r (Table 4.4.4.1). From a model including the REGA GIS, patients who had a virus that showed sensitivity to the PI/r experienced a 0.12 greater $\log_{10}$ reduction in HIV RNA between baseline and week 4 compared to patients who had a virus that exhibited full resistance, but this trend no longer reached statistical significance. The main factor confounding the association between PI/r resistance levels and virological response was the baseline HIV RNA level. This is because PI-naïve patients (i.e. those with few resistance mutations in their predominant viral species) are also likely to be those with higher baseline HIV RNA levels. In a model

including the REGA GIS there was a 0.34 (0.23 to 0.46) log_{10} larger reduction in HIV RNA from baseline for every log_{10} higher baseline HIV RNA ($p<0.001$).

Similar patterns are seen when a GSS is calculated for the whole regimen (i.e. incorporating the level of resistance to the PI/r into the sensitivity score) to those seen when looking at the level of resistance to the PI/r alone. After adjustment for the baseline HIV RNA level all associations become non-significant (data not shown).

Table 4.4.4.1: Differences in HIV RNA change (ΔVL: BL to week 4) for each GIS According to the PI/r resistance level and number of active ARVs in the regimen

Interpretation System	**Unadjusted Analysis** Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	**P-value**	**Adjusted Analysis** Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	**P-value**
ANRS				
PI/r resistance (R → I → S)	0.19 (0.01, 0.38)	0.04	0.04 (-0.13, 0.22)	0.63
GSS to the rest of the regimen				
For every additional active ARV	0.10 (-0.06, 0.25)	0.21	-0.04 (-0.18, 0.10)	0.59
REGA				
PI/r resistance (R → I → S)	0.20 (-0.01, 0.40)	0.06	0.06 (-0.14, 0.26)	0.54
GSS to the rest of the regimen				
For every additional active ARV	0.11 (-0.03, 0.25)	0.14	-0.04 (-0.18, 0.10)	0.55
DMC				
PI/r resistance (R → I → S)	0.18 (0.03, 0.34)	0.02	0.08 (-0.07, 0.24)	0.29
GSS to the rest of the regimen				
For every additional active ARV	0.10 (-0.03, 0.23)	0.15	-0.05 (-0.19, 0.08)	0.44
Stanford				
PI/r resistance (R → I → S)	0.21 (0.02, 0.40)	0.03	0.07 (-0.12, 0.27)	0.48
GSS to the rest of the regimen				
For every additional active ARV	0.11 (-0.04, 0.25)	0.15	-0.06 (-0.21, 0.09)	0.43

- Coefficients for PI/r resistance represent the average change in HIV RNA per increase in the level of sensitivity to the PI/r (i.e. from resistant to intermediate to sensitive viruses). Therefore patients with a virus that is sensitive to the PI/r have a 0.04x2=0.08 greater log_{10} reduction from baseline to week 4 than patients with a virus resistant to the PI/r using the ANRS GIS (i.e. a difference of 0.04 log_{10} between resistant and intermediate and another 0.04 log_{10} between intermediate and sensitive)
- Coefficients for the number of active drugs in the rest of the regimen represent the average change in HIV RNA reduction for each additional active drug in the regimen
- Adjustments were made for the PI/r used and baseline HIV RNA levels

4.4.5. Prognostic value of GISs for predicting viral load decline at week 12

By week 12 the median (IQR) HIV RNA level was 2.0 (1.7 to 2.8) $\log_{10}$ copies/mL and the median (IQR) HIV RNA reduction from baseline to week 12 was 2.3 (1.4 to 3.0) $\log_{10}$ copies/mL, which was greater than at week 4. These reductions were similar for all PI/rs: 2.2 (1.3 to 2.9) $\log_{10}$ copies/mL (IDV/r), 2.4 (1.4 to 3.1) $\log_{10}$ cps/mL (LPV/r) and 2.3 (1.2 to 3.0) $\log_{10}$ copies/mL (SQV/r). There were 40 (48%), 47 (37%) and 61 (43%) patients who received IDV/r, LPV/r and SQV/r, respectively, who had an undetectable HIV RNA (<50 copies/mL) whose observations have been censored in the censored regression analysis.

Table 4.4.5.1 shows that week 12 HIV RNA reductions were crudely associated with PI/r resistance levels ($p<0.0001$) for all of the GISs and for the number of other active drugs in the regimen ($p<0.01$). All of the GISs showed greater HIV RNA reductions with increasing sensitivity to the PI/r and with an increasing number of other active drugs in the regimen. Patients with a virus considered to be resistant to the PI/r, using the REGA GIS, experienced a mean (95% CI) 1.6 (0.8 to 2.4) $\log_{10}$ decline to week 12, patients harbouring a virus with intermediate resistance had a mean 1.9 (1.2 to 2.6) $\log_{10}$ decline and patients with a sensitive virus had a 2.8 (2.6 to 3.0) $\log_{10}$ copies/mL decline.

In a multivariable analysis, adjusted for baseline HIV RNA levels, the PI/r used and the GSS to the rest of the regimen (using the same GIS that was used to calculate the level of resistance to PI/r), all of the GISs still showed significantly greater HIV RNA reductions as sensitivity to the PI/r increased (Table 4.4.5.1). Baseline HIV RNA levels also confounded the association between PI/r resistance and HIV RNA changes in this comparison, however, adjustment for the baseline HIV RNA did not remove the association between PI/r sensitivity and HIV RNA changes.

In a multivariable model including the REGA GIS there was a 0.57 (0.37 to 0.77) $\log_{10}$, $p<0.0001$, larger reduction in HIV RNA from baseline for every $\log_{10}$ higher baseline HIV RNA. From the model including the REGA GIS, patients with a virus that showed sensitivity to the PI/r had a 0.82 greater $\log_{10}$ reduction between baseline and week 12 compared to patients with a virus that showed full resistance. The number of other active drugs in the regimen did not predict response further for any GIS after adjustments.

When the level of resistance to the PI/r was incorporated into an overall genotypic sensitivity score, there was a 0.13 (-0.03 to 0.29) greater $\log_{10}$ reduction from baseline to week 12 for each unit higher GSS score calculated with ANRS, a 0.10 (-0.04 to 0.25)

greater log_{10} reduction per unit higher GSS score using REGA, a 0.12 (-0.01 to 0.25) greater log_{10} reduction per unit higher GSS score using DMC and a 0.13 (-0.02 to 0.28) greater log_{10} reduction per unit higher GSS score using Stanford, after adjustment for the baseline HIV RNA levels and the PI/r used.

Table 4.4.5.1: Differences in HIV RNA change (ΔVL: BL to week 12) for each GIS According to the PI/r resistance level and number of active ARVs in the regimen

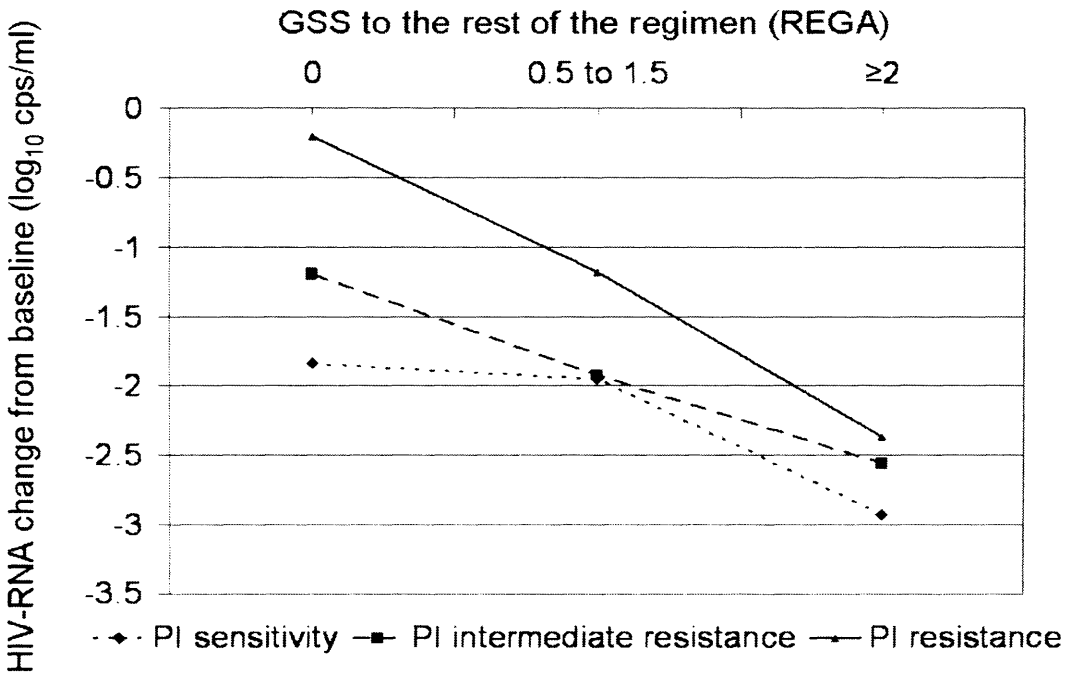
Interpretation System	**Unadjusted Analysis** Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	**P-value**	**Adjusted Analysis** Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	**P-value**
ANRS				
PI/r resistance (R → I → S)	0.60 (0.27, 0.93)	<0.0001	0.37 (0.05, 0.68)	0.02
GSS to the rest of the regimen				
For every additional active ARV	0.40 (0.12, 0.68)	0.006	0.11 (-0.14, 0.37)	0.38
REGA				
PI/r resistance (R → I → S)	0.69 (0.32, 1.06)	<0.0001	0.41 (0.06, 0.77)	0.02
GSS to the rest of the regimen				
For every additional active ARV	0.36 (0.09, 0.63)	0.008	0.04 (-0.22, 0.29)	0.78
DMC				
PI/r resistance (R → I → S)	0.62 (0.34, 0.91)	<0.0001	0.42 (0.14, 0.70)	0.004
GSS to the rest of the regimen				
For every additional active ARV	0.37 (0.12, 0.62)	0.004	0.01 (-0.23, 0.26)	0.91
Stanford				
PI/r resistance (R → I → S)	0.81 (0.47, 1.15)	<0.0001	0.49 (0.14, 0.83)	0.006
GSS to the rest of the regimen				
For every additional active ARV	0.43 (0.16, 0.70)	0.002	0.04 (-0.23, 0.30)	0.78

- Coefficients are interpreted in the same way as in Table 4.4.4.1
- Adjustments were made for the PI/r used and baseline HIV RNA levels

Although the GSS to the rest of the regimen was not related to virological response at week 4 or week 12 for any of the GISs, there were still differences between GISs in the magnitude of their predicted effects (Table 4.4.4.1 and Table 4.4.5.1). At week 4 there was a trend for smaller HIV RNA reductions with an increasing GSS to the rest of the regimen after adjustment. Conversely there was a trend for greater HIV RNA reductions with increasing GSS to the rest of the regimen by week 12. At week 12 each GIS showed greater HIV RNA declines with increasing PI/r sensitivity and with increasing GSS to the

rest of the regimen, although sensitivity to the PI/r was more strongly related to HIV RNA decline. This is illustrated using the REGA GIS in Figure 4.4.5.1.

Figure 4.4.5.1: HIV RNA declines at week 12, using the REGA GIS
According to PI/r resistance levels and the GSS to the rest of the regimen



Number of patients:

PI resistance	3	11	4
PI intermediate resistance	3	14	7
PI sensitivity	13	83	230

4.4.6. Using a fixed GIS to calculate the GSS to the rest of the regimen

Each GIS varies in the way it relates resistance mutations to each ARV, which has resulted in differences between their predicted PI/r resistance levels and their inferred levels of resistance to the other ARVs in the regimen. So far I have adjusted each PI/r model for resistance to the background regimen ascribed by the corresponding GIS. To reduce the noise generated by these background variations I decided to examine the relationship between PI/r resistance levels and virological response after 12 weeks of follow-up, after adjusting for a fixed GSS to the background regimen whilst varying the system used for the PI/r. An analysis adjusted for the REGA GIS rather than each corresponding GIS, in addition to baseline HIV RNA levels and the PI/r used, does not substantially affect the magnitude of the overall estimates (Table 4.4.6.1).

Table 4.4.6.1: Differences in HIV RNA change (ΔVL: BL to week 12) for each GIS: adjusted for the REGA GSS

Interpretation System	Adjusted Analysis Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	P-value
ANRS PI/r resistance (R → I → S)	0.37 (0.05, 0.70)	0.02
GSS to the rest of the regimen (REGA) For every additional active ARV	0.04 (-0.22, 0.29)	0.77
REGA PI/r resistance (R → I → S)	0.41 (0.06, 0.77)	0.02
GSS to the rest of the regimen (REGA) For every additional active ARV	0.04 (-0.22, 0.29)	0.78
DMC PI/r resistance (R → I → S)	0.43 (0.15, 0.71)	0.003
GSS to the rest of the regimen (REGA) For every additional active ARV	0.00 (-0.26, 0.25)	0.98
Stanford PI/r resistance (R → I → S)	0.51 (0.16, 0.86)	0.004
GSS to the rest of the regimen (REGA) For every additional active ARV	-0.01 (-0.27, 0.25)	0.92

- Coefficients are interpreted in the same way as in Table 4.4.4.1
- Adjustments were made for the PI/r used and baseline HIV RNA levels

4.4.7. Sensitivity analysis excluding PI-naïve patients

Since the GISs were created using resistance data from drug-experienced patients I repeated this analysis on a subgroup of 201 PI-experienced patients to see whether our results were consistent with those in this population. PI-experienced patients display higher levels of baseline resistance in their virus than PI-naïve patients; however, there was still one (1%) PI-naïve patient who had a virus with a resistance mutation to the PI/r they received at baseline. Conversely, 40 (20%) PI-experienced patients had a virus with a major IAS-USA PI-resistance mutation to a PI/r they were receiving at baseline. There were 117 (58%), 106 (53%), 108 (54%) and 99 (59%) patients with a virus that was susceptible to ≥2 ARVs in their background regimen using ANRS, REGA, DMC and Stanford, respectively. Although PI-experienced patients had higher average levels of resistance in their virus to all of the ARVs they were receiving at baseline compared to those in our previous analyses, these comparisons were performed on a reduced number of patients and therefore they have limited power.

Overall, the magnitude of the difference in HIV RNA reductions between the ascribed PI/r resistance levels for each GIS was similar to the comparisons including both PI-experienced and PI-naïve patients. However, these differences no longer reached statistical significance (Table 4.4.7.1).

Table 4.4.7.1: Differences in HIV RNA change (ΔVL: BL to week 12) for each GIS: PI-experienced patients

Interpretation System	**Unadjusted Analysis** Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	**P-value**	**Adjusted Analysis** Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	**P-value**
ANRS				
PI/r resistance (R → I → S)	0.40 (0.04, 0.76)	0.03	0.30 (-0.04, 0.64)	0.08
GSS to the rest of the regimen				
For every additional active ARV	0.32 (-0.02, 0.65)	0.06	0.07 (-0.23, 0.38)	0.63
REGA				
PI/r resistance (R → I → S)	0.46 (0.06, 0.87)	0.03	0.30 (-0.08, 0.69)	0.13
GSS to the rest of the regimen				
For every additional active ARV	0.24 (-0.09, 0.57)	0.15	-0.04 (-0.35, 0.27)	0.78
DMC				
PI/r resistance (R → I → S)	0.46 (0.13, 0.79)	0.007	0.31 (-0.02, 0.64)	0.07
GSS to the rest of the regimen				
For every additional active ARV	0.32 (0.01, 0.63)	0.04	0.00 (-0.31, 0.31)	0.99
Stanford				
PI/r resistance (R → I → S)	0.60 (0.21, 0.98)	0.003	0.33 (-0.06, 0.73)	0.10
GSS to the rest of the regimen				
For every additional active ARV	0.36 (0.02, 0.70)	0.04	0.00 (-0.33, 0.34)	0.98

- Coefficients are interpreted in the same way as in Table 4.4.4.1

4.4.8. Other mutations and virological response at week 4 and week 12

Although there is an issue of multiple testing, I then looked at the relationship between individual protease mutations (according to the IAS-USA 2006 guidelines[233]) and HIV RNA response at both time points to see if these trends can be attributed to the presence of any specific mutation. In this comparison I found strong associations between mutations at positions 10, 24, 46, 47, 54, 82 and 90 with HIV RNA declines (Table 4.4.8.1). The presence of these mutations was associated with smaller decreases in HIV RNA, but prevalence rates of these mutations were still low in the virus populations of these patients. After adjustment for baseline HIV RNA, many of these trends disappeared.

Table 4.4.8.1: Relationship between individual PR mutations and viral load change

	Predicts week 4 response				Predicts week 12 response			
Mutation Present	**Unadjusted Analysis** Additional HIV RNA reduction (log_{10} cps/mL)	**P-value**	**Adjusted Analysis** Additional HIV RNA reduction (log_{10} cps/mL)	**P-value**	**Unadjusted Analysis** Additional HIV RNA reduction (log_{10} cps/mL)	**P-value**	**Adjusted Analysis** Additional HIV RNA reduction (log_{10} cps/mL)	**P-value**
IAS-USA mutations								
L10F/I/R/V	-0.10 (-0.33, 0.13)	0.38	-		-0.52 (-0.82, -0.21)	0.001	-0.38 (-0.64, -0.12)	0.004
K20R/M/I/L/T	-0.17 (-0.44 0.10)	0.22	-		-0.23 (-0.59 0.14)	0.23	-	
L24I	-1.12 (-2.18, -0.07)	0.03	-		-1.80 (-3.22, -0.39)	0.01	-	-
D30N	-0.30 (-0.66, 0.06)	0.10	-		-0.36 (-0.85, 0.13)	0.15	-	
V32I	-0.97 (-2.03, 0.08)	0.07	-		-1.19 (-2.61, 0.23)	0.10	-	
L33I/F	0.61 (-0.15, 1.37)	0.11	0.67 (-0.01, 1.35)	0.05	0.29 (-0.73, 1.31)	0.57	-	
M36I/L/V	-0.02 (-0.22, 0.18)	0.85	-		-0.09 (-0.36, 0.19)	0.53	-	
M46I/L	-0.23 (-0.55, 0.09)	0.17	-		-0.79 (-1.22, -0.36)	<0.001	-0.33 (-0.70, 0.05)	0.09
I47A/V	-1.17 (-2.47, 0.12)	0.08	-		-1.73 (-3.47, 0.00)	0.05	-1.26 (-2.71, 0.19)	0.09
G48V	-0.18 (-1.60, 1.25)	0.81	-		-0.78 (-2.69, 1.13)	0.42	-	
F53L	-0.19 (-1.11, 0.73)	0.69	-		-0.41 (-1.65, 0.83)	0.51	-	
I54V/L/A/M/T/S	-0.19 (-0.57, 0.19)	0.33	-		-0.64 (-1.15, 0.13)	0.02	-	-
L63A/P	-0.06 (-0.26, 0.14)	0.58	-		0.02 (-0.25, 0.30)	0.86	-	
A71V/T	0.01 (-0.24, 0.26)	0.93	-		-0.31 (-0.65, 0.03)	0.07	-	
G73C/S/T/A	0.11 (-0.56, 0.77)	0.75	-		0.24 (-0.66, 1.13)	0.61	-	
V77I	0.05 (-0.16, 0.27)	0.63	-		0.04 (-0.33, 0.26)	0.82	-	
V82A/F/T/S	-0.30 (-0.67, 0.08)	0.12	-		-0.71 (-1.21, -0.22)	0.005	-	-
I84V/A/C	-0.09 (-0.73, 0.55)	0.77	-		-0.46 (-1.32, 0.39)	0.29	-	-
N88D/S	-0.18 (-0.60, 0.24)	0.40	-		-0.41 (-0.98, 0.16)	0.16	-	-
L90M	-0.23 (-0.55, 0.08)	0.14	-		-0.73 (-1.15, -0.30)	0.001	-0.38 (-0.74, -0.02)	0.04
Other PR mutations (found in >5% of patients)								
S37D	-0.13 (-0.43, 0.16)	0.37	-		-0.45 (-0.85, -0.04)	0.03	-0.36 (-0.69, -0.02)	0.04

- Mutations in **bold** indicate primary PI mutations to one of the three randomised PIs according to the IAS-USA mutational lists[233]
- Analyses are adjusted for the PI/r received and baseline HIV RNA

I then identified other protease mutations that were not included in any of the GISs, but were observed in the sampled virus of > 5% of our patients: Table 4.4.8.1 shows that the

only additional mutation associated with virological response was the S37D mutation. Since the number of patients who had a virus containing each mutation was small, I investigated the combined number of protease mutations (including mutations listed in the IAS-USA guidelines in addition to the S37D mutation) to see whether an accumulation of mutations has a significant effect on HIV RNA reduction.

The number of PI mutations did not have a predictive effect on HIV RNA change after four weeks of follow-up (at week four there was a 0.01 (-0.06 to 0.08) $\log_{10}$ cps/mL greater reduction in HIV RNA for each additional PI mutation, p=0.83), but there was a strong effect after 12 weeks (at week 12 there was a -0.19 (-0.06 to -0.31) $\log_{10}$ copies/mL greater reduction i(i.e. a smaller reduction) in HIV RNA for each additional PI mutation, p=0.004), after adjustment.

4.5. Discussion

4.5.1. Summary of main results

Drug resistant HIV can adversely impact on virological and clinical outcomes in both treated and untreated individuals[196;348;349]. ARV-experienced patients may have resistance from a previous regimen whereas untreated individuals may be infected with a virus that already contains mutations. Genotyping tells us the genetic code of the virus, which is conveyed for analysis in the form of a list of mutations that are present in the majority virus of a patient, so they can be translated into a sensitivity score using a GIS.

In this analysis I compared four GISs, using data from three trials, where each GIS contained three resistance levels (i.e. resistant, intermediate resistance or sensitive). Randomisation in randomised controlled trials (RCTs) ensures there are no systematic differences between treatment arms apart from the treatment received, so any differences observed in the outcome can be attributed to the treatment specifically[350]. However, any balances stemming from the randomised nature of the trials do not extend to patients with resistance testing. Moderate concordance was seen between GISs in their predicted levels of resistance to a PI/r (kappas ranged from 0.37 to 0.75 for IDV/r; 0.46 to 0.93 for LPV/r; and 0.38 to 0.68 for SQV/r).

I then examined whether any interpretational differences impacted on the ability of each system to predict short term virological response. In this comparison none of the GISs were associated with significantly greater HIV RNA reductions as sensitivity to the PI/r

increased after four weeks of follow-up, but after 12 weeks the ascribed level of sensitivity to the PI/r was related to HIV RNA change for all of the GISs. From the model including the REGA GIS, patients who had a virus that showed sensitivity to the PI/r had a 0.82 greater $\log_{10}$ reduction between baseline and week 12 compared to patients who had a virus that showed full resistance, p=0.02. The GSS to the rest of the regimen was not associated with virological response at either time point.

4.5.2. Comparison with other studies

Other studies that have evaluated concordance performed their analysis on more GISs, investigated a larger number of ARVs or compared GISs containing additional levels of resistance, which resulted in more discordance overall[228;229]. Ravela *et al* investigated concordance between ANRS, REGA, Stanford and Visible Genetics (VGI) and found discordance between 33.6% of interpretations, mainly driven by the NRTIs. Amongst the PI/rs, they found similar kappas for IDV/r (from 0.61 (REGA *vs* Stanford) to 0.80 (REGA *vs* ANRS)), lower kappas for LPV/r (from 0.40 (ANRS *vs* Stanford) to 0.67 (REGA *vs* ANRS)), but higher kappas for SQV/r (from 0.63 (REGA *vs* Stanford) to 0.77 (ANRS *vs* Stanford))[230] compared to this study. However, they defined discordance as one GIS assigning a resistant score and another a sensitive score. If a patient showed intermediate viral resistance they were considered neutral and no discordance between either resistant or sensitive strains was noted. This re-categorisation would lead to larger kappa values in the Ravela study compared to ours.

The Ravela comparison uses GISs that have since been updated to account for the latest developments in research and, in some cases, to account for the use of ritonavir boosting. Originally GISs were created from the same data, some with overlapping expert interpreters, so interpretations were comparable. As time progressed more drugs entered the market, more resistance mutations were identified and opinions on what mutations contributed towards resistance started to diverge. In addition only some GISs took account of the clinically derived GSSs that were published in recent years (i.e. those that looked at viral load response)[330;333;351;352]. ARVs are now used more frequently so there has been more of a consensus on what mutations indicate resistance. There has also been a concerted effort to improve agreement between GISs. This converging of opinions could have resulted in higher kappa values in our study, but that was not the case either.

Our analysis includes the DMC GIS, the system that caused most of the disagreements between GISs. Removing DMC from the comparisons increases our overall concordance

to 89%, bringing our findings closer to the Ravela study. If I consider 'intermediate resistance' to be consistent with either sensitive or resistant interpretations then concordance increases further to >98%. The higher concordance found when our comparisons are identical to the Ravela comparisons may reflect a converging of opinions regarding resistance within experts. This is especially true for LPV/r which has become more widely used since the Ravela study, possibly reflected by the larger kappa values in our comparisons. The lower concordance for SQV/r in our analysis may reflect uncertainty on how to interpret the impact of ritonavir boosting on resistance.

In a recent study by Maillard *et al*, the GSSs as defined by Stanford and ANRS were predictive of the 24-week virological response to LPV/r in an ARV-experienced population[353]. Since virological outcomes are pivotal for testing the clinical use of GISs I examined the relationship between PI/r resistance levels and HIV RNA change at two time points: week four and week 12. In these analyses, differences in HIV RNA reductions between ascribed resistance levels did not become apparent until week 12. The majority of patients in these comparisons initiated a completely new regimen at baseline so the majority virus should have been susceptible to most of the ARVs in the regimen. As a result, it may take longer than four weeks for the impact of baseline mutations on viral replication to become apparent. Since HIV RNA does not decline linearly with time, in fact it starts to increase in some patients on salvage therapy after 4 weeks of follow-up, it would be useful to reach a consensus on when to expect a virological response[193].

HIV RNA was reduced by 1.8 (1.2 to 2.3) and 2.3 (1.4 to 3.0) log copies/mL from baseline after 4 and 12 weeks, respectively. These reductions are larger than in other studies comparing GISs for predicting viral load decline[193;197;354], but these other studies were conducted on patients who were receiving salvage therapy and were less likely to experience large reductions. The HIV RNA reductions are greater in this analysis because this population contains more varied patients, including patients who were naïve to ARV therapy, failing a first-line regimen or failing a later regimen. When I examined HIV RNA declines at week 12 according to previous PI exposure, HIV RNA was reduced by 2.7 (2.0 to 3.2) and 1.9 (1.0 to 2.7) log copies/mL from baseline among patients who were PI-naïve and those who were PI-experienced, respectively.

4.5.3. Interpretation of results

Although each GIS considers different mutations to be relevant for each ARV (Figure 4.4.2.1 and Figure 4.4.2.2), they all predicted virological response to a similar extent.

None of the GISs showed significantly greater HIV RNA reductions as sensitivity to the PI/r increased by four weeks of follow-up, but after 12 weeks the ascribed level of sensitivity to the PI/r was related to HIV RNA change for all of the GISs. In multivariable analyses the GSS to the rest of the regimen was not associated with virological response at either time point, although at week 12 there was a trend for greater reductions with an increasing number of active drugs in the background regimen. These results are consistent with a study by Miller *et al* and a recent study by Rhee *et al* where resistance to a PI/r was shown to be a stronger predictor for virological failure at week 24 than NRTI resistance or NNRTI resistance[297;355].

On the other hand, De Luca *et al* found that the GSS to the whole regimen (including the PI/r) did not predict response to a PI/r-containing regimen[354], but the independent effect of PI/r sensitivity may have been overshadowed by sensitivity to the rest of the regimen in that study since the regimen was studied as a whole rather than PI/rs separately. This is similar to the findings of this study when I combined the PI/r resistance levels with the GSS for the rest of the regimen, after adjustment for the baseline HIV RNA.

Although the current GISs are able to identify differences in viral load response according to their ascribed PI/r resistance levels, patients with a virus that was deemed to be fully resistant to the PI/r still experienced large HIV RNA reductions (i.e. a reduction of >1 $\log_{10}$ copies/mL). PI/rs may exert antiviral effects in the presence of resistance; however, this observation is probably, in part, due to susceptibility to the nucleoside backbone. Using the REGA GIS, the mean (95% CI) GSS to the rest of the regimen for patients with a virus that was considered to be resistant to the PI/r was 0.9 (0.5 to 1.3), N=18. Indeed, if a patient had a high GSS to the other drugs in their regimen they still experienced large HIV RNA reductions despite the presence of PI/r resistance (Figure 4.4.5.1), but this trend was only seen in a small number of patients (N=4). Patients with a virus that was fully resistant to all ARVs in their regimen (i.e. the background ARVs and the PI/r) only experienced small HIV RNA declines consistent with the predicted activity of the regimen.

In our analysis >70% of patients had a virus that was susceptible to the PI/r they were receiving at baseline. In these patients the GSS to the rest of the regimen was not an important determinant of HIV RNA decline, probably because sensitivity to the PI/r was the major factor influencing response. Even though GISs vary on what resistance mutations they consider to be important, as reflected by the low-to-moderate kappa values, the relationship between resistance and HIV RNA change for each GIS illustrates that GISs

effectively discriminate viruses according to sensitivity to PI/rs. This is somewhat surprising considering that the DMC GIS had not yet been updated to take into account ritonavir-boosting.

I assessed the relationship between individual mutations and virological response for all mutations that were included in any of the GISs. The presence of these mutations was associated with a poorer virological response in most cases; however, not all associations were significant, possibly because some mutations were too infrequent to quantify their effects accurately. At week 4 no other protease mutations than those included in the GISs were related to virological response, but by week 12 the S37D mutation was significantly associated with a poorer response in both unadjusted and adjusted analysis (Table 4.4.8.1).

Marcelin *et al* found a strong association between the decrease in HIV RNA and the number of mutations observed at codons 24, 62, 82, 84 and 90 for SQV/r[330]. In this analysis I found mutations at positions 10, 24, 46, 47, 54, 82 and 90 to be significantly associated with response. Since there were a limited number of people who had a virus containing each mutation, I investigated a combination of protease mutations (i.e. the total number of IAS-USA protease mutations plus the S37D mutation) to see whether accumulation of mutations had a significant effect on HIV RNA reduction. The overall number of protease mutations was significantly associated with response at week 12, but not at week 4. This is consistent with other studies where the number of protease mutations has been associated with response to PI/rs[330;335;356].

4.5.4. Limitations of analysis

This analysis has several limitations: I do not have a measure of adherence in these patients and since the predictive ability of the GISs is stronger in adherent patients[354] non-adherence could dilute the strength of association between each GIS and response. Pharmacokinetic analysis after four weeks of follow-up revealed high PI PK drug levels in the majority of patients in these trials (Table 3.4.2.1), reflecting good adherence overall; however, adherence rates are known to diminish with time on a regimen[357]. I examined virological response after 4 and 12 weeks follow-up and found a stronger association between the PI/r resistance levels and the 12 week HIV RNA decline compared to week 4. This indicates continuously high levels of adherence in these patients. Another limitation is that a large proportion of our study population is PI-naïve and will respond to therapy differently to PI-experienced patients. This analysis probably should have been restricted

to PI-experienced patients because the GISs were created from data derived from patients who were receiving PIs and are designed for use in PI-experienced patients. However, when I repeated the analysis in the subgroup of PI-experienced patients I found similar trends.

A wider limitation of genotypic resistance testing concerns the practical implications of dealing with consensus sequences rather than clones (i.e. a procedure of obtaining the sequences of a single cloned viral genome rather than the consensus sequences derived from multiple genomes). Use of consensus sequencing produces a consensus sequence from the dominant quasi-species in circulation; therefore some of the clinically significant resistant mutations present at baseline, at levels below 20 to 25% of the virus population, may be missed. This could also be a problem with clones unless a large enough number of clones from different PCR reactions are sequenced. The presence of low-frequency or archived species is a major problem with building GISs in general.

Treatment success beyond the first few weeks of the start of a regimen is likely to be influenced by the low frequency viral populations and therefore these may need to be incorporated into a GIS in the future if sufficiently sensitive resistance testing approaches can be developed (e.g. using ultrasentive detection methods for resistance for low frequency mutations and testing PBMC as well as plasma for archived resistance). Alternatively, another resistance test could be performed after four weeks of follow-up when the minority species has had more chance to replicate. A more simple approach to maximise the benefit of GISs is to combine resistance history and treatment history into the interpretation system[223]. It may be necessary to consider all previous causes of treatment failure in order to optimise treatment. This is currently done in routine clinical practice.

4.5.5. Conclusion

To conclude, differences exist between GISs in their ascribed levels of resistance to a PI/r; however, the level of disagreement for predicting short-term HIV RNA change is relatively minor. Although patients with a virus that is deemed to be sensitive to the drugs in their regimen experience the largest HIV RNA declines for all of the interpretation systems, GISs still need further refinement to improve discrimination and concordance. Observational data from cohort studies will enable us to repeat these analyses on the newer PI/rs (i.e. atazanavir, darunavir, tipranavir and fos-amprenavir) to see whether the same trends are seen in patients receiving these drugs.

CHAPTER 5: THE ABILITY OF FOUR GISs TO PREDICT VIRAL LOAD RESPONSE TO RITONAVIR-BOOSTED PIS, USING OBSERVATIONAL DATA

5. Introduction

Data from observational studies differ to data from randomised controlled trials (RCTs) in many ways. The main advantage of RCTs is that they remove bias from treatment comparisons and ensure comparable groups at study entry. A major advantage of observational studies is that they best reflect what happens in clinical practice[350;358]. In the previous chapter I compared the prognostic value of four GISs for predicting viral load response to three PI/rs using data from three RCTs. RCTs include pre-scheduled follow-up visits and, as a result, the HIV RNA declines seen in the trials may not reflect those seen in clinical practice. The ascribed PI/r resistance levels were related to virological response after 12 weeks of follow-up, but not after four weeks. A key limitation of the results in the last chapter was that only a low number of patients had a virus containing resistance mutations at baseline.

5.1. Aims of the chapter

To increase the number of patients who could be included in these comparisons I repeated the 12 week analysis on patients from EuroSIDA to see whether the same patterns are observed in patients who are followed up less frequently, due to the nature of the study. If no major differences are observed, it will be possible to combine datasets and increase the overall power of these comparisons. As a result, I will refer back to the previous chapter throughout the results section of this chapter. This chapter examines whether GISs can predict viral load response to a PI/r containing regimen in both an observational study (EuroSIDA) and the trials, combined.

5.2. Literature review

In the previous chapter, the resistance data from the trials can be considered to be pseudo-observational because the balances stemming from the randomised nature of the trials do not extend to patients with resistance testing. So, repeating this analysis on observational data is unlikely to amount in additional bias. In an observational setting, it is likely that systematic differences exist between the groups being compared and, even though variations between known factors can be adjusted for in a multivariable analysis, it is not always possible to account for any unknown or unmeasured confounding factors[359-362].

There are some advantages to analysing observational data: patients are usually representative of those seen in routine clinical practice so they reflect what happens in real clinical situations[363-365]. In addition, patient characteristics are monitored over a longer period of time than in most trials, so observational data also enable us to investigate the long term response to treatment. Although follow-up is longer in observational studies than in trials, patients are usually not followed up as frequently or regularly. This can result in differences between observational studies and trials in their reported incidence and prevalence rates of virological and immunological events[366-369].

Irregular follow-up visits also pose a problem for choosing a time point to best measure virological response to a regimen because, even though linear interpolation or other methods can be used to account for missing measurements at various time points[366;370-376], they may not accurately reflect the actual viral load values at these different times. In the MaxCmin and COLATE trials, the clinical visits were scheduled after four and 12 weeks and then at 12 week intervals thereafter. This analysis investigates the virological response after 12 weeks of follow-up (range: 4 to 24 weeks) for patients who started a PI/r containing regimen in EuroSIDA, because PI/r resistance levels were more powerful at predicting the response at week 12 compared to week 4 in the previous chapter.

5.3. Methods

Since these data are observational there were no restrictive entry criteria for inclusion in the study, so patients are likely to have more heterogeneous characteristics (i.e. the number and type of background ARVs is likely to be more varied and patients are likely to have a virus that shows a larger degree of resistance to all of the ARVs in their regimen) than the trial patients. Repeating the analysis on patients who have a virus that contains a higher level of resistance will provide more power to determine whether there are real differences in viral load response according to the ascribed PI/r resistance levels and whether differences do, in fact, exist between GISs.

Repeating this analysis on observational data also meant it was possible to include more up-to-date regimens. This analysis involves a greater number of PI/rs than in the previous chapter, some of which have recently entered the market. Patients in EuroSIDA who started APV/r, ATV/r, IDV/r, LPV/r or SQV/r were included in these comparisons.

5.3.1. EuroSIDA

Details of EuroSIDA have been described in section 2.2.1. At each visit, all $CD4^+$ T cell counts and HIV RNA levels taken since the last follow-up visit were collected and dates of starting and stopping each ARV were recorded. Out of the 11928 patients with available clinical data when the database was closed for analysis, in July 2005, there were 3893 (32.6%) who had ever started a PI/r.

5.3.2. Virological response

Since HIV RNA measurements are taken less frequently in observational studies compared to the trials I considered any HIV RNA value recorded in the three months prior to and up to one week after starting a PI/r to correspond to the baseline HIV RNA level. The HIV RNA value that was recorded closest to baseline was considered to be the baseline HIV RNA as long as it was measured before PI/r initiation. For seven patients, no HIV RNA data was recorded in the three months preceding the start of a PI/r, but was available in the week after. Since HIV RNA is likely to change in the week following initiation of a new regimen, the HIV RNA measured in the week following initiation was taken as the baseline HIV RNA, under the assumption that it was actually taken before the start of the regimen, but the dates of entry were inaccurate in the database.

HIV RNA response was measured 12 weeks (range: 4 to 24 weeks) after PI/r initiation. If a patient had more than one HIV RNA measurement in the follow-up window then the value taken closest to 12 weeks was included in this analysis.

Since resistance testing is unlikely to coincide with the date that the PI/r was started, the latest resistance test taken in the year prior to starting a PI/r was included as the baseline resistance test in this analysis. A sensitivity analysis was performed where this restriction was reduced to six months.

5.3.3. Laboratory methods

In EuroSIDA, virological sequencing was either performed at the IrsiCaixa Foundation (Badalona, Spain) or at the International Clinical Virology Center, depending on the time of the resistance test (section 2.2.1.3). Sample mix-up or lab-contaminated samples were identified by generating phylogenetic trees.

5.3.4. Genotypic interpretation systems

Genotypes were provided as amino acid substitutions from HXB2 and the GISs used to ascribe resistance levels were the same as in 4.3.2

5.3.5. Statistical analysis

Concordance between ascribed PI/r resistance levels for each GIS was evaluated using kappa statistics in the same way as 4.3.4. I related these ascribed PI/r resistance levels to HIV RNA reductions from baseline after 12 weeks follow-up. In EuroSIDA the most commonly used HIV RNA assay has a lower limit of quantification of 50 copies/mL; however, some sites use a more sensitive HIV RNA assay that can quantify virus below that threshold. In these cases I modelled the full HIV RNA reductions and did not consider the patient to have a censored HIV RNA measurement. All variables associated with HIV RNA decline in an unadjusted censored regression analysis were entered into separate multivariable models for each GIS. Each multivariable model is adjusted for the GSS to the rest of the regimen calculated using the same GIS.

5.4. Results

5.4.1. Patient characteristics

Out of the 11928 patients who were enrolled into EuroSIDA, there were 4968 (42%) patients who had plasma samples stored for genotypic resistance testing at some point during follow-up. Overall, 3893 patients received a PI/r and of these, 475 (12%) had a resistance test in the year prior to PI/r initiation. A total of 418 of 475 (88%) patients had HIV RNA values available at baseline (-3 months to +1 week) and 376 (79%) also had HIV RNA values available between 4 and 24 weeks after starting the PI/r. The 376 patients who were included in these comparisons started one of the following single PI/rs (Table 5.4.1.1): APV/r (N=28, 7%), ATV/r (N=29, 8%), IDV/r (N=55, 15%), LPV/r (N=231, 61%) and SQV/r (N=33, 9%). Of these, 301 (80%) patients were PI-experienced and the other 75 (20%) were PI-naïve.

Patients were primarily male (77%) and were a median age of 41 years (IQR: 37 to 48 years). Patients in EuroSIDA had been infected with HIV for longer than those in the trials: more patients had an AIDS defining event (34% versus 29%) and a greater number had been exposed to PIs prior to entry (80% versus 55%). This wider treatment use resulted in patients having their HIV-infection more under control, as reflected by the higher $CD4^+$ cell counts and lower HIV RNA levels. Median $CD4^+$ cell counts and HIV RNA levels in the

EuroSIDA population were 260 (IQR: 160 to 406) cells/μL and 4.0 (IQR: 3.2 to 4.9) log_{10} cps/mL, respectively, whereas in the trials the equivalent baseline $CD4^+$ cell counts and HIV RNA levels were 223 (IQR: 100 to 360) cells/μL and 4.7 (IQR: 3.9 to 5.2) log_{10} cps/mL, respectively.

Table 5.4.1.1: Baseline characteristics for the 376 patients in EuroSIDA

Baseline parameter	APV/r N=28	ATV/r N=29	IDV/r N=55	LPV/r N=231	SQV/r N=33
Gender No. Male, (%)	24 (86)	17 (59)	50 (91)	179 (77)	21 (63)
Age Med yrs (IQR)	39 (35, 49)	41 (35, 44)	40 (35, 42)	42 (38, 49)	41 (36, 47)
Antiretroviral naïve N, (%)	0 (0)	0 (0)	7 (13)	7 (3)	9 (27)
PI-naïve N, (%)	0 (0)	5 (17)	12 (22)	29 (13)	6 (18)
PI-experienced N, (%)	28 (100)	24 (83)	36 (65)	195 (84)	18 (55)
CDC, cat. C N, (%)	16 (57)	6 (21)	19 (35)	77 (33)	8 (24)
Calendar year of starting the PI/r Med month, yr (IQR)	May 00 Dec 99, Jan 02	Aug 04 Oct 03, Sep 04	Apr 00 Jul 99, Jan 01	Aug 02 May 01, Oct 03	Jun 00 Jun 99, Jan 01
HIV RNA (cps/mL log_{10}) Med (IQR)	4.0 (3.5, 5.0)	4.2 (2.8, 5.0)	4.3 (3.2, 4.8)	4.0 (3.2, 4.9)	3.7 (3.1, 4.9)
CD4+ (10^6/l)* Med (IQR)	198 (72, 357)	317 (174, 435)	255 (170, 392)	260 (153, 410)	249 (192, 406)
CD4+ nadir (10^6/l) Med (IQR)	20 (9, 77)	116 (30, 160)	150 (69, 224)	81 (26, 180)	143 (85, 202)
HIV exposure group N, (%)					
Homosexual/bisexual	14 (50)	15 (52)	29 (53)	112 (48)	14 (42)
IVDU	6 (21)	6 (21)	10 (18)	43 (19)	7 (21)
Heterosexual	4 (14)	8 (28)	14 (25)	61 (26)	9 (27)
Other/unknown	4 (14)	0 (0)	2 (4)	15 (6)	3 (9)
Race N, (%)					
White	24 (86)	20 (69)	46 (84)	187 (81)	25 (76)
Black	1 (4)	2 (7)	4 (7)	16 (7)	3 (9)
Other/unknown	3 (11)	7 (24)	5 (9)	28 (12)	5 (15)
Any NRTI mutation* N, (%)	27 (96)	19 (66)	35 (64)	189 (82)	18 (55)
Any NNRTI mutation* N, (%)	17 (61)	21 (72)	18 (33)	106 (46)	7 (21)
Any primary PI mutation* N (%)	24 (86)	9 (31)	17 (31)	121 (52)	10 (30)

* Refers to any major mutation as defined in the IAS-USA 2006 tables[233]

Overall, 288 (77%), 169 (45%) and 181 (48%) patients had a virus showing a major IAS-USA resistance mutation (Sept 2006)[233] to an NRTI, NNRTI or PI, respectively. Patients started a variable number of ARVs in addition to the PI/r component of the regimen, with the majority (N=231, 61%) starting two other ARVs. There were 35 (9%) patients who received fewer than two background ARVs and the remaining 110 (29%) patients received ≥3 background ARVS.

5.4.2. Concordance of the interpretation of genotypic data by all GISs

At baseline, 363 patients received an NRTI, 100 received an NNRTI and 376 received a PI/r. There were 220 (61%) patients with a virus that showed an IAS-USA (Sept 2006)[233] resistance mutation to one of their baseline NRTIs, 27 (27%) with a virus that showed a resistance mutation to a baseline NNRTI and 92 (24%) with a virus that showed a major resistance mutation to a baseline PI/r. These levels of resistance are higher than those seen in the trials where there were 151 (41%), 4 (11%) and 41 (11%) patients with a virus that showed a major resistance mutation to an NRTI, NNRTI or PI they were receiving at baseline, respectively.

Using the REGA GIS (version 6.4), there were 68 (18%) patients with a virus that showed full or intermediate resistance to the PI/r they were receiving at baseline (N=9 (32%), N=2 (7%), N=10 (18%), N=44 (19%) and N=3 (9%) patients on APV/r, ATV/r, IDV/r, LPV/r and SQV/r, respectively). In the same way as for the trial data ANRS (version 13) calculated comparable levels of resistance to REGA (version 6.4) but both DMC (October 2004) and Stanford (version 4.2.0) predicted over double the number of patients with a virus exhibiting full or intermediate resistance to their baseline PI/r. This comparison involves a greater number of PI/rs than in the previous chapter. Large differences occur between GISs in the ascribed level of resistance to the newer PI/rs, although recently there is a growing consensus on which mutations indicate resistance to these drugs. The breakdown of resistance by PI/r and GIS is shown in Table 5.4.2.1.

Table 5.4.2.1: Patients with a virus exhibiting full or intermediate baseline resistance to the PI/r according to each GIS

	Genotypic Interpretation System			
	ANRS N (%)	**DMC** N (%)	**REGA** N (%)	**Stanford** N (%)
PI/r				
APV/r (N=28)	3 (11)	17 (61)	9 (32)	23 (82)
ATV/r (N=29)	2 (7)	9 (31)	2 (7)	8 (28)
IDV/r (N=55)	14 (25)	16 (29)	10 (18)	12 (22)
LPV/r (N=231)	31 (13)	80 (35)	44 (19)	96 (42)
SQV/r (N=33)	2 (6)	9 (27)	3 (9)	7 (21)
Any PI/r (N=376)	52 (14)	131 (35)	68 (18)	146 (39)

Overall concordance between GISs on the predicted level of resistance to the combined PI/rs was low-to-moderate. Kappas ranged from 0.01 to 0.38 for APV/r; 0.31 to 1.00 for ATV/r; 0.48 to 0.79 for IDV/r; 0.34 to 0.77 for LPV/r; and 0.30 to 0.57 for SQV/r (Table 5.4.2.2).

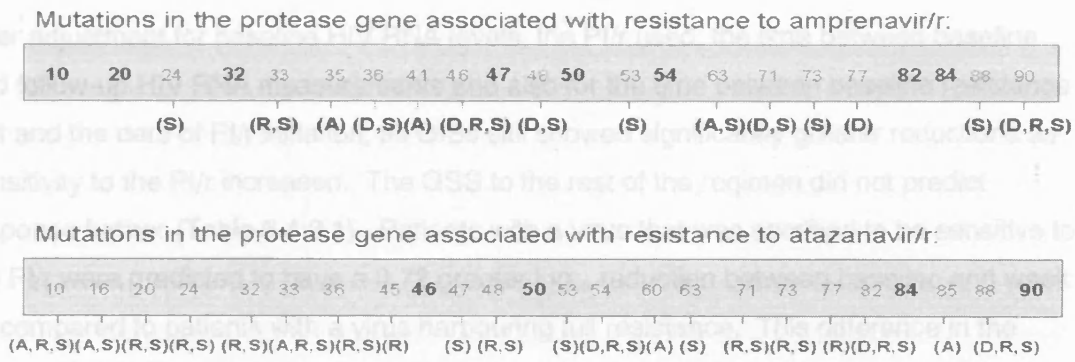
Table 5.4.2.2: Kappa values to illustrate concordance between GISs

		REGA	DMC	Stanford
APV/r	**ANRS**	0.18	0.17	0.08
	REGA	-	0.06	0.01
	DMC	-	-	0.38
ATV/r	**ANRS**	1.00	0.31	0.35
	REGA	-	0.31	0.35
	DMC	-	-	0.92
IDV/r	**ANRS**	0.65	0.48	0.68
	REGA	-	0.67	0.79
	DMC	-	-	0.52
LPV/r	**ANRS**	0.77	0.36	0.34
	REGA	-	0.50	0.48
	DMC	-	-	0.65
SQV/r	**ANRS**	0.57	0.31	0.30
	REGA	-	0.35	0.45
	DMC	-	-	0.47

Poor $\kappa \leq 0.2$; Fair $0.21 \leq \kappa \leq 0.40$; Moderate $0.41 \leq \kappa \leq 0.60$; Good $0.61 \leq \kappa \leq 0.80$ and Very Good $\kappa > 0.80$

You will recall that Figure 4.4.2.2 illustrates the codon positions that cause discordance between resistance levels for IDV/r, SQV/r and LPV/r. Figure 5.4.2.1 shows that there is also a high level of discordance between GISs when examining interpretations for APV/r and ATV/r separately. The GISs vary on what codons and what amino acid substitutions they consider to be associated with resistance to each PI/r and they also account for the mutations differently, by assigning them different weights. This has not been accounted for in these figures.

Figure 5.4.2.1: Mutational discrepancies between GISs: for APV/r and ATV/r



5.4.3. Prognostic value of GISs for predicting viral load decline

The median (IQR) baseline HIV RNA was 4.0 (3.2 to 4.9) and after a median 13 (IQR: 9 to 17) weeks from the start of a PI/r containing regimen this was reduced by a mean 1.8 (95% CI: 1.7 to 1.9) $\log_{10}$ cps/mL. This reduction is smaller than that seen in the trials, presumably because more patients in EuroSIDA had a virus with resistance mutations to their ARVs, so it is expected that their virus would not be as susceptible to treatment.

The HIV RNA reduction was similar for patients using each PI/r: 1.5 (0.9 to 2.0) $\log_{10}$ (APV/r); 1.9 (1.3 to 2.4) $\log_{10}$ cps/mL (ATV/r); 1.6 (1.3 to 2.0) $\log_{10}$ cps/mL (IDV/r); 1.9 (1.7 to 2.1) $\log_{10}$ (LPV/r); and 1.7 (1.3 to 2.2) $\log_{10}$ (SQV/r); (p=0.42 from a censored regression analysis). There were 88 (23%) patients with an HIV RNA recorded as 50 cps/mL in whom the reduction was censored in the regression analysis (4 (14%), 7 (24%), 7 (13%), 63 (27%) and 7 (21%) patients receiving APV/r, ATV/r, IDV/r, LPV/r or SQV/r, respectively).

In unadjusted analysis, the level of resistance to the PI/r (all PI/rs combined) was significantly related to HIV RNA reductions for all GISs ($p \leq 0.01$). The GSS to the rest of the regimen did not predict virological response using either the ANRS GIS or the REGA GIS (Table 5.4.3.1). There was a trend towards a relationship between the GSS to the rest of the regimen with viral load response for both DMC and Stanford, but these trends went in the opposite direction to what was expected. For the DMC GIS there was a -0.11 (-0.26 to 0.02) larger decline from baseline in HIV RNA for each additional active drug a patient had in their background regimen (i.e. a smaller decline). Similarly, for the Stanford GIS there was a -0.13 (-0.28 to 0.02) larger decline in HIV RNA from baseline for every additional active drug a patient had in their background regimen.

After adjustment for baseline HIV RNA levels, the PI/r used, the time between baseline and follow-up HIV RNA measurements and also for the time between baseline resistance test and the date of PI/r initiation, all GISs still showed significantly greater reductions as sensitivity to the PI/r increased. The GSS to the rest of the regimen did not predict response further (Table 5.4.3.1). Patients with a virus that was ascribed to be sensitive to the PI/r were predicted to have a 0.72 greater $\log_{10}$ reduction between baseline and week 12 compared to patients with a virus harbouring full resistance. This difference in the magnitude of effect sizes is comparable to the patterns we saw in the trials where there was predicted to be a 0.82 greater $\log_{10}$ reduction between baseline and week 12 for

patients with a virus that showed sensitivity to the PI/r compared to patients with a virus exhibiting full resistance.

Table 5.4.3.1: Differences in HIV RNA change (ΔVL: BL to week 12) for each GIS According to the PI/r resistance level and number of active ARVs in the regimen

Interpretation System	Unadjusted Analysis Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	P-value	Adjusted Analysis Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	P-value
ANRS				
PI/r resistance (R → I → S)	0.34 (0.11, 0.58)	0.003	0.36 (0.15, 0.56)	0.001
GSS to the rest of the regimen				
For every additional active ARV	-0.02 (-0.16, 0.12)	0.78	0.04 (-0.08, 0.16)	0.56
REGA				
PI/r resistance (R → I → S)	0.37 (0.13, 0.60)	0.003	0.36 (0.15, 0.58)	0.001
GSS to the rest of the regimen				
For every additional active ARV	-0.04 (-0.19, 0.11)	0.58	0.04 (-0.09, 0.18)	0.53
DMC				
PI/r resistance (R → I → S)	0.30 (0.12, 0.47)	0.001	0.22 (0.06, 0.38)	0.008
GSS to the rest of the regimen				
For every additional active ARV	-0.11 (-0.26, 0.02)	0.10	-0.05 (-0.18, 0.08)	0.45
Stanford				
PI/r resistance (R → I → S)	0.42 (0.20, 0.63)	<0.0001	0.42 (0.21, 0.63)	<0.0001
GSS to the rest of the regimen				
For every additional active ARV	-0.13 (-0.28, 0.02)	0.09	0.05 (-0.09, 0.19)	0.51

- Coefficients are interpreted in the same way as in Table 4.4.4.1
- Adjustments were made for baseline HIV RNA levels, the PI/r used, the time between baseline and follow-up HIV RNA measurements and also for the time between baseline resistance test and the date of PI/r initiation

The results of the multivariable model including the REGA GIS show: a 0.52 (0.41 to 0.63) log_{10} larger reduction in HIV RNA from baseline for every log_{10} higher baseline HIV RNA ($p<0.0001$); a 0.02 (0.00 to 0.04) log_{10} larger reduction in HIV RNA from baseline for every week longer between the baseline and the follow-up HIV RNA measurements ($p=0.06$); and a -0.006 (-0.014 to 0.003) log_{10} smaller reduction in HIV RNA from baseline for every additional week prior to baseline that the resistance test occurred on ($p=0.20$). Similar results were seen in multivariable models including all of the other GISs (data not shown).

5.4.4. Investigating each PI/r separately

I investigated HIV RNA reductions according to the level of resistance to each PI/r separately to see whether the above trends were driven by a specific PI/r. Even though individual comparisons in each treatment arm resulted in reduced power, the levels of resistance to ATV/r, LPV/r and to SQV/r were significantly associated with virological response for each GIS: with more viral sensitivity related to larger HIV RNA reductions ($p<0.01$). The same trends were not seen in the subgroups of patients receiving IDV/r or APV/r. For APV/r this could be partly due to small numbers. This is illustrated using the REGA GIS in Table 5.4.4.1.

Table 5.4.4.1: Differences in HIV RNA change (ΔVL: BL to week 12): for each PI/r Using the REGA interpretation system

Interpretation System	**Unadjusted Analysis** Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	**P-value**	**Adjusted Analysis** Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	**P-value**
APV/r resistance (R → I → S)	0.42 (-0.55, 1.40)	0.38	0.65 (-0.36, 1.66)	0.20
GSS to the rest of the regimen For every additional active ARV	0.46 (-0.29, 1.21)	0.22	0.62 (-0.18, 1.42)	0.12
ATV/r resistance (R → I → S)	0.88 (-0.04, 1.81)	0.06	1.22 (0.42, 2.02)	0.004
GSS to the rest of the regimen For every additional active ARV	-0.47 (-1.56, 0.61)	0.38	-0.56 (-1.42, 0.29)	0.19
IDV/r resistance (R → I → S)	-0.33 (-0.91, 0.24)	0.25	-0.17 (-0.61, 0.28)	0.46
GSS to the rest of the regimen For every additional active ARV	-0.04 (-0.67, 0.59)	0.90	-0.11 (-0.58, 0.37)	0.65
LPV/r resistance (R → I → S)	0.45 (0.15, 0.75)	0.003	0.48 (0.21, 0.75)	<0.0001
GSS to the rest of the regimen For every additional active ARV	0.02 (-0.25, 0.30)	0.87	0.13 (-0.10, 0.37)	0.27
SQV/r resistance (R → I → S)	1.09 (0.23, 1.96)	0.02	0.95 (0.25, 1.64)	0.009
GSS to the rest of the regimen For every additional active ARV	0.10 (-0.63, 0.82)	0.78	-0.29 (-0.81, 0.23)	0.27

- Coefficients are interpreted in the same way as in Table 4.4.4.1

5.4.5. The combined trials/EuroSIDA dataset

Although EuroSIDA contained a more treatment experienced population compared to the trials, separate analysis of the two datasets provided consistent results. Therefore, I merged the datasets to improve power because the frequency of resistance to the PI/r in each study individually was relatively low. To my knowledge this is the largest combined database of patients receiving a PI/r in which it is possible to examine the relationship between ascribed resistance levels and viral load outcome.

In this comparison I also investigated the GSS to the rest of the regimen as a categorical variable with three categories (i.e. no other active drugs; 0.5 to 1.5 other active drugs; and ≥2 other active drugs) instead of as a continuous variable. This was because I wanted to distinguish between patients who had a virus that showed resistance to all of their background drugs from patients who had a virus that showed susceptibility to all of their background drugs.

The median (IQR) baseline HIV RNA was 4.4 (3.5 to 5.1) $\log_{10}$ copies/mL, after a median (IQR) 12 (9 to 13) weeks from the start of a PI/r containing regimen this was reduced by a mean (95% CI) 2.2 (2.1 to 2.3) $\log_{10}$ copies/mL. The level of resistance to the PI/r (all PI/rs combined) was related to HIV RNA reductions for all GISs ($p \leq 0.0001$).

After adjusting for the baseline HIV RNA level, the PI/r used, the time between baseline and follow-up HIV RNA measurement and the GSS to the rest of the regimen (calculated using the same GIS that was used to calculate the level of PI/r resistance), all GISs still showed significantly greater reductions as sensitivity to the PI/r increased. Patients with a virus that showed sensitivity to the PI/r experience a 0.82 greater $\log_{10}$ reduction between baseline and week 12 compared to patients with a virus exhibiting full resistance using the REGA GIS (Table 5.4.5.1).

When I included the GSS to the rest of the regimen in the model as a categorical variable instead of as a continuous variable I found that differences existed between GISs. The resistance levels ascribed by two GISs were significantly related to HIV RNA declines. There was a trend for greater HIV RNA reductions with increasing PI/r sensitivity and with increasing GSS to the rest of the regimen in both unadjusted and adjusted analysis for REGA and ANRS (Table 5.4.5.1).

Table 5.4.5.1: Differences in change (ΔVL: BL to week 12), for the trials and EuroSIDA combined

Interpretation System	Unadjusted Analysis Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	P-value	Adjusted Analysis Additional HIV RNA reduction per higher level of sensitivity (log_{10} cps/mL)	P-value
ANRS				
PI/r resistance (R → I → S)	0.49 (0.29, 0.68)	<0.0001	0.39 (0.22, 0.56)	<0.0001
GSS to the rest of the regimen				
0	Ref	0.003	Ref	0.04
0.5 to 1.5	0.33 (-0.15, 0.82)		0.46 (0.05, 0.87)	
≥2	0.66 (0.21, 1.11)		0.49 (0.11, 0.86)	
REGA				
PI/r resistance (R → I → S)	0.56 (0.35, 0.76)	<0.0001	0.41 (0.23, 0.59)	<0.0001
GSS to the rest of the regimen				
0	Ref	0.004	Ref	0.07
0.5 to 1.5	0.25 (-0.23, 0.73)		0.39 (-0.01, 0.79)	
≥2	0.59 (0.13, 1.06)		0.45 (0.06, 0.83)	
DMC				
PI/r resistance (R → I → S)	0.49 (0.33, 0.65)	<0.0001	0.33 (0.19, 0.47)	<0.0001
GSS to the rest of the regimen				
0	Ref	0.20	Ref	0.99
0.5 to 1.5	-0.15 (-0.62, 0.32)		-0.02 (-0.41, 0.37)	
≥2	0.10 (-0.34, 0.53)		-0.01 (-0.37, 0.35)	
Stanford				
PI/r resistance (R → I → S)	0.67 (0.49, 0.86)	<0.0001	0.46 (0.29, 0.63)	<0.0001
GSS to the rest of the regimen				
0	Ref	0.55	Ref	0.67
0.5 to 1.5	0.12 (-0.43, 0.67)		0.20 (-0.25, 0.65)	
≥2	0.23 (-0.30, 0.75)		0.18 (-0.25, 0.61)	

- Coefficients are interpreted in the same way as in Table 4.4.4.1
- Adjustments are made for the PI/r used, baseline HIV RNA and the time between PI/r initiation and follow-up HIV RNA measurement

For both of these GISs, the relationship between the GSS to the rest of the regimen and HIV RNA declines was driven by patients who had a virus exhibiting full resistance to all of the other drugs in the regimen compared to those with a virus that showed susceptibility to at least one drug. Neither DMC nor Stanford showed a relationship between the GSS to the rest of the regimen and HIV RNA response.

5.4.6. Investigating other mutations and virological response

I then looked at the relationship between individual protease mutations (according to the IAS-USA September 2006 guidelines[233]) and viral load response in EuroSIDA, to see whether the same mutations predicted response as I found in the trial data (section 4.4.8). In contrast to the comparisons performed in the trials, the presence of the F53L mutation was associated with a worse virological response, and the I47A/V mutation was not related to HIV RNA decline in either unadjusted or adjusted analysis. This is somewhat surprising given the impact of I47A on LPV/r, for example. To assess whether the effect was masked by the fact that the two mutations I47V and I47A were considered together in Table 5.4.6.1, I investigated the relationship between each mutation and HIV RNA reductions separately. No relationship was identified in either of these analyses (data not shown). Mutations at positions 10, 46, 54, 82 and 90 were consistently associated with smaller HIV RNA declines (Table 4.4.8.1 and Table 5.4.6.1).

Table 5.4.6.1: Relationship between individual mutations and virological response

Mutation Present	**Unadjusted Analysis** Additional HIV RNA reduction with presence of mutation (log_{10} cps/mL)	**P-value**	**Adjusted Analysis** Additional HIV RNA reduction with presence of mutation (log_{10} cps/mL)	**P-value**
L10F/I/R/V	-0.35 (-0.64, -0.04)	0.03	-0.34 (-0.60, -0.07)	0.01
K20R/M/I/L/T	-0.15 (-0.51, 0.22)	0.43	-	-
L24I	-0.52 (-1.27, 0.24)	0.18	-	-
D30N	0.05 (-0.50, 0.59)	0.87	-	-
V32I	-0.10 (-1.12, 0.92)	0.84	-	-
L33I/F	-0.18 (-0.83, 0.47)	0.58	-	-
M36I/L/V	0.10 (-0.21, 0.40)	0.54	-	-
R41K	-0.15 (-2.78, 2.48)	0.91	-	-
M46I/L	-0.37 (-0.70, -0.04)	0.03	-0.38 (-0.68, -0.09)	0.01
I47A/V	0.34 (-0.88, 1.56)	0.58	-	-
G48V	0.05 (-0.77, 0.86)	0.91	-	-
I50L/V	0.45 (-2.18, 3.08)	0.74	-	-
F53L	-0.78 (-1.42, -0.14)	0.02	-0.68 (-1.24, -0.13)	0.02
I54V/L/A/M/T/S	-0.57 (-0.98, -0.16)	0.007	-0.62 (-0.98, -0.26)	0.001
L63A/P	-0.21 (-0.51, 0.08)	0.15	-0.24 (-0.49, 0.01)	0.06
A71V/T	-0.39 (-0.69, -0.09)	0.01	-0.42 (-0.68, -0.16)	0.002
G73C/S/T/A	-0.39 (-0.86, 0.09)	0.11	-	-
V77I	-0.13 (-0.43, 0.18)	0.42	-	-
V82A/F/T/S	-0.65 (-1.02, -0.27)	0.001	-0.52 (-0.84, -0.19)	0.002
I84V/A/C	-0.33 (-0.75, 0.09)	0.13	-0.39 (-0.76, -0.03)	0.04
N88D/S	0.21 (-0.37, 0.79)	0.48	-	-
L90M	-0.58 (-0.89, -0.27)	<0.0001	-0.56 (-0.83, -0.29)	<0.0001

* Analyses are adjusted for the PI/r received and baseline HIV RNA

• Mutations in bold indicate primary PI mutations to one of the five randomised PIs according to the IAS-USA mutational lists[233]

I combined the EuroSIDA and trials datasets and investigated HIV RNA reductions according to the overall number of IAS-USA September 2006 primary PI mutations in the virus. In unadjusted analysis there was a -0.14 (-0.18 to -0.10) $\log_{10}$ cps/mL larger HIV RNA reduction (i.e. a smaller reduction) for each additional primary PI mutation present, $p<0.0001$. After adjustment for the baseline HIV RNA and the PI/r used there was a -0.11 (-0.15 to -0.07) $\log_{10}$ cps/mL smaller reduction over 12 weeks of follow-up for each additional primary PI mutation, $p<0.0001$.

5.5. Discussion

In this chapter I compared the ascribed levels of resistance to five PI/rs using four GISs and evaluated the relationship between resistance and viral load response using data from the EuroSIDA study. Patients in EuroSIDA are more representative of HIV infected patients than those in the trials because they have less restrictive entry and follow-up procedures. In this comparison, patients had more exposure to ARVs prior to the start of a PI/r and consequently had more resistance mutations in their virus at baseline compared to the trial patients.

5.5.1. Summary of main results

When I evaluated concordance between GISs I found similar levels of concordance (i.e. low-to-moderate) between ascribed PI/r resistance levels in EuroSIDA and in the trials. In EuroSIDA, kappas ranged from 0.01 to 0.38 for APV/r; 0.31 to 1.00 for ATV/r; 0.48 to 0.79 for IDV/r; 0.34 to 0.77 for LPV/r; and 0.30 to 0.57 for SQV/r. In the trials, kappas ranged from 0.37 to 0.75 for IDV/r; 0.46 to 0.93 for LPV/r; and 0.38 to 0.68 for SQV/r. In EuroSIDA, the kappa statistics for IDV/r were slightly higher whereas, in contrast, they were moderately lower for SQV/r and LPV/r compared to those seen in the trials. This indicates that there is more of a consensus between GISs on mutations that confer resistance to IDV/r, because the EuroSIDA comparisons are conducted on isolates containing a greater number of mutations overall. However, variations on the weight assigned to these mutations exist for both LPV/r and SQV/r. It may be better to consider the number in addition to the type of protease mutations for these PI/rs rather than individual mutations specifically. For example, the total number of IAS-USA mutations could be considered where primary and secondary mutations are both assigned a different weight.

I also found high levels of discordance for APV/r (i.e. low kappa values), which could result from the use of different formulations of amprenavir when each GIS was initially created (i.e. amprenavir boosted with ritonavir, amprenavir alone or fosamprenavir). Different formulations may lead to different rates of non-compliance and to disagreements between experts on the degree of resistance inferred by each mutation. There is a consensus on whether most mutations affect susceptibility to APV/r, but the weight attributed to each mutation differs according to the GIS. These differences resulted in high levels of discordance between GISs. More genotype-to-viral-load-response data, on a larger group of patients receiving APV/r, would improve these comparisons.

There are minor differences between GISs in the way they assign resistance levels (i.e. the presence of the L10F mutation when combined with other mutations may indicate high-level resistance using one GIS but using another GIS it may only correlate to intermediate resistance). To see if these differences in ascribed resistance levels impacted on the viral load response I related baseline resistance levels to the 12 week HIV RNA decline. HIV RNA was reduced by 1.8 (1.7 to 1.9) log cps/mL a median of 13 (IQR: 9 to 17) weeks after starting a PI/r. This reduction is smaller than the HIV RNA decline seen in the trials because EuroSIDA patients had a virus with more resistance to the ARVs in their regimen and are therefore not expected to respond as well. In addition, they started their PI/r with a lower HIV RNA so they have less scope for large reductions. EuroSIDA patients are also followed less intensively (i.e. this HIV RNA value is likely to be recorded at the first visit after starting the PI/r in EuroSIDA whereas in the trials patients will have been followed up at week four as well as at week 12). As a result, early increases in HIV RNA that would require treatment changes may be missed or only identified at a later stage in EuroSIDA. Due to this delay in response times the virological profiles in observational studies may be worse.

Although EuroSIDA patients have a larger number of resistance mutations in their predominant virus at the start of a PI/r compared to patients in the trials, some of these patients will have had a resistance test performed before starting a new regimen. It is therefore unlikely that they will have been assigned an ARV which they will be resistant to. As a result fairly low levels of resistance were still seen at PI/r initiation in EuroSIDA.

In both unadjusted and adjusted analysis, the level of resistance to the PI/r was significantly related to HIV RNA declines, and all of the GISs predicted HIV RNA declines to a similar extent. The relationships between PI/r resistance levels and HIV RNA declines

in EuroSIDA were more significant, but of a similar magnitude, to those observed in the trials. This could be because there was a higher amount of baseline resistance seen in patients in EuroSIDA therefore more power to identify any differences if they did exist.

To see whether the relationship between PI/r resistance levels and viral load reductions was driven by a specific PI/r, I quantified the relationship between PI/r resistance levels and HIV RNA changes for each PI/r individually. Although this resulted in comparisons on a smaller number of patients and therefore less power to show any differences, resistance levels to ATV/r, LPV/r and to SQV/r were significantly associated with viral load changes for every GIS. The magnitude of the effect (i.e. the reduction in HIV RNA from baseline) differed significantly between PI/rs: from a 0.96 $\log_{10}$ cps/mL greater reduction in patients with a virus that was ascribed to be sensitive to LPV/r compared to patients with a virus ascribed to be resistant to LPV/r to a 2.44 $\log_{10}$ cps/mL greater reduction in patients with a virus ascribed to be sensitive to ATV/r compared to patients with a virus ascribed to be resistant to ATV/r, using the REGA GIS. This illustrates that the GISs are able to discriminate between patients who have a virus that contains PI/r resistance mutations and are not expected to have favourable HIV RNA reductions from baseline, and patients who have a virus that exhibits sensitivity to the PI/r and are expected to respond well to the PI/r. However, since these analyses were performed on a sub-group of patients, the differences in coefficient estimates between PI/rs may not actually reflect those that would be seen in clinical practice.

5.5.2. Interpretation of results

Although GISs do effectively predict differences in HIV RNA declines according to the ascribed resistance category, these trends are not seen for all of the PI/rs separately (Table 5.4.4.1). The lack of a difference for APV/r and IDV/r could be due to the small numbers, but it could also be because only a few specific primary PI mutations are thought to indicate resistance to these PI/rs. Instead, resistance may need to be thought of as a continuum (including a wider variety of mutations) rather than as a binary variable according to the presence or absence of a specific mutation. Most of the existing GISs include all mutations they consider to confer resistance in their algorithms, in order to calculate the overall resistance score for each drug. However, many less frequently occurring mutations may be overlooked, or have unknown impact because they are less common and/or less well studied. These mutations may be on the pathway for the emergence of further resistance. The resistance output for the Stanford GIS is actually provided as a continuous score that accounts for every possible PR mutation, but it is

rarely used on a continuous level in clinical practice. Furthermore, the Stanford GIS does not account for combinations of mutations but only specific mutations individually. Some mutations may need to be assigned different weights if they are present alongside other resistance mutations.

5.5.3. The combined dataset

I combined EuroSIDA data with the trial data to increase the number of patients in whom I could investigate the GSS to the rest of the regimen in more detail. Yet still, in this combined study population there were low prevalence rates of some mutations. Although the prognostic effect of the GSS to the rest of the regimen partly disappeared after adjustment for the PI/r resistance level, baseline HIV RNA and the time between PI/r initiation and follow-up HIV RNA measurement, there was a trend for greater HIV RNA declines if the virus also showed some activity to the background regimen compared to patients who had a virus that showed no activity to their background ARVs. This trend was not seen for DMC or Stanford where no relationship between the number of active background ARVs and viral load response was seen. In all of these comparisons, PI/r sensitivity was much more predictive of HIV RNA declines than the GSS to the rest of the regimen. This may be because the main drug in the regimen (i.e. the PI/r) is more potent than the background drugs and is required (to a greater extent than the background drugs) to maximise virological suppression.

I then repeated the analysis in 4.4.8, exploring the relationship between individual mutations and viral load response in the EuroSIDA population. The presence of mutations at positions 10, 46, 54, 82 and 90 were associated with smaller HIV RNA declines in both EuroSIDA and the trials, even after adjustment for the PI/r used. I then looked at primary protease mutations as a continuum (in all of the studies combined) and, consistent with other studies, I found that the number of protease mutations was significantly associated with response[330;335;356]. There was a 0.11 (0.07 to 0.15) $\log_{10}$ cps/mL smaller HIV RNA reduction over follow-up for each additional primary PI mutation, $p<0.0001$.

5.5.4. Other resistance approaches

Various alternative approaches for quantifying the effect of resistance have been suggested. Marcelin *et al* proposed integrating the ARV-specific drug levels with the genotypic resistance score to quantify the combined effect of drug exposure and viral

susceptibility. The ratio of the PI C_{min} to the number of baseline PI mutations was shown to predict virological response significantly for a number of different ARVs[330;377;378]. Resistance levels derived using other bioinformatic approaches, some of which employ the use of fuzzy logic (a mathematical approach used to represent knowledge affected by uncertainty), have also been related to viral load response[216;379]. Machine learning techniques have also been employed, such as support vector machines (SVMs), artificial neural networks (ANNs), super-learner prediction algorithms in addition to other genotypic resistance algorithms. Some of their resulting interpretations have been related to viral load response[380-383].

SVMs are a set of supervised learning methods that are used to classify isolates as being resistant or sensitive to a particular ARV. SVMs attach a weight to each point mutation so they capture all resistance mutations in the overall sequence background and not just mutations that are linked to a few prominent sequence positions. SVMs have been shown to reliably predict phenotypes from genotypes for most of the existing ARVs[384;385], but limited data are available showing the value of these interpretations for predicting viral load response.

On reflection, mutations that are present in low level resistant strains are likely to become dominant again if a drug that selects for these mutations is re-started. In this analysis, the majority of patients started a new PI/r containing regimen at baseline, so low-level resistant strains may start to outgrow the virus that was dominant at baseline if the patient fails to suppress replication on the new regimen. As a result, a method that takes into account archived mutations or mutations that are present in low levels may be a better indication of the baseline resistance profiles of patients in these comparisons.
ANNs or neural networks (NNs) are mathematical or computational models that are based on biological neural networks. In the Copenhagen HIV Programme (CHIP), we have recently used ANNs to identify resistance patterns in 1507 matched phenotypic and genotypic resistance pairs that were obtained from the Stanford HIV-DB database. We used these data to train and validate ANNs to reliably predict susceptibility to 16 ARVs using knowledge of the physiochemical descriptors of the amino acid mutations in the complete enzyme's primary structure for the RT- and PR- gene, rather than only examining a subset of mutations. Now we plan to use these ANNs to see if we can model the complex relationship between resistance mutations and viral load response in patients from EuroSIDA and from the trials combined. If these ANNs are shown to be clinically useful for predicting viral load outcome in patients, they will be implemented as tools on

the world wide web to provide a rapid and free alternative to both virtual and real phenotypic testing as well as genotypic testing (J Kjær *et al*: resubmitted to HIV medicine).

5.5.5. Conclusion

To conclude, differences were observed between GISs using both trial data and observational data as illustrated in this subgroup of EuroSIDA study participants and in the previous chapter looking at trial patients. Although differences exist in the resistance levels ascribed by each GIS, the GISs were comparable in the extent to which they predicted virological response after 12 weeks of follow-up.

By combining study populations I was able to increase power and investigate the relationship between GIS resistance levels, both to the PI/r and to the other ARVs in the regimen, and virological response in more detail. In this analysis there was a trend for greater HIV RNA reductions with increasing PI/r sensitivity and with increasing GSS to the rest of the regimen for the ANRS and REGA GISs. For these GISs, the relationship between the GSS and HIV RNA decline was driven by patients who had a virus that did not show sensitivity to any other drugs in the regimen compared to those who had a virus that showed sensitivity to at least one other drug. All of these GISs need to be refined further to enable them to predict HIV RNA changes more efficiently and eventually a consensus on what resistance mutations contribute to viral load response may be reached.

CHAPTER 6: EVOLUTIONARY DISTANCES IN COLATE

6. Introduction

In chapter 3, I investigated the emergence of drug resistance mutations between baseline and the time of virological failure by identifying all new mutations that emerged in the predominant virus of patients treated with a PI/r. In chapters 4 and 5, I compared the ascribed PI/r resistance levels at baseline, generated with each of four genotypic interpretation systems (GISs), for predicting viral load response to a PI/r containing regimen. In these chapters, there was moderate concordance between GISs on how best to interpret genotypic data for PI/rs, and a trend towards larger viral load declines with increasing sensitivity to the PI/r. When I investigated the prognosis of a genotypic sensitivity score (GSS) to the other drugs in the regimen for predicting viral load response, I found a weaker association for all GISs. Since the level of resistance to the PI/r is a stronger predictor of viral load response than the GSS to the other drugs in the regimen, measuring the level of viral susceptibility to a regimen by summing the predicted levels of susceptibility to each of the component drugs may not yet be a reliable approach for quantifying resistance for the whole regimen.

Phylogeny aims to provide us with an understanding of the molecular changes that influence viral evolution and gives us a more precise picture of how the virus evolves in general (both under pressure from, and in absence of, ARVs) (section 1.6.6). Phylogenetic analysis involves the collection of information on specific nucleotide changes rather than solely looking at changes to the nucleotide-triplet encoded amino acids. Data are available at the nucleotide level in all of the studies in this thesis, so it would be possible to calculate crude distances between sequences to estimate molecular changes on a nucleotide level. However, PAUP (Phylogenetic Analysis Using Parsimony)[263] is an alternative approach that can be used to identify clusters of similar viral strains easily, and to calculate the distances between them, using different approaches to model the nucleotide changes.

6.1. Aims of the chapter

PAUP was used in this chapter to compare sequence data, obtained from the *pol* gene of the virus, in patients from the COLATE trial. The phylogenetic analyses were conducted on consensus sequences and not on individual clones; so all nucleotide distances are calculated between viruses that were sampled through the use of population sequencing. As a result, a mutation that is only present in a minority viral strain at baseline, or during

follow up, will not be accounted for in these comparisons. I will use these data to evaluate nucleotide changes in the predominant virus of patients who were receiving cART, and to examine the potential benefits of preserving the M184I/V mutation in the dominant virus population.

6.2. Literature review

HIV drug resistance can have a negative impact on the response to cART in both previously naïve and in ARV-experienced patients[188;189;386]. The continued use of an antiretroviral, to which an HIV infected patient is harbouring a resistant viral strain, remains controversial. Current guidelines recommend that following virological failure at least two active ARVs should be started, because resistance mutations to the drugs in the failing regimen are likely to be present in the predominant virus[33]. Alternative strategies are to add on an active ARV or switch some ARVs in the regimen, especially in patients with few treatment options available (section 1.6.9).

With the recent introduction of integrase inhibitors, CCR5 inhibitors and other new drugs from existing classes, there should be other active drugs around for the foreseeable future. Yet, resistance to these new drugs and their relationship with the other, older drugs still needs to be understood. Given the potential emergence of resistance, patients in need of salvage therapy may wish to postpone starting one of these newer drugs until a fully active regimen containing at least two and preferably three fully active drugs can be prescribed simultaneously.

HIV infected patients are also living for longer due to the improvements in therapy, and this has led to the growth of a highly treatment experienced population who have an array of resistance mutations in their virus populations. Current UK data suggest that the prevalence of triple class resistance is approximately 10% in all patients who have resistance testing available. In order to preserve treatment options in patients who have high levels of resistance in their virus populations, the need for recycling ARVs that a patient has already been exposed to, is likely to persist despite the expanding number of antiretroviral drug classes.

Lamivudine (3TC) is a commonly used NRTI, currently used in >65% of patients receiving cART in EuroSIDA. Virological failure on a 3TC-containing regimen is associated with the emergence of the point mutation M184I or M184V[387], both of which confer high-levels of resistance to 3TC[328;388], but conversely hypersusceptibility to zidovudine, stavudine and

tenofovir[389;390]. Viruses containing these mutations are associated with >100-fold and >500-fold reduced susceptibility to 3TC respectively (i.e. M184V is associated with a 500 to 1000 fold increase of the 50% inhibitory concentration (IC_{50}) compared to wild-type virus – see 1.6.3.2 for more information on the IC_{50})[387;391].

Although the largest benefits from cART are seen if a patient has a virus that is fully-susceptible to the ARVs in their regimen, it has been hypothesized that retaining 3TC in a failing 3TC-containing regimen and consequently retaining the M184V mutation could be virologically beneficial[272;389]. Evidence from *in vitro* experiments suggests that viruses containing the M184V mutation may have a lower replication capacity and increased fidelity compared to wild-type viruses[271-278]. Alternatively, lamivudine may retain some residual activity against a virus containing the M184V mutation.

These results have been confirmed, in part, in clinical studies where plasma HIV RNA has remained below baseline values by approximately 0.5 $\log_{10}$ copies/mL in 3TC-treated patients despite the detection of M184V[391;392]. The clinical benefit of adding 3TC to other NRTIs was also seen beyond the first few months in CAESAR, i.e. after 3TC resistance had emerged[94], adding further support to the hypothesis that the presence of M184V may lead to better efficacy of other ARVs[393].

In the E-184V study, all patients who were on a 3TC containing regimen, in whom the M184V mutation was detected by population sequencing, and who requested a treatment interruption, were randomised to either interrupt all ARVs or to continue receiving 3TC alone. By week 48, 20 (69%) patients in the treatment interruption arm and 12 (41%) patients in the 3TC arm had discontinued the study due to immunological or clinical failure (i.e. a difference of 28%), illustrating the protective ability of continuing 3TC despite the presence of high-level resistance. Further, the mean decline in $CD4^+$ T cell percentage, viral rebound and recovery of replication capacity were significantly lower in the 3TC group compared to the no-3TC group in this study[279].

Although an immunological, virological and clinical benefit associated with the detection of the M184V mutation has been shown *in vivo* in the E-184V study[279], these patients were highly treatment experienced and received lamivudine mono-therapy. Resistance data showed that the continuation of lamivudine preserved viruses with M184V as well as other resistance mutations, probably due to their linkage with M184V on the same virus genome.

Thus the beneficial effects in terms of reduced replication capacity cannot be ascribed entirely to the selective pressure on M184V mutants.

The COLATE study (appendix V) was set up to examine the possible beneficial effects of maintaining selective pressure on M184V, induced by continuation of 3TC, on virological and immunological response in treatment-experienced patients who were receiving cART rather than 3TC alone. A study by Miller MD *et al,* examined intensification trials of tenofovir in treatment-experienced patients, in order to determine the effects of resistance at baseline on HIV RNA response. In these trials, HIV RNA response to tenofovir was reduced among patients with ≥3 TAMs inclusive of either the M41L or L210W, or in patients who had a preexisting K65R mutation, but slightly increased treatment responses were observed when the M184V mutation was present.

In this chapter I will report the main results of the COLATE trial (i.e. HIV RNA change according to the use of 3TC), and evaluate resistance patterns in patients with viraemia during follow-up. The rationale behind COLATE was described in 2.1.2. At screening, patients were divided in two strata: stratum A, including only patients who experiencied virological failure on their first 3TC-containing cART regimen; and stratum B, including those who were failing a second or later 3TC-containing cART regimen. Even if no difference in HIV RNA reductions is seen between intervention arms, it remains of interest to test whether the repeat detection of M184V over time is associated with a slower accumulation of additional mutations in the predominant virus of patients not achieving complete viral suppression. If this is the case, maintaining 3TC may be a viable approach for preserving future treatment options.

I will use phylogenetic analysis to monitor nucleotide distances over time, and assess whether there were accelerated rates of viral evolution amongst patients who discontinued 3TC (Off-3TC) compared to those who continued on it (On-3TC) as part of COLATE. In addition, I will compare nucleotide distances from baseline at each time-point, according to the presence of M184V, to evaluate the virological benefit of retaining M184V in the predominant virus, through follow-up.

6.3. Methods

6.3.1. The COLATE study

Details of entry into COLATE have been described in 2.1.2. In brief, all randomised patients, irrespective of their treatment status, were followed from baseline (the day of starting the optimised regimen plus 3TC in the On-3TC arm and the day of starting the optimised regimen alone in the Off-3TC arm) to week 48. Follow-up visits were scheduled at weeks 4, 12, 24, 36, and 48. During follow-up, the following procedures were performed: clinical evaluation, blood safety analyses, HIV RNA, $CD4^+$ T cell count and plasma storage.

6.3.2. PCR and sequencing

Genotypic resistance testing and virological sequencing was performed centrally in batches using cryopreserved plasma samples. ABL sequenced the protease gene and the first 1005 nucleotides of the reverse transcriptase gene, spanning a total of 1302 nucleotides. Plasma samples were used for sequencing at baseline and at each visit during follow-up if HIV RNA >500 copies/mL.

6.3.3. Statistical methods for this chapter

Similarly to the MaxCmin trials, the per-protocol population for analysis of the main COLATE trial included all patients who initiated their assigned treatment. The primary efficacy measure was the average-area-under-the-curve-minus-baseline (AAUCMB) reduction in $\log_{10}$ HIV RNA after 48 weeks. Observations from patients who withdrew consent, were lost to follow-up or died were censored at their last follow-up visit. The definition of treatment failure included observed virological failure (see section 2.1.2.2.1), patients who withdrew consent, were lost to follow-up or died.

Chi-squared and Fisher's exact tests were used to compare categorical variables. Continuous variables were analysed using Student's *t*-tests or Kruskal-Wallis tests depending on the distribution. Cox analysis was performed and Kaplan-Meier plots were produced for analyses investigating the time to: virological failure (section 2.1.2.2.1); virological suppression (i.e. HIV RNA < 50 copies/mL); treatment discontinuation and to a $CD4^+$ T cell count increase >100 cells/μl, for comparisons that contained a sufficient number of events (n>25). Multivariable models were developed to identify independent predictors of virological failure.

Patients were included in the nucleotide distances sub-study if they had a baseline sample and at least one follow-up sample available for sequencing. Sequencing was only possible at study visits if a patient had an HIV RNA >500 cps/mL. Some patients had an HIV RNA <500 cps/mL soon after baseline so follow-up resistance testing could not be performed on their virus. This resulted in a different number of sequences coming from each patient. I wanted to investigate all nucleotide changes from baseline, so patients with resistance data at baseline and at at least one other time point were included in the primary comparison (i.e. at the baseline visit and at, at least, one follow-up visit). I also performed a sensitivity analysis looking at all nucleotide changes between sequences at consecutive time points, irrespective of whether the first sequence in the pair was the baseline sequence.

In the main resistance analysis, nucleotide distances from baseline were estimated by comparing all available sequences to the baseline sequence for each patient and calculating the number of nucleotide changes that occurred, using PAUP. Non-parametric tests were used to test whether differences existed in these distances between treatment arms at all scheduled visits. Using the Bonferroni correction for multiple testing a p-value of ≤0.01 was considered significant (i.e. 5 tests, one per visit, therefore 0.05/5=0.01).

Generalised linear models (appendix IV) were then used as a more sensitive measure to assess the association between treatment arms and nucleotide distances and between the presence of M184V and nucleotide distances. Since each patient could have more than one observation (i.e. more than one follow-up resistance test so more than one measurement of their nucleotide distances from baseline), I used generalised estimating equations (GEEs) to account for the non-independence of some observations. I selected an autoregressive correlation structure to account for the fact that distances between sequences taken close to each other in time (i.e. closer to baseline) are likely to be shorter than distances between sequences measured with a long gap between them. GEEs use an adjusted standard error to account for non-independence among repeat observations (i.e. nucleotide distances) from the same patient. Adjustments were made for other confounding factors. All statistical analyses were performed in STATA, version 8.2.

6.3.4. Genetic distances and phylogenetic reconstruction

In order to match sequences from an individual, Jesper Kjær at CHIP performed pairwise genetic distance calculations between all sequences using the following three evolutionary nucleotide approaches: Jukes-Cantor[394]; Kimura 2-parameter methods[395]; and SynScan[396].

Jukes-Cantor allows us to model substitutions at each nucleotide position assuming an an equal rate of substitution for each nucleotide change (i.e. a within purine transition A ←→ G has the same probability as a within pyrimidine transition T ←→ C, and similarly this is equal to the rate of transversions (A or G ←→ C or T)). For example, if valine (say the nucleotide triplet GTC) was present at codon position 184 at baseline, and methionine (say the nucleotide triplet ATG) was present in the follow-up resistance test, the change from G to A (i.e. a transition) at the first position would be modelled with the same likelihood as the nucleotide change from C to G (i.e. a transversion) in the third position.

The Kimura 2-parameter method is a more sensitive approach and measures distances after accounting for the transversion/transition ratio. Since transitions are more likely to occur than transversions, because transversions cause problems in hydrogen bonds and in geometrical structures, they are modelled using different likelihoods using the Kimura 2-parameter approach. So, in the previous example the change from G to A at the first position would be modelled with a higher likelihood than the nucleotide change from C to G in the third position.

The SynScan evolutionary nucleotide model is the most sensitive evolutionary nucleotide approach to take for detection of evolution because it uses the approach described by Nei and Gobori[397] to take into account both synonymous and non-synonymous changes as well as accounting for information held by ambiguous nucleotides (i.e. positions that code for a mixture of A, C, T and G). A synonymous mutation is a 'silent' nucleotide change that does not result in an amino acid substitution and always occurs in the third position of a codon. A non-synonymous mutation is a nucleotide change that results in an amino acid substitution. In the above example, valine may have changed to methionine using two different routes: either the synonymous route, GTC → GTG → ATG or the non-synonymous route GTC → ATC → ATG. In the first case the amino-acid has changed from valine → valine → methionine and in the second case, the substitution route is valine → isoleucine → methionine. These are accounted for using different weights using the SynScan approach.

To build phylogenetic trees we imported pairwise distances into PAUP[263]. Trees were created using Neighbour-Joining distance methods and the robustness of the trees was evaluated using bootstrap analysis with 1000 rounds of replication. Sequences from a single individual should be closely related to each other therefore they are expected to group together rather than sequences from different patients. If the sequences from a

single individual did not group together, I assumed the sequences were not actually from the same patient, so all sequences from these patients were excluded from the analysis.

6.4. Results

6.4.1. Patient characteristics

From May 1999 to May 2002, there were 136 patients who were receiving a lamivudine containing regimen and were enrolled into the COLATE trial. Of these, 131 (96.3%) initiated their optimised regimen in addition to their randomised treatment: 65 continued 3TC in the On-3TC arm and 66 discontinued 3TC in the Off-3TC arm. There were a total of 124 patients (N=60 in the On-3TC arm and N=64 in the Off-3TC arm) who were followed up for the entire 48 weeks of the study or have outcome data available after 48 weeks of follow-up.

Out of the 131 patients who initiated their randomised regimen there were 17 (13%) who permanently discontinued it during follow-up (11 patients stopped 3TC in the On-3TC arm and six re-started 3TC in the Off-3TC arm, p=0.18). The primary reason for stopping 3TC for patients in the On-3TC arm was due to clinical non-fatal adverse events; similarly clinical events due to other drugs in the regimen were amongst the main reasons for re-initiating 3TC in patients who discontinued it as part of randomisation. These patients are not counted as failures when looking at the effect of treatment on viral load. The disposition of patients through week 48 is shown in Table 6.4.1.1.

Table 6.4.1.1: Disposition of patients through week 48 in the main COLATE trial

	On-3TC N (%)	**Off-3TC** N (%)	**Total** N (%)
Randomised:	65 (100)	71 (100)	136 (100)
Initiated assigned treatment (ITT/e population)	65 (100)	66 (93)	131 (96)
Initiated but permanently discontinued treatment [1)]	11 (17)	6 (9)	17 (13)
Reason:			
Virological failure	3	1	4
Death	0	1	1
Clinical non-fatal adverse event	4	2	6
Patient choice	1	0	1
Lost to follow-up	3	2	5
Patients with an outcome at week 48	60 (92)	64 (97)	124 (95)

1) Discontinuation in the On-3TC arm is equal to stopping 3TC; in the Off-3TC arm it is equal to starting 3TC

At baseline, no differences were observed between treatment arms in medical history, demographics, clinical and laboratory parameters or in exposure to ARVs prior to baseline, with the exception of a higher $CD4^+$ T cell count in patients in the On-3TC arm compared to patients in the Off-3TC arm (Table 6.4.1.2; p=0.01).

There were 28 (43%) and 27 (41%) patients in stratum A who were randomised to the On-3TC and Off-3TC arms, respectively. The remaining 76 patients entered the study after failing a second or later 3TC-containing cART regimen and were included in stratum B (N=37 (57%) in the On-3TC arm and N=39 (59%) in the Off-3TC arm). Due to the entry criteria (see 2.1.2.2) patients in stratum A (n=55) had less advanced HIV-disease compared to patients in stratum B (n=76); those in stratum B were older and exposure to more ARVs prior to baseline. There were no other baseline differences between strata in medical history, demographics or in clinical and laboratory parameters (Table 6.4.1.2).

Table 6.4.1.2: Baseline characteristics according to treatment group and strata

Baseline parameter	**On-3TC N =65**	**Off-3TC N =66**	**Stratum A [a] N =55**	**Stratum B [b] N =76**	**P-value[c]**
Gender No. male (%)	53 (82)	54 (82)	43 (78)	64 (84)	0.38
Age Median yrs, (IQR)	42.0 (36.5, 50.0)	40.0 (36.0, 47.3)	39.0 (34.0, 43.0)	42.5 (37.3, 51.8)	0.005
HIV exposure group No. (%)					0.48
Men who have sex with men	39 (60)	37 (56)	29 (52)	47 (62)	
Heterosexual	18 (28)	20 (30)	19 (35)	19 (25)	
Intravenous drug use	2 (3)	1 (2)	2 (4)	1 (1)	
Unknown & other	6 (9)	8 (12)	5 (9)	9 (12)	
Race No. (%)					0.32
White	55 (85)	53 (80)	43 (78)	65 (86)	
Black	8 (12)	10 (15)	10 (18)	8 (11)	
Asian	2 (3)	1 (2)	2 (4)	1 (1)	
Other	0 (0)	2 (3)	0 (0)	2 (3)	
CDC, Category C No. (%)	23 (35)	20 (30)	14 (25)	29 (38)	0.13
HIV RNA $\log_{10}$ cps/mL; median (IQR)	3.9 (3.3, 4.4)	4.0 (3.4, 4.6)	3.5 (3.1, 4.3)	4.1 (3.7, 4.7)	0.002
$CD4^+$ T cell count 10^6/l; median (IQR)	360 (257, 481)	280 (180, 376)	327 (204, 451)	300 (199, 425)	0.57
Nadir $CD4^+$ T cell count 10^6/l; median (IQR)	133 (38, 198)	122 (61, 200)	150 (53, 208)	121 (43, 193)	0.92
ARVs used prior to baseline median number (IQR)	5 (4, 8)	5 (3, 8)	4 (3, 5)	7 (5, 9)	<0.0001

a) Stratum A = patients enrolled following their 1st virological failure on a 3TC-containing regimen

b) Stratum B = patients enrolled following their 2nd or more failure on a 3TC-containing regimen

c) P-value for the comparison between stratum A versus stratum B

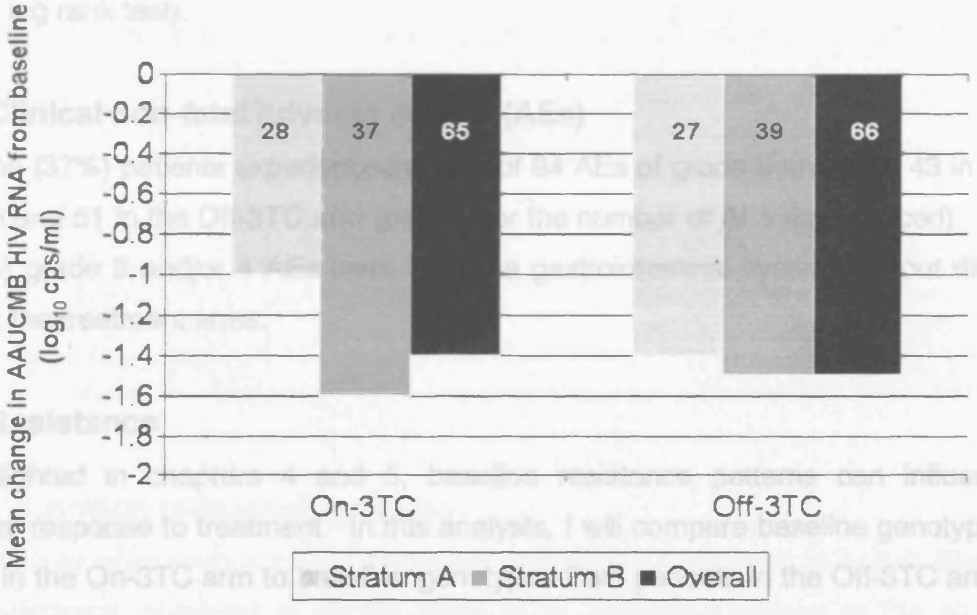
There were no differences in the planned number of ARVs or ARV drug classes between treatment arms. Further, patients in each treatment arm initiated a comparable number of ARVs at baseline, negating the possibility that clinicians may have prescribed extra ARVs in the Off-3TC arm to combat the additional use of 3TC in the On-3TC arm. The mean (95% CI) number of ARVs other than 3TC that were received was 3.5 (3.2 to 3.8) and 3.4 (3.1 to 3.7) in the On-3TC and Off-3TC arms, respectively. The most common combination of NRTIs used at baseline was d4T plus ddI (N=25 (38%) in the On-3TC arm and N=27 (41%) in the Off-3TC arm) and ABC plus ddI (N=9 (14%) in the On-3TC arm and N=9 (14%) in the Off-3TC arm).

No significant differences were observed between treatment arms in the proportion of patients who switched to, or at week 48, received a PI, NNRTI or an abacavir-containing regimen. Further, there was no difference in the time to first change of assigned treatment between treatment arms with regard to the 3TC component (i.e. the time to stopping 3TC in the On-3TC arm and re-starting 3TC in the Off-3TC arm, p=0.20, log rank test).

6.4.2. Virological response

No significant differences were observed between treatment arms in the primary efficacy outcome (i.e. the AAUCMB reduction in HIV RNA - Figure 6.4.2.1). Mean HIV RNA reductions from baseline were 1.4 (95% CI: 1.1 to 1.6) log_{10} cps/mL in the On-3TC arm and 1.5 (95% CI: 1.2 to 1.7) log_{10} cps/mL in the Off-3TC arm (p=0.51).

Figure 6.4.2.1: AAUCMB analysis according to treatment arm and strata



Similarly, no differences were seen in the primary efficacy outcome between treatment arms within strata (Stratum A: 1.0 (95% CI: 0.7 to 1.4) $\log_{10}$ cps/mL for On-3TC versus 1.4 (95% CI: 1.0 to 1.8) $\log_{10}$ cps/mL for Off-3TC (p=0.21); Stratum B: 1.6 (95% CI: 1.3 to 1.9) $\log_{10}$ cps/mL for On-3TC versus 1.5 (95% CI: 1.3 to 1.8) $\log_{10}$ cps/mL for Off-3TC (p=0.75)). The mean HIV RNA AAUCMB reductions did differ between strata, HIV RNA reductions from baseline were 1.2 (95% CI: 0.9 to 1.5) $\log_{10}$ cps/mL in stratum A and 1.6 (95% CI: 1.4 to 1.8) $\log_{10}$ cps/mL in stratum B, (p=0.02). However, there was no evidence of a significant interaction between treatment and strata on the AAUCMB reductions (test for interaction, p=0.22).

No difference was seen between treatment arms in the time to protocol-defined virological failure (p=0.65, log rank test), time to achieving a >1.0 $\log_{10}$ cps/mL reduction in HIV RNA from baseline or time to viral suppression for patients who had an HIV RNA above the level of detection (>50/>400 cps/mL). The overall proportion of patients with HIV RNA <50 copies/mL increased from 0% at baseline to 59% in the On-3TC arm and to 46% in the Off-3TC arm by week 48 (i.e. 12.6% (95% CI: -5.1% to 30.3%) more patients had HIV RNA <50 cps/mL at week 48 in the On-3TC arm compared to the Off-3TC arm, p=0.17).

6.4.3. Immunological response

The median (IQR) increase from baseline in $CD4^+$ T cell counts was 87 cells/µl (25 to 153) in the On-3TC arm and 76 cells/µl (-32 to 186) in the Off-3TC arm (p=0.41). A rise of ≥100 $CD4^+$ T cells/µl at any time during follow-up was seen in 42 patients in the On-3TC arm and 44 patients in the Off-3TC arm with no difference in the time to achieve this increase (p=0.86, log rank test).

6.4.4. Clinical non-fatal adverse events (AEs)

Forty-nine (37%) patients experienced a total of 94 AEs of grade 3 and/or 4; 43 in the On-3TC arm and 51 in the Off-3TC arm (p=0.25 for the number of AEs experienced). Overall, 16 (17%) grade 3 and/or 4 AEs were from the gastrointestinal system without difference between the treatment arms.

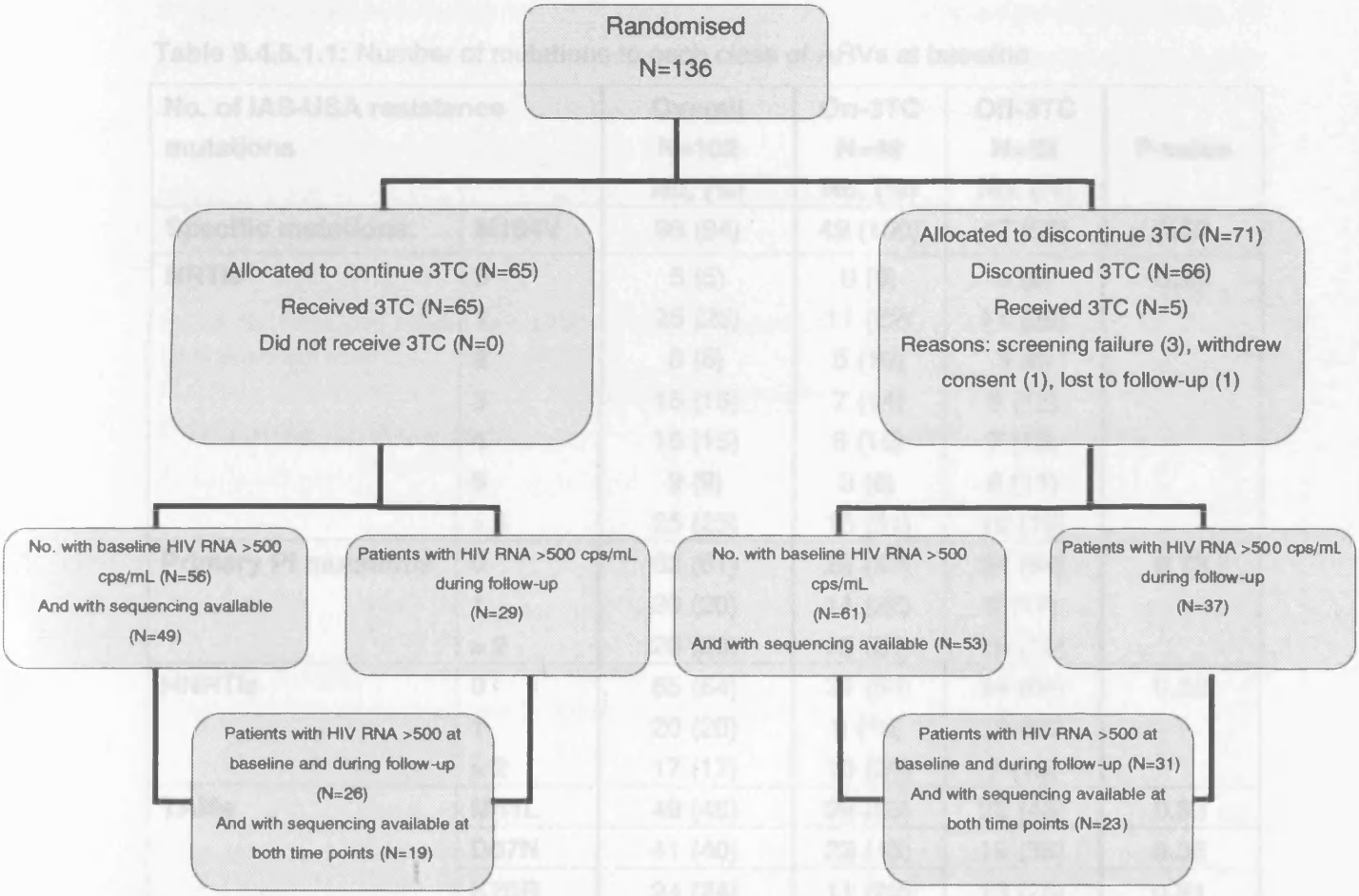
6.4.5. Resistance

As highlighted in chapters 4 and 5, baseline resistance patterns can influence the virological response to treatment. In this analysis, I will compare baseline genotypes from patients in the On-3TC arm to baseline genotypes from patients in the Off-3TC arm. This

analysis will highlight any initial differences that occur in the predominant virus populations of patients in COLATE that may impact on virological response. Since this analysis is restricted to patients who have a baseline sequence available, the availability of a follow-up sample is not a necessity for inclusion in these comparisons.

There were a total of 117 patients who were enrolled into COLATE and had a baseline HIV RNA >500 cps/mL (N=56 in the On-3TC arm and N=61 in the Off-3TC arm). From these, genotypic resistance test results were available from 102 (87%) patients at baseline; 49 (88%) in the On-3TC arm and 53 (87%) in the Off-3TC arm. Patients who contribute data to all of the resistance analyses in this chapter are outlined in Figure 6.4.5.1.

Figure 6.4.5.1: Patient flow through the study



6.4.5.1. Baseline resistance patterns

No differences were seen between treatment arms in the number of IAS-USA (September 2006) resistance mutations to NRTIs, NNRTIs, or primary mutations to PIs at baseline

(Table 6.4.5.1.1). In addition, the baseline genotypic sensitivity score (GSS) to all of the drugs received at baseline was comparable between arms: using the REGA GIS (version 6.4.0), there was a mean GSS of 1.9 (95% CI: 1.6 to 2.2) in the On-3TC arm and 2.1 (95% CI: 1.9 to 2.4) in the Off-3TC arm, p=0.34.

All 49 patients harboured M184V in the On-3TC arm, but in the Off-3TC arm M184V was only detected in 47 out of 53 (89%) patients, p=0.03. No differences were seen between treatment arms in the presence of TAMs at baseline, although marginally more patients in the On-3TC arm had the L210W mutation at baseline compared to those in the Off-3TC arm, p=0.03. Since these groups make up a sub-group of the COLATE trial, patients are no longer randomised in these comparisons, so imbalances may be seen between treatment arms.

Table 6.4.5.1.1: Number of mutations to each class of ARVs at baseline

No. of IAS-USA resistance mutations		**Overall N=102 No, (%)**	**On-3TC N=49 No, (%)**	**Off-3TC N=53 No, (%)**	**P-value**
Specific mutations:	**M184V**	96 (94)	49 (100)	47 (89)	0.02
NRTIs	**0**	5 (5)	0 (0)	5 (9)	0.25
	1	25 (25)	11 (22)	14 (26)	
	2	8 (8)	5 (10)	3 (6)	
	3	15 (15)	7 (14)	8 (15)	
	4	15 (15)	8 (16)	7 (13)	
	5	9 (9)	3 (6)	6 (11)	
	≥ 6	25 (25)	15 (31)	10 (19)	
Primary PI mutations	**0**	62 (61)	28 (57)	34 (64)	0.73
	1	20 (20)	11 (22)	9 (17)	
	≥ 2	20 (20)	10 (20)	10 (19)	
NNRTIs	**0**	65 (64)	31 (63)	34 (64)	0.52
	1	20 (20)	8 (16)	12 (23)	
	≥ 2	17 (17)	10 (20)	7 (13)	
TAMs	**M41L**	49 (48)	26 (53)	23 (43)	0.33
	D67N	41 (40)	22 (45)	19 (36)	0.35
	K70R	24 (24)	11 (22)	13 (25)	0.81
	L210W	29 (28)	19 (39)	10 (19)	0.03
	T215Y	42 (41)	22 (45)	20 (38)	0.46
	K219E/Q	25 (25)	12 (24)	13 (25)	0.99

6.4.5.2. Nucleotide distances

To see whether the use of 3TC influenced nucleotide changes during follow-up, I compared all available sequences to the baseline sequence and calculated the number of nucleotide changes that occurred. Patients with a baseline sequence and at least one follow-up sequence were included in these comparisons.

There were 57 patients (26 (40.0%) and 31 (47.0%) in the On-3TC and Off-3TC arms respectively) with HIV RNA >500 copies/mL at baseline and at some point during follow-up. From these, there were 42 (73.7%) patients who had samples that were possible to sequence at both baseline and follow-up (19 (73.1%) and 23 (74.2%) in the On-3TC and Off-3TC arms respectively) (Figure 6.4.5.1 and Table 6.4.5.2.1).

It was not possible to sequence both baseline and follow-up samples for the remaining 15 patients due to insufficient plasma concentrations, sample contamination or due to a lack of an available sample at either the baseline or follow-up time point.

Table 6.4.5.2.1: Disposition of patients in the nucleotide distances sub-study.

	On-3TC No. (%)	**Off-3TC** No. (%)	**Total** No. (%)
Patients with HIV RNA >500 c/ml at baseline and at ≥1 follow-up visit:	26 (100)	31 (100)	57 (100)
Patients with baseline and ≥1 follow-up sequences:	19 (73)	23 (74)	42 (74)
Total number of sequences:	**67**	**67**	**134**
Baseline	19	23	42
Week 4	12	10	22
Week 12	9	7	16
Week 24	9	10	19
Week 36	8	7	15
Week 48	10	10	20
Patients included in the final analysis:	18 (69)	19 (61)	37 (65)
Total number of sequences in the final analysis:	**63**	**54**	**117**
Baseline	18	19	37
Week 4	12	9	21
Week 12	9	6	15
Week 24	8	9	17
Week 36	7	4	11
Week 48	9	7	16

Patients who had a baseline and at least one follow up sequence available (N=42) were compared to the remaining patients in COLATE (N=89). Similar proportions of patients with sequencing information came from stratum A (N=17, 31%) and stratum B (N=25,

32%). At baseline, patients who had their virus sequenced had a higher HIV RNA (median (IQR): 4.1 (3.7 to 4.9) *vs.* 3.9 (3.2 to 4.4) log_{10} copies/mL, p=0.005), a lower $CD4^+$ T cell count (median (IQR): 203.0 (113.0 to 355.0) *vs.* 360.0 (247.0 to 458.0) cells/µl, p<0.0001), a lower $CD4^+$ nadir (median (IQR): 75.0 (32.3 to 162.5) *vs.* 140.0 (64.5 to 210.0) cells/µl, p=0.002) and were significantly more likely to have an AIDS-defining illness prior to study entry (21 (50.0%) *vs.* 22 (24.7%), p=0.004) compared to patients in COLATE who did not have both baseline and follow-up samples available. Since patients who had their virus sequenced at both baseline and follow-up were virologically worse off than those who did not (i.e. they have an HIV RNA >500 copies/mL during follow-up), a larger number of nucleotide substitutions is likely to be detected than in the average patient enrolled into COLATE, due to the higher viral replication rates in these patients.

A total of 67 sequences from 19 patients in the On-3TC arm and 67 sequences from 23 patients in the Off-3TC arm were obtained (Table 6.4.5.2.1). Sequences were analysed for each patient using population sequencing. Patients could have a maximum of one sequence per follow up visit, resulting in differences between the numbers of sequences for each patient. If sequences stemming from each patient did not group together in the phylogenetic trees, and grouped more closely with sequences from another patient, I suspected that there could be an error in either the matching of the sequences or in the sequence itself. This is because all of the sequences stemming from a particular patient should be more similar to each other than sequences from other patients, so I would expect them to group together in the phylogenetic trees. All patients who had sequences that did not group together were excluded from the analyses.

Four sequences from one patient in the On-3TC arm (hollow circles, Figure 6.4.5.2.1A) and thirteen sequences from four patients in the Off-3TC arm (diamonds, squares, circles and triangles, Figure 6.4.5.2.1B) were suspicious sequences and have been marked in the phylogenetic trees. These patients, and all their sequences, were excluded from the analyses so that I could study nucleotide distances between viral populations that are closely linked without interference from patients who experience major changes to their predominant virus (as indicated by a jump in the phylogenetic trees), or more likely, a mix up of sequences or sample contamination.

After the exclusions of patients with suspicious sequences, 63 sequences from 18 patients in the On-3TC arm and 54 sequences from 19 patients in the Off-3TC arm were included

in the analysis (Table 6.4.5.2.1). Phylogenetic trees were created for the protease and RT genes separately and were similar to the trees for the whole sequence (data not shown).

Figure 6.4.5.2.1: Neighbour-Joining distance trees for sequences in the On-3TC arm (A) and Off-3TC arm (B) using distances calculated through the Jukes-Cantor method

Figure A:

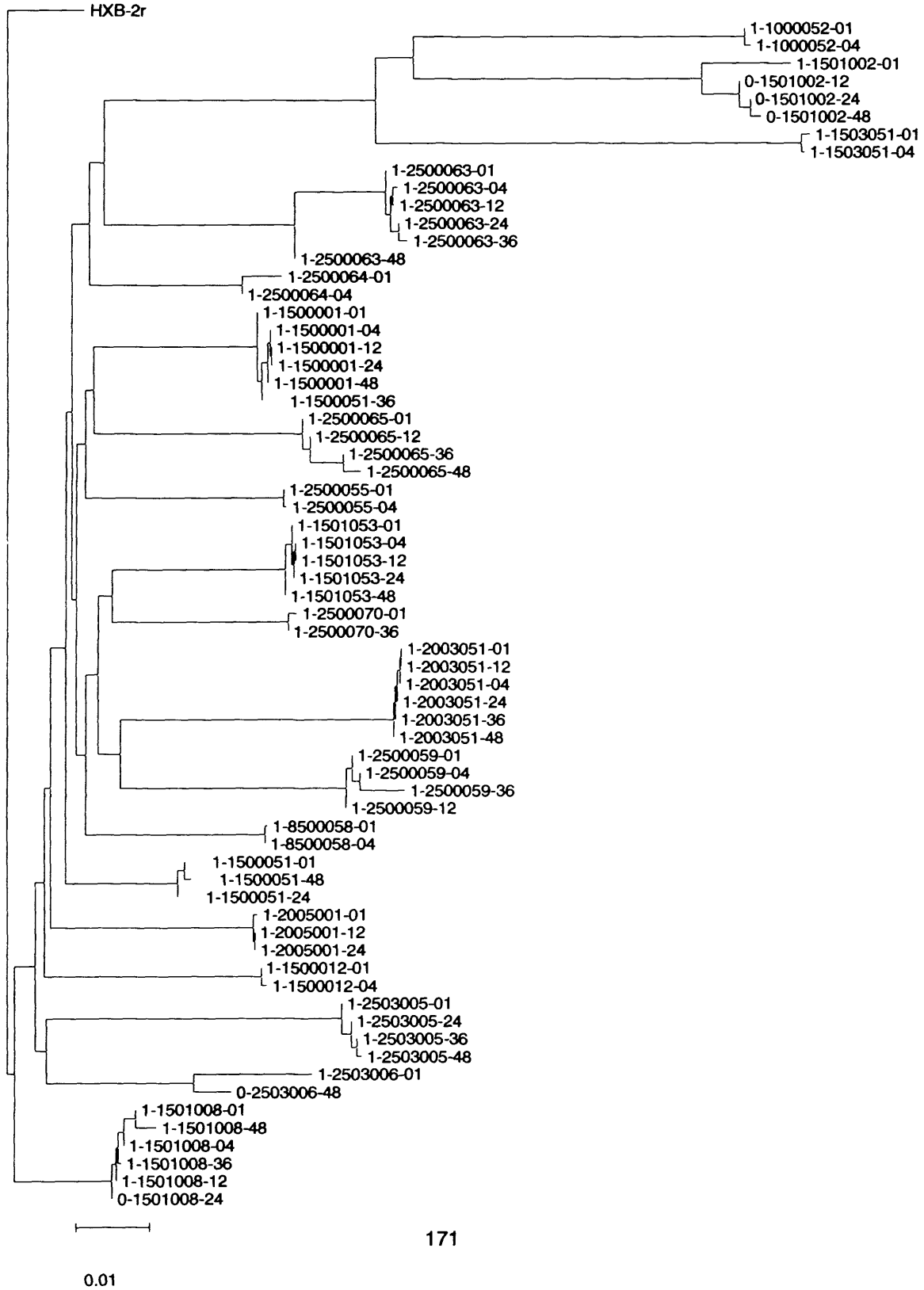
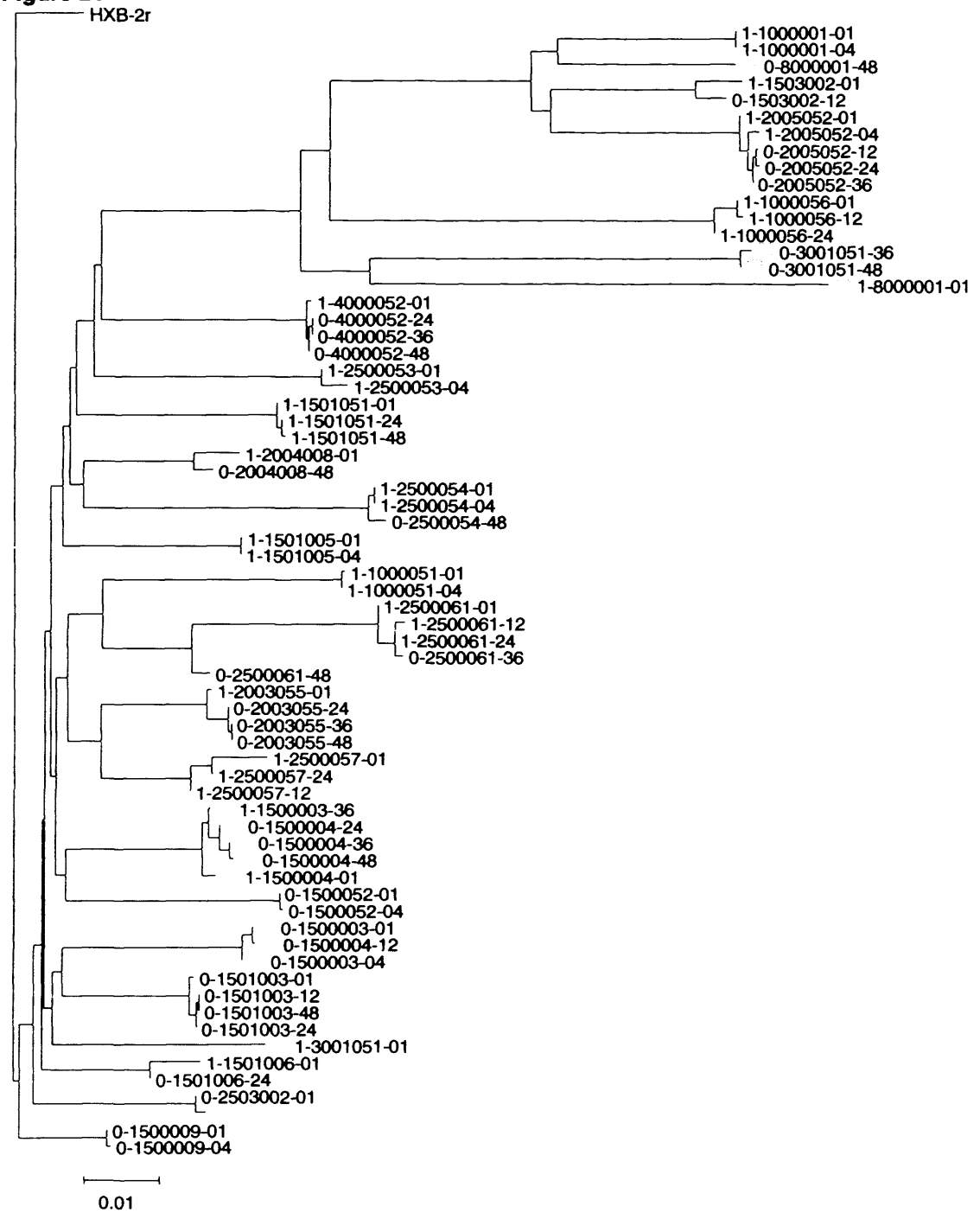


Figure B:



Legend: The first number in the leaf name indicates the presence of the M184V mutation (1 = present, 0 = not present). Leaves marked with a symbol have been excluded from the analysis. The number between the two hyphens is the patient identifier. The last two-digit number is the week visit to which the sequence belongs: 01 = Baseline; 04 = Week 04; 12 = Week 12; 24 = Week 24; 36 = Week 36; 48 = Week 48

6.4.5.2.1. Characteristics of patients in the nucleotide distances sub-study

The 37 patients who were included in this final resistance analysis were primarily male (78.4%) and white (78.4%). They had relatively low $CD4^+$ T cell counts (median: 175, IQR: 111 to 324 cells/μl) with a median (IQR) viral load of 4.1 (3.8 to 4.9) $\log_{10}$ copies/mL. There were 15 patients (41%) who reached virological suppression at some point during follow-up. No significant differences were observed in baseline demographics between treatment arms, but differences did exist in prior treatment use (Table 6.4.5.2.1.1).

Approximately the same proportions of patients in both treatment arms had previously used PIs, 88.9% (On-3TC) and 84.2% (Off-3TC), but patients randomised to On-3TC had been exposed to this drug class for longer, a median duration (IQR) of 51.6 (38.2 to 55.5) months (On-3TC) vs. 33.7 (18.6 to 49.0) months (Off-3TC), p=0.02. When looking at the AAUCMB analysis for patients who had their virus sequenced the results were similar to those obtained in COLATE overall: a mean 1.6 (95% CI: 1.3 to 1.8) $\log_{10}$ cps/mL reduction in HIV RNA for patients in the On-3TC arm compared to 1.5 (95% CI: 1.3 to 1.8) $\log_{10}$ cps/mL for patients in the Off-3TC arm (p=0.78).

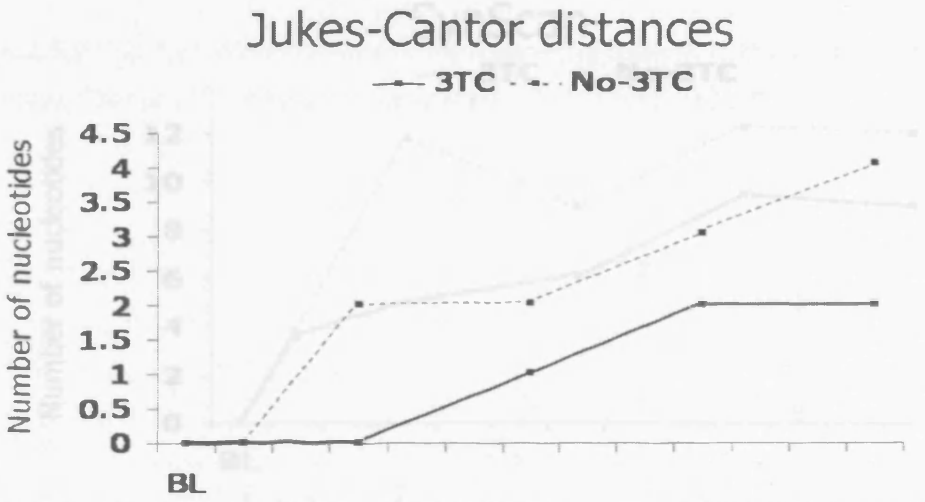
Table 6.4.5.2.1.1: Baseline characteristics of the patients included in the analysis.

Baseline parameter		**On-3TC N =18**	**Off-3TC N =19**
Gender	No. male, (%)	15 (83)	14 (74)
Age	Median years (IQR)	41.5 (32.5, 51.3)	39.0 (37.0, 43.0)
CDC, category C	No. Yes, (%)	9 (50)	11 (58)
Viral load	copies/mL $\log_{10}$ (IQR)	4.4 (3.7, 4.9)	4.0 (3.8, 5.0)
$CD4^+$ count	10^6/l; median (IQR)	170 (111, 284)	180 (116, 351)
$CD4^+$ nadir count	10^6/l; median (IQR)	42.5 (5.8, 97)	80 (39, 180)
HIV exposure group	Homosexual/bisexual	11 (61)	11 (58)
	Heterosexual	5 (28)	7 (37)
	IVDU	1 (6)	0 (0)
	Unknown & other	1 (6)	1 (5)
Race:	White	14 (78)	15 (79)
	Black	3 (17)	3 (16)
	Asian	1 (6)	0 (0)
	Other	0 (0)	1 (5)
Previous NRTI use	No. (%)	18 (100)	19 (100)
	Median months, (IQR)	56.2 (24.7, 99.9)	49.5 (29.3, 77.2)
Previous PI use	No. (%)	16 (88.9)	16 (84.2)
	Median months, (IQR)	51.6 (38.2, 55.5)	33.7 (18.6, 49.0)
Previous NNRTI use	No. (%)	9 (50.0)	13 (68.4)
	Median months, (IQR)	6.9 (5.4, 27.0)	15.2 (0.6, 20.5)

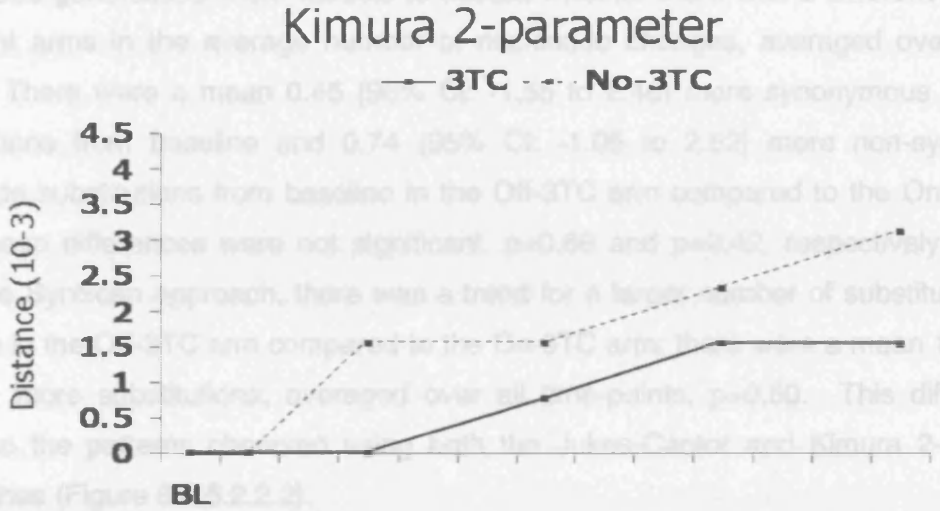
6.4.5.2.2. Nucleotide distances according to treatment arm

For each patient, nucleotide distances from baseline were estimated by comparing all available sequences to the baseline sequence and calculating the number of nucleotide changes that occurred, using each of the three approaches described in section 6.3.4. The patterns observed in Figure 6.4.5.2.2.1 are consistent with the sequences illustrated in the phylogenetic trees (Figure 6.4.5.2.1), where samples taken during follow-up are progressively more distant from the baseline sample. Distances appear to increase more quickly for patients in the Off-3TC arm compared to patients in the On-3TC arm (Figure 6.4.5.2.2.1). However, due to the small number of patients and consequently low power at each visit, no statistically significant differences were observed using Kruskal-Wallis tests. At Week 48 the median number of nucleotide changes from baseline was 9.00 (IQR: 5.00 to 28.38) in the On-3TC arm and 12.00 (IQR: 8.50 to 20.00) in the Off-3TC arm using the SynScan approach, p=0.71. A lower number of substitutions was seen using both the Jukes-Cantor and the Kimura 2-parameter approaches (Figure 6.4.5.2.2.1).

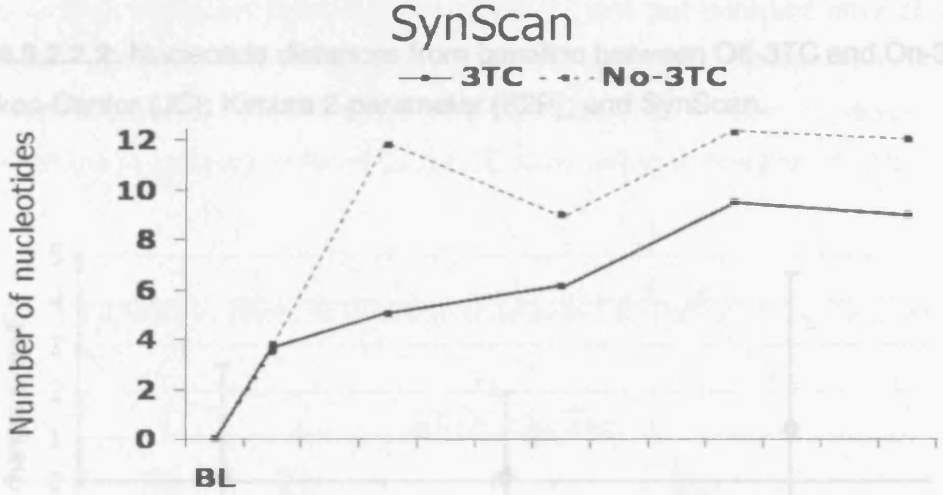
Figure 6.4.5.2.2.1: The distance between baseline and each follow-up visit using: A) Jukes-Cantor; B) Kimura 2-parameter and C) SynScan.



Week	4	12	24	36	48
3TC Median (IQR)	0.00 (0.00, 1.01) N=12	0.00 (0.00, 1.01) N=9	1.01 (0.00, 2.01) N=8	2.01 (1.00, 5.05) N=7	2.01 (0.00, 17.41) N=9
No-3TC Median (IQR)	0.00 (0.00, 0.00) N=9	2.02 (1.01, 6.35) N=6	2.03 (0.51, 4.55) N=9	3.04 (1.27, 5.53) N=4	4.05 (1.01, 11.22) N=7
P-value	0.39	0.04	0.21	0.45	0.49



Week	4	12	24	36	48
3TC Median (IQR)	0.00 (0.00, 0.78) N=12	0.00 (0.00, 0.78) N=9	0.76 (0.00, 1.55) N=8	1.55 (0.77, 3.88) N=7	1.54 (0.00, 13.39) N=9
No-3TC Median (IQR)	0.00 (0.00, 0.00) N=9	1.55 (0.77, 4.88) N=6	1.56 (0.39, 3.49) N=9	2.33 (0.98, 4.25) N=4	3.11 (0.78, 8.63) N=7
P-value	0.39	0.04	0.21	0.45	0.49

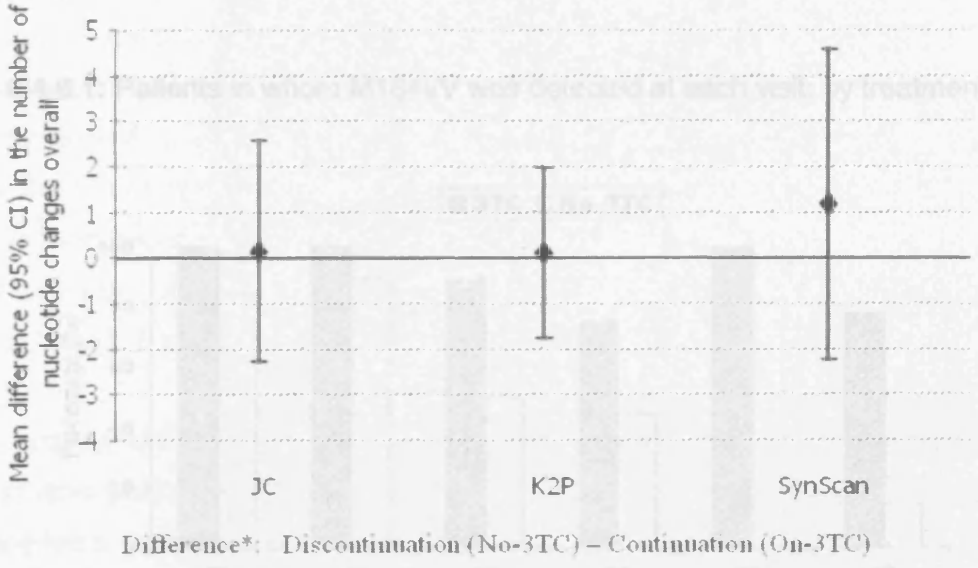


Week :		4	12	24	36	48
Median (IQR) dS + dN	3TC	3.8 (1.5, 5.2) N=12	5.0 (3.8, 7.1) N=9	6.1 (4.8, 11.6) N=8	9.5 (5.0, 13.0) N=7	9.0 (5.0, 28.4) N=9
Median (IQR) dS + dN	No-3TC	3.5 (2.4, 5.8) N=9	11.8 (6.8, 21.0) N=6	9.0 (8.0, 15.3) N=9	12.3 (11.6, 13.6) N=4	12.0 (8.5, 20.0) N=7
P-value		0.86	0.13	0.21	0.22	0.71
dS / dN	3TC	1.3 (0.8, 1.6)	1.0 (0.5, 1.9)	1.2 (0.4, 1.7)	0.6 (0.2, 1.3)	0.9 (0.3, 1.4)
dS / dN	No-3TC	1.9 (1.2, 3.4)	0.9 (0.6, 2.2)	1.3 (0.4, 2.1)	0.4 (0.3, 1.2)	0.7 (0.4, 1.0)

I then used generalised linear models to assess whether there was a difference between treatment arms in the average number of nucleotide changes, averaged over all time-points. There were a mean 0.45 (95% CI: -1.55 to 2.46) more synonymous nucleotide substitutions from baseline and 0.74 (95% CI: -1.05 to 2.52) more non-synonymous nucleotide substitutions from baseline in the Off-3TC arm compared to the On-3TC arm; albeit these differences were not significant, p=0.66 and p=0.42, respectively. Overall, using the SynScan approach, there was a trend for a larger number of substitutions from baseline in the Off-3TC arm compared to the On-3TC arm: there were a mean 1.16 (-2.24 to 4.56) more substitutions, averaged over all time-points, p=0.50. This difference is similar to the patterns observed using both the Jukes-Cantor and Kimura 2-parameter approaches (Figure 6.4.5.2.2.2).

Nucleotide distances did not differ between strata: there were a mean 0.47 (-2.67 to 3.61) more substitutions in stratum A compared to stratum B, averaged over all time-points, p=0.77. Patients who experienced increases in HIV RNA experienced significantly larger nucleotide distances: 2.42 (0.80 to 4.05) more substitutions for every $\log_{10}$ cps/mL increase in HIV RNA over the period (p=0.003).

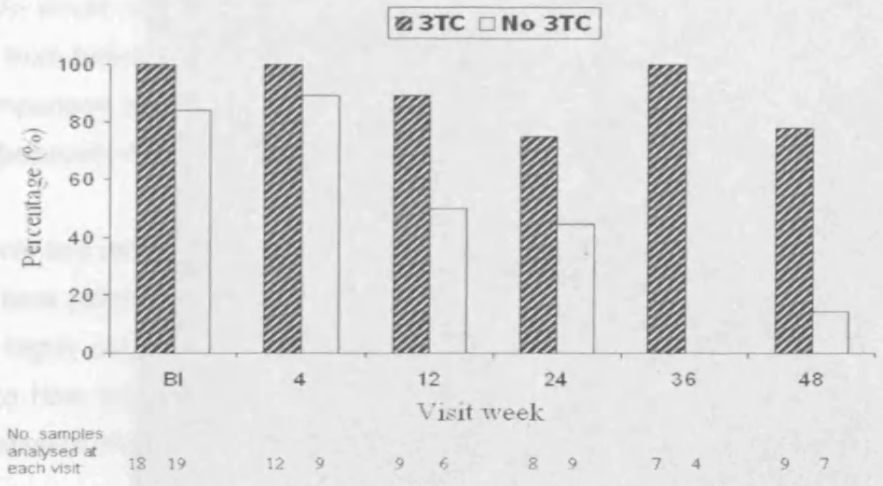
Figure 6.4.5.2.2.2: Nucleotide distances from baseline between Off-3TC and On-3TC Using: Jukes-Cantor (JC); Kimura 2-parameter (K2P); and SynScan.



6.4.6. Pattern of decline in the prevalence of M184I/V

To investigate whether the lack of a difference in the average number of accumulated nucleotide changes between treatment arms could be due to the fact that the M184I/V mutation remained present in the dominant viral population of patients who discontinued 3TC, I decided to look at the prevalence of M184I/V throughout follow-up. By examining the proportion of patients with mutations at genotypic testing, the prevalence of M184I/V showed a decline over time in the predominant virus of patients who discontinued 3TC (Figure 6.4.6.1). By week 24, M184I/V was detected in the predominant virus of four of nine (44.4%) patients in the Off-3TC arm; by week 48 this had decreased to 14.3% (i.e. one of seven patients in the Off-3TC arm who had resistance tests available at week 48). The patient in the Off-3TC arm, in whom the M184I/V mutation was detected by population sequencing at week 48, re-started 3TC a week into the trial. This may explain why M184I/V remained present in the dominant viral species. In contrast, in patients in the On-3TC arm, M184I/V was still detected in the dominant virus of six of eight (75.0%) and seven of nine (77.8%) patients at weeks 24 and 48, respectively. Of note, Figure 6.4.6.1 only reflects the pattern of decline in patients with successful sequencing and, for obvious reasons, does not include patients who were virologically suppressed. The characteristics of patients in each treatment arm, in whom M184I/V was not detected after 24 weeks of follow-up, were comparable to patients in whom M184I/V was detected after 24 weeks of follow-up, with respect to baseline NNRTI use and baseline PI use. In addition, similar proportions of these patients entered COLATE after failing a first-line regimen (data not shown).

Figure 6.4.6.1: Patients in whom M184I/V was detected at each visit: by treatment arm.



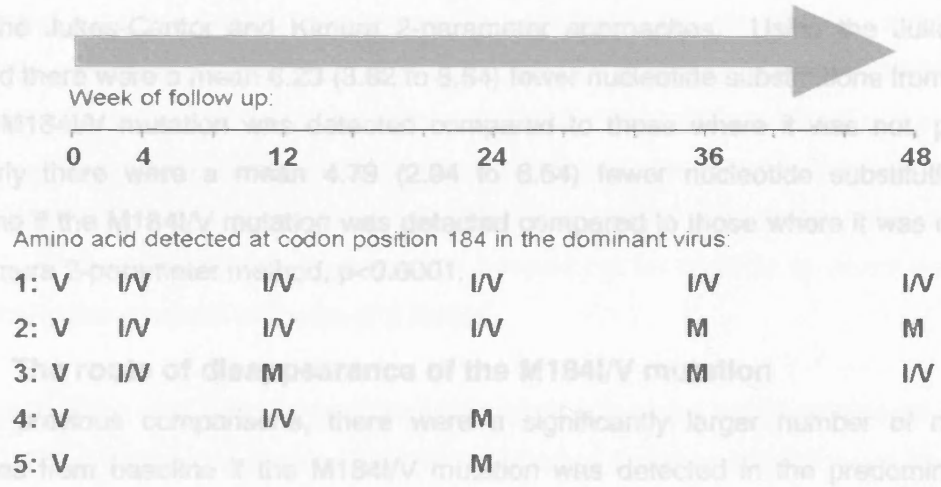
6.4.7. Nucleotide distances according to presence of M184I/V

As depicted in Figure 6.4.6.1, there was an appreciable amount of time in the Off-3TC arm where the M184I/V mutation remained present even though patients had discontinued 3TC. Conversely, the 184I/V appeared to have reverted back to 184M in some patients in the On-3TC arm even under selection pressure from continued use of 3TC. To account for the fact that M184I/V could be detected in the predominant virus of some patients in the Off-3TC arm during follow-up, and M184I/V could not be detected in the predominant virus of some patients in the On-3TC arm, I identified whether the M184I/V mutation was present in the predominant virus at each visit and compared nucleotide distances from baseline, according to the presence of this mutation.

So, for example, Figure 6.4.7.1 illustrates four possible scenarios for the disappearance and re-appearance of the M184I/V mutation at each of the follow-up visits. The distance between baseline and each of the five follow-up time points would contribute data to the '184I/V comparison arm' for patient 1. Since the 184I/V mutation is continuously detected in the predominant virus of this patient, they would not contribute any data to the '184M comparison arm'. In patient 2, 184I/V is detected at each visit from baseline until week 36. At week 36 the 184I/V mutation is no longer detected, instead it appears to have reverted back to 184M. The first three distance calculations (i.e. nucleotide distances from baseline to weeks 4, 12 and 24) would contribute data to the '184I/V comparison arm' whereas the last two distances (i.e. nucleotide distances from baseline to weeks 36 and 48) would contribute data to the '184M comparison arm'. The 184I/V mutation disappears from the predominant virus of patient 3 after four weeks of follow-up. However, it is detected again in the predominant virus afer 48 weeks of follow-up. In this situation, the first and last distance calculations (i.e. nucleotide distances from baseline to week 4 and from baseline to week 48) would contribute data to the '184I/V comparison arm' whereas the nucleotide distances from baseline to week 12 and baseline to week 36 would contribute data to the '184M comparison arm'. Patient 3 would not contribute any data to these comparisons at week 24, because no resistance data is available at this time point for this patient.

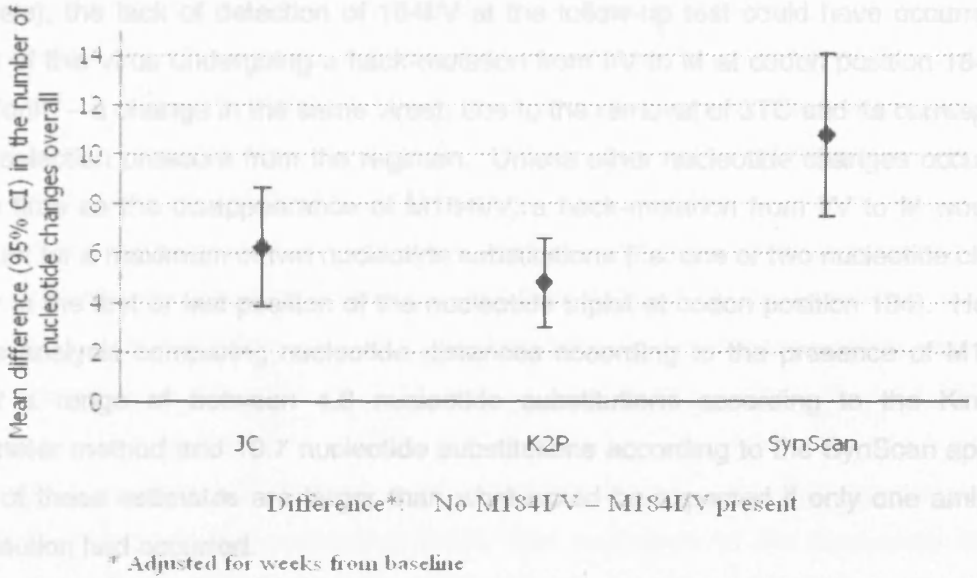
Of note, only two patients had plasma samples available for sequencing at each of the five follow-up time points and at baseline, and, since some of the situations outlined in the figure are highly unlikely to occur, this diagram is just provided as a hypothetical example to illustrate how this analysis was performed. Patients 4 and 5 are likely to be more representative of the patients in our study than patients 1 to 3.

Figure 6.4.7.1: Hypothetical presence of mutations in the dominant virus, over follow-up



When I modelled nucleotide distances from baseline using this approach, there were a mean 10.74 (7.46 to 14.02) fewer nucleotide substitutions from baseline in follow-up samples where 184I/V was detected compared to those where 184M was detected, averaged over all time-points, using the SynScan method (synonymous and non-synonymous combined), p<0.0001 (Figure 6.4.7.2).

Figure 6.4.7.2: Nucleotide distances from baseline between M184I/V and no M184I/V
Using: Jukes-Cantor (JC); Kimura 2-parameter (K2P); and SynScan.



Larger nucleotide distances were also seen from baseline in patients who had a follow-up virus in which 184M was detected compared to those in which 184I/V was detected, using both the Jukes-Cantor and Kimura 2-parameter approaches. Using the Jukes-Cantor method there were a mean 6.23 (3.82 to 8.64) fewer nucleotide substitutions from baseline if the M184I/V mutation was detected compared to those where it was not, $p<0.0001$. Similarly there were a mean 4.79 (2.94 to 6.64) fewer nucleotide substitutions from baseline if the M184I/V mutation was detected compared to those where it was not, using the Kimura 2-parameter method, $p<0.0001$.

6.4.8. The route of disappearance of the M184I/V mutation

In the previous comparisons, there were a significantly larger number of nucleotide changes from baseline if the M184I/V mutation was detected in the predominant virus during follow-up compared to when it was not, using all three evolutionary nucleotide approaches (i.e. from 4.8 to 10.7 nucleotide changes). These differences may arise due to the actual loss of the M184I/V mutation (i.e. a back-mutation from isoleucine or valine to methionine in the same virus), in combination with a possible increase in viral replication and a reduced fidelity of the new virus. Or, potentially, it may be due to other viral changes that occur when I/V reverts back to M (i.e. the virus population that was sampled at baseline may no longer be the dominant viral species, instead an archived sub-species may have outgrown it and become dominant).

In the first case (i.e. assuming that the same virus was sampled at the baseline and follow-up tests), the lack of detection of 184I/V at the follow-up test could have occurred as a result of this virus undergoing a back-mutation from I/V to M at codon position 184 (i.e. a viral "drift" – a change in the same virus), due to the removal of 3TC and its corresponding drug selection pressure from the regimen. Unless other nucleotide changes occur at the same time as the disappearance of M184I/V, a back-mutation from I/V to M would only account for a maximum of two nucleotide substitutions (i.e. one or two nucleotide changes, either in the first or last position of the nucleotide triplet at codon position 184). However, in the analysis comparing nucleotide distances according to the presence of M184I/V I found a range of between 4.8 nucleotide substitutions according to the Kimura 2-parameter method and 10.7 nucleotide substitutions according to the SynScan approach. Both of these estimates are larger than what would be expected if only one amino acid substitution had occurred.

Alternatively, and possibly more realistically, the virus that was sampled during follow-up may actually come from a different virus population to the one that was sampled at baseline (i.e. a viral "shift" – a change in the dominant virus population). If this was the case, the detection of 184I/V at baseline followed by a lack of detection of 184I/V at the follow-up test, may arise because a virus containing the wild-type 184M amino acid may be more 'fit' under the conditions at the time of follow-up (for example because lamivudine was discontinued) and so it may out-compete the baseline virus that contains the mutant 184I/V. Under both of these assumptions it would not be possible to detect the M184I/V mutation in the predominant virus any longer.

In the case of a viral shift, the number of nucleotide changes would provide an erroneous picture of the mutational changes that occur between baseline and follow-up because this approach involves comparing the baseline virus to a virus that was only present in minor populations at baseline (i.e. a different virus) and so the virus would appear to have undergone more nucleotide changes than it actually had. As a result, elevated estimates of nucleotide distances would be obtained. The resistance methods in COLATE do not allow us to distinguish between viral drifts and viral shifts, with respect to the disappearance of M184I/V, in our patients.

6.4.8.1. Distances within samples containing 184M or containing 184I/V

In order to overcome the issue of viral shifts, I designed an alternative analysis. First, I assumed that all viruses containing 184M came from the same virus population and that all viruses containing 184I/V came from the same virus population. Then, in order to see how much of the difference in distances between viruses in which M184I/V was detected compared to viruses where M184I/V was not detected, may be explained by an amino acid substitution from I/V to M (i.e. a back-mutation) I excluded information on nucleotide changes that occurred at the time of the reversion back to 184M. Instead, I estimated the distance-per-unit-time from the first sequence that contained 184M to following samples where 184M was present, and compared this distance to the distance-per-unit-time between viruses that contained 184I/V (i.e. from baseline to other time points where 184I/V was present).

In essence, nucleotide distances were calculated between the first sample containing 184M to other samples containing 184M, and compared to the nucleotide distances between the first sample containing 184I/V (i.e. the baseline sample) and other samples containing 184I/V. Patients could contribute sequence data to both arms of this sub-

analysis if they had ≥1 sequence where 184I/V was present as well as ≥1 sequence where 184M was present. The nucleotide changes that occur at the time of the amino acid reversion from I/V to M will not contribute to the distance calculations in this analysis because distances are not calculated between viruses containing 184I/V and viruses containing 184M. Instead, this analysis investigates whether distances among patients with a virus containing 184M are different from those with a virus containing 184I/V. Then, by definition, only nucleotide changes other than those seen at codon position 184 will be accounted for in these comparisons.

So, if I refer back to the example in Figure 6.4.7.1, the distance between baseline and each of the five follow-up time points would still contribute data to the '184I/V comparison arm' for patient 1 and this patient would not contribute any data to the '184M comparison arm'. In patient 2, the first three distance calculations (i.e. nucleotide distances from baseline to weeks 4, 12 and 24) would contribute data to the '184I/V comparison arm' whereas the nucletode distance between the last two samples (i.e. the nucleotide distance from week 36 to week 48) would contribute data to the '184M comparison arm'. Similarly, in patient 3, the first and last distance calculations (i.e. nucleotide distances from baseline to weeks 4 and from baseline to week 48) would contribute data to the '184I/V comparison arm' whereas the nucleotide distances from week 12 to week 36 would contribute data to the '184M comparison arm'. Patient 4 would only contribute data to '184I/V comparison arm' (i.e. the distance between baseline and week 12), and patient 5 would contribute no data to these comparisons. All of the distances were standardised for the time between measurements.

Using this approach, there was a mean: 2.58 (-0.49 to 5.64), p=0.10; 1.98 (-0.38 to 4.34), p=0.10; and 3.47 (-1.22 to 8.16), p=0.15, fewer nucleotide substitutions in viruses where M184I/V was detected compared to those in which it was not, after adjustment for the time between measurements, using the Jukes-Cantor; Kimura 2-parameter; and SynScan methods, respectively. In this analysis, the magnitude of the difference in the number of nucleotide substitutions between samples where 184I/V was detected compared to those where 184M was detected, was reduced from 10.74 (7.46 to 14.02) to 3.47 (-1.22 to 8.16), using the SynScan approach. This may suggest that the trend for smaller distances observed in the main analysis, for patients where 184I/V was detected, can be partly explained by the actual loss of the 184I/V mutation (or other mutational changes that occur simultaneously). However, there still appears to be a marginal difference in the number of nucleotide changes between viruses containing M184I/V and viruses that do not.

6.4.8.2. Comparing distances from samples where M184I/V is present, missing or changed (M→ I/V or I/V→M) between visits:

As a further way to try to understand whether the reversion of I/V back to M, or the re-emergence of 184I/V, happened as a consequence of a shift to a different viral sub-population (as opposed to a viral drift) I compared nucleotide distances between patients who had a virus with the same mutation at consecutive visits (i.e. either 184M was present at both visits or 184I/V was present at both visits) to patients who had a virus that underwent an amino acid substitution in between visits.

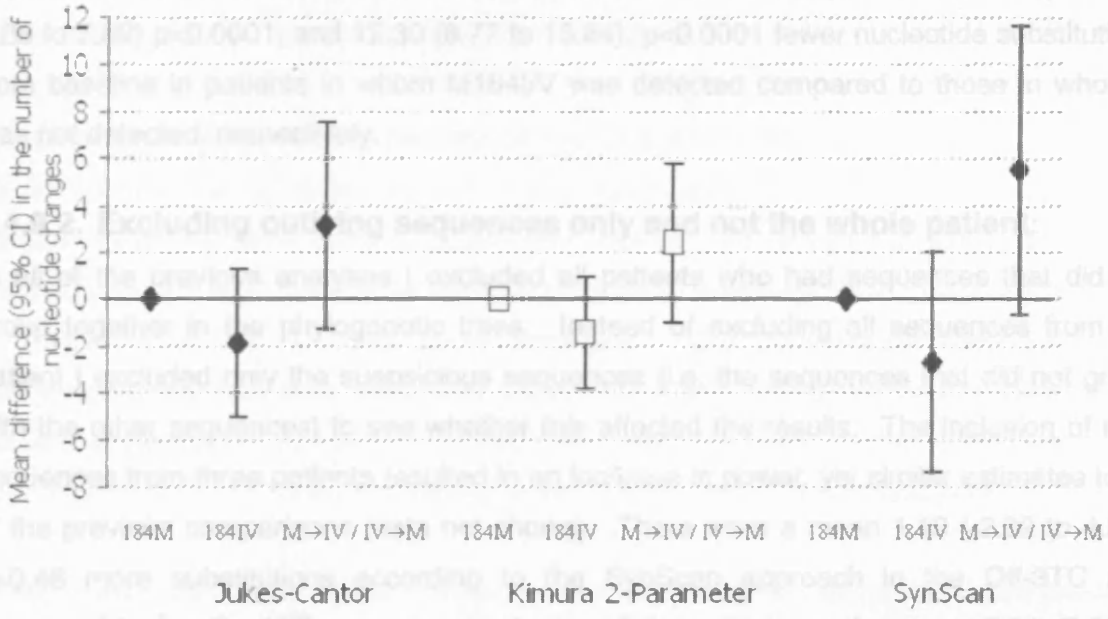
If I refer back to Figure 6.4.7.1 in this case, patient 1 would still contribute five sets of observations to these comparisons, but they now reflect the distance between visits rather than the distances from baseline. For patient 1, the data that would now be included in the '184I/V comparison arm' includes the distance from baseline to week 4, from week 4 to week 12, from week 12 to week 24, from week 24 to week 36 and from week 36 to week 48). In patient 2, the nucleotide distances from baseline to week 4, from week 4 to week 12 and from week 12 and 24 would contribute data to the '184I/V comparison arm'; the nucletode distance between week 24 and week 36 would contribute data to the 'change from M to I/V or I/V to M arm'; and the nucletode distance from week 36 to week 48 would contribute data to the '184M comparison arm'. In patient 3, the nucleotide distances from baseline to week 4 would contribute data to the '184I/V comparison arm'; the nucleotide distances from week 4 to week 12 and from week 36 to week 48 would contribute data to the 'change from M to I/V or I/V to M arm'; and the nucleotide distances from week 12 to week 36 would contribute data to the '184M comparison arm'. Patient 4 would contribute data to '184I/V comparison arm' (i.e. the distance between baseline and week 12) and to the 'change from M to I/V or I/V to M arm' (i.e. the distance between week 12 and week 24) and patient 5 would also contribute data to the 'change from M to I/V or I/V to M arm'. Distances were again standardised for the time between measurements.

If the reversion of I/V back to M was due to a shift in the dominant virus population, the largest nucleotide distances would be expected to be seen in patients who had a virus that underwent an amino acid substitution at position 184. This is because, under our assumption, the nucleotide distance would be calculated between two different viruses. In fact, I would expect the nucleotide distances in this group to be substantially greater than the nucleotide distances in the other two groups.

Although the trends were in the anticipated direction - patients who had a virus that underwent an amino acid change from M to I/V or I/V to M experienced the largest distances and patients who retained 184I/V the smallest - I did not find any significant differences between the three groups (Figure 6.4.8.2.1).

Using Jukes-Cantor, Kimura 2-parameter and SynScan there were a mean 1.85 (95% CI: -1.29 to 4.98), 1.42 (95% CI: -0.99 to 3.83) and 2.70 (95% CI: -2.00 to 7.39) fewer nucleotide substitutions in patients who had a virus in which 184I/V was detected at consecutive visits respectively, and 3.17 (-1.25 to 7.60), 2.44 (95% CI: -0.96 to 5.84) and 5.51 (95% CI: -0.63 to 11.66) more nucleotide substitutions in patients with an amino acid change, compared to patients who had a virus in which 184M was detected at consecutive visits.

Figure 6.4.8.2.1: Comparison of distances between samples where M184I/V is present, missing or changed (M→ I/V or I/V→M) between consecutive visits



6.4.9. Sensitivity analyses

6.4.9.1. Excluding patients who did not have M184I/V at baseline:

When I excluded the three patients who did not have a virus showing M184I/V at baseline, the results of the main analysis comparing nucleotide distances between treatment-arms (section 6.4.5.2.2) did not change substantially. There was still a trend for a larger number of substitutions in the Off-3TC arm compared to the On-3TC arm: in the Off-3TC arm a mean 0.34 (-2.30 to 2.97) more substitutions were seen according to the Jukes-Cantor approach, p=0.80; a mean 0.26 (-1.77 to 2.28) more substitutions according to the Kimura 2-parameter approach, p=0.80; and a mean 1.39 (-2.28 to 5.06) more substitutions according to the SynScan approach, p=0.46, averaged over all time-points.

In this same subset of patients, there were slightly larger differences in the nucleotide distance from baseline than that observed in the main analysis, when we grouped patients according to the detection of M184I/V instead of the COLATE randomisation group (section 6.4.7), after exclusion of the three patients who did not have a virus containing M184I/V at baseline. Using the Jukes-Cantor, Kimura 2-parameter and SynScan approaches there were a mean 7.65 (95% CI: 5.04 to 10.26), p<0.0001; 5.88 (95% CI: 3.88 to 7.89) p<0.0001; and 12.30 (8.77 to 15.84), p<0.0001 fewer nucleotide substitutions from baseline in patients in whom M184I/V was detected compared to those in whom it was not detected, respectively.

6.4.9.2. Excluding outlying sequences only and not the whole patient:

In all of the previous analyses I excluded all patients who had sequences that did not group together in the phylogenetic trees. Instead of excluding all sequences from the patient I excluded only the suspsicious sequences (i.e. the sequences that did not group with the other sequences) to see whether this affected the results. The inclusion of nine sequences from three patients resulted in an increase in power, yet similar estimates to all of the previous comparisons (data not shown). There were a mean 1.13 (-2.02 to 4.29), p=0.48 more substitutions according to the SynScan approach in the Off-3TC arm compared to the On-3TC arm, averaged over all time points, and a mean 9.91 (6.88 to 12.94) fewer nucleotide substitutions from baseline if the M184I/V mutation was detected in the predominant virus population compared to those in whom it was not, p<0.0001. The results using the Jukes-Cantor and Kimura 2-parameter approaches were consistent with the above (data not shown).

6.5. Discussion

6.5.1. Summary of main results

The aim of COLATE was to assess whether retaining 3TC in a new regimen after failing cART, in patients in whom the M184V mutation was present in their predominant viral population, could be virologically or immunologically beneficial in clinical practice. This is the first study to address the effect of retaining the M184V mutation during cART *in vivo*. COLATE was designed with the assumptions that i) M184V would be present at baseline ii) M184V would gradually disappear in the Off-3TC arm iii) the background cART would consist of at least three ARVs throughout follow-up and iv) knowledge of whether each patient was receiving 3TC, due to the open-label design of the trial, would not result in additional ARVs being added to the pre-selected background regimen or to ARVs used during follow-up. All of these expectations were met in this study.

All patients with baseline resistance tests in the On-3TC arm and 89% of patients in the Off-3TC arm harboured M184V. In the On-3TC arm this mutation was detected throughout the whole of follow-up whereas it gradually disappeared in the Off-3TC arm. The mean number of ARVs received other than 3TC was >3 throughout follow-up, with no differences in the use of individual drugs between treatment arms.

6.5.2. Interpretation of the results of the COLATE trial

As far as the results of the main trial are concerned, no differences were observed between treatment arms in the AAUCMB HIV RNA $\log_{10}$ reductions (On-3TC arm: 1.4 and Off-3TC arm: 1.5 $\log_{10}$; p=0.51), in the ability or time to suppress HIV RNA, or in the time to or magnitude of $CD4^+$ T cell count increases. Our observations differ from (the interpretation of) data from laboratory experiments and non-randomised studies, which indicate a postulated benefit of retaining the M184V mutation in the predominant viral population[272;393;398;390]. Several potential explanations exist for this discordance.

Firstly, most clinical studies that have addressed this issue had limited statistical power to draw firm conclusions. With a sample size of 160 the COLATE trial was designed with 90% power to show a difference in HIV RNA reductions between treatment arms of ≥0.5 $\log_{10}$ cps/mL. Even though the final sample size was only 131 patients, the trial retained >80% power to detect a difference in the primary analysis.

Secondly, use of different ARVs and/or ARV drug class during follow-up, or different baseline resistance patterns in the predominant viral populations of these patients, could have influenced the overall outcome of the trial. In this study, no difference was seen in use or duration of treatment with individual ARVs or drug class prior to baseline, or during follow-up, between patients in the On-3TC and those in the Off-3TC arm. In addition, there were no differences at baseline in the number of primary mutations to NRTIs, NNRTIs or PIs (Table 6.4.5.1.1) and there were no differences in the baseline GSS between treatment arms. However, for all patients in stratum A and the majority of patients in stratum B a new regimen consisting of ≥2 drugs to which the virus was susceptible to was initiated at baseline. The residual viral effect of 3TC in the On-3TC arm could have been indiscernible because patients were receiving other active drugs. Since the predominant virus at baseline was reasonably susceptible to the other ARVs in the regimen, it may not have been possible to tease out an added virological benefit of continuing 3TC.

The results of the COLATE study can only be applied to patients who have the potential to initiate three new ARVs and cannot be extended to patients with multi-drug resistance who have few treatment options available. The continued use of 3TC alone has already been addressed in the E-184V pilot study[279], with results consistent with both an impaired fitness of M184V containing viruses or residual activity of 3TC against viruses harbouring the M184V mutation, although these results still need to be reproduced using a larger dataset.

One concern about the re-use of drugs, which, in many cases would be an add-on to a 3- or 4-ARV regimen, is the potential risk of side effects associated with multi-drug regimens[399]. Lamivudine is a very well tolearated drug and I did not observe any statistically significant differences in adverse events leading to premature interruption of the assigned treatment strategy between patients in the On-3TC and those in the Off-3TC arm.

6.5.3. Interpretation of the results investigating nucleotide distances

In this chapter, I described a sub-group of patients who had genotypic data available at baseline and during follow-up. This was done because I wanted to evaluate whether patients who discontinue 3TC after failing a 3TC-containing cART regimen experience more nucleotide substitutions in their dominant virus population compared to patients who maintain it in their regimen. This is the first study that has addressed this question *in vivo,* thereby enabling us to assess whether adding 3TC to a new cART regimen for a patient, in

whom M184V is detected in their predominant viral population, is likely to be worthwhile in clinical practice.

Based on the hypothesis of reduced replication capacity and increased fidelity of viruses containing M184V, larger evolutionary distances were expected in patients who discontinued 3TC compared to those who retained it over time. I examined nucleotide distances for patients with HIV RNA >500 cps/mL and a genotypic test, both at baseline and at ≥1 visit during follow-up. Although these patients did not reach virological suppression and may have different rates of viral evolution compared to those who reached virological suppression (and for whom resistance could not be measured), they should still reflect the changes that occurred in the predominant virus populations during follow-up.

Using the Jukes-Cantor, Kimura 2-parameter and SynScan evolutionary nucleotide approaches I found comparable mutation rates through 48 weeks of follow-up in patients in both arms of the study. Nucleotide distances increased from baseline throughout follow-up (Figure 6.4.5.2.1 and Figure 6.4.5.2.2.1) indicating that the virus population progressively evolved away from the population seen at baseline. In the Off-3TC arm a large number of nucleotide substitutions occurred between weeks 4 and 12, whereas substitutions started to appear later on in the On-3TC arm, although none of the differences were statistically significant at any individual time point. When I estimated the overall mean difference in nucleotide distances between treatment arms, averaged over all time-points, I saw 1.2 more changes in patients who discontinued 3TC. This is consistent with our *a priori* expectations because patients in the Off-3TC arm are expected to lose M184V after discontinuing 3TC and this reversion back to wild-type would entail at least a one nucleotide non-synonymous change at codon 184 of the reverse transcriptase gene.

Although I did not find an appreciable effect of discontinuing 3TC on nucleotide distances in some patients in the Off-3TC arm, M184V was detected in a number of tests for an appreciable period of time after stopping 3TC. Therefore, this may have eclipsed the detection of an effect associated with the presence of this mutation if it did exist. Using the proportion of patients with mutations at genotypic testing, the rates of decline of the M184V mutation were in concomitance to the increases in nucleotide distances that we saw from baseline. Since I analysed patients who initiated a cART regimen simultaneously to their 3TC-protocol strategy, other ARVs may have influenced nucleotide distances. Certain drugs could block the evolutionary pathway of the virus or delay the

loss of M184V, which could explain why, in some patients who discontinued 3TC, M184V could be detected throughout follow-up. Adjustment for individual drugs was not possible in the multivariable analyses because it would have resulted in groups containing too few patients. Four patients in each treatment arm stopped their randomised regimen before week 48 and one patient in the Off-3TC arm experienced a treatment interruption.

To account for the fact that, in some patients in the Off-3TC arm, M184V could still be detected in the predominant virus during follow-up, and, in some patients in the On-3TC arm, M184V could not be detected, I compared evolutionary distances from baseline according to whether M184I/V had been detected or not. I used generalised linear models to compare nucleotide distances from baseline between these two groups, and to take into account the fact that, on average, the time between tests was longer in patients in whom M184V was detected at baseline but not in the follow-up test compared to those where M184V was detected at both time points. In these comparisons, I found highly significant differences: patients who had a virus showing M184I/V in their follow-up resistance tests had significantly fewer nucleotide substitutions than those in whom the M814V mutation had reverted back to M at some point.

It is possible that these differences occur because the loss of M184I/V results from a viral drift (i.e. the reversion of 184I/V back to M in the dominant virus). However, it is more likely that this change reflects a shift in the dominant virus population – from a virus that contains M184V to a different virus that does not. In an alternative analysis, I compared the distance-per-unit-time among viruses not showing the M184I/V mutation during follow-up to viruses where M184I/V was detected during follow-up, under the assumption that these sequences stem from the same virus population. Compared to viruses showing the M184I/V mutation, I saw larger distances in those without it, but these trends were only marginally significant (Jukes-Cantor: p=0.10, Kimura 2-parameter: p=0.10, SynScan: p=0.15). If the assumption is true then this would imply that the difference I saw in the main analysis may be only partly explained by the actual loss of M184I/V and that other changes are occuring.

I then compared nucleotide distances between consecutive tests for patients in whom the same mutation was detected at codon position 184 at each visit (i.e. either 184M was present at both visits or 184I/V was present at both visits) to those of patients in whom 184I/V ws detected at one test but not at the following test (or vice-versa). If the same mutation is detected at consecutive visits then the distance between sequences from

these visits is likely to reflect the distance between viruses sampled from the same virus population. This comparison was performed to highlight whether the distance between consecutive sequences, in which there was an amino acid change, are substantially larger than the distance between viruses sampled from the same viral population.

As expected, patients who had a virus that underwent an amino acid substitution between visits experienced larger distances, but they were not significantly different to patients in whom the same mutation was detected at codon position 184 at consecutive visits. Therefore, if our assumptions are correct, a shift to a different viral sub-population is improbable. Furthermore, using the SynScan approach, I only saw 10.7 more nucleotide changes from baseline in viruses in which the M184I/V mutation was not detected compared to viruses where M184I/V was detected, so a shift to a different viral sub-population is unlikely because more than ten nucleotide changes (from 1302 nucleotide positions studied) would be expected if a new viral sub-population had become dominant.

I used the Jukes-Cantor, the Kimura 2-parameter and the SynScan approaches to model the number of substitutions over 48 weeks of follow-up. Although I saw similar trends in nucleotide distances using all three different nucleotide methods, the magnitude of the effect size varied according to the method used. The SynScan approach provided estimates that were two to three fold higher than either the Jukes-Cantor or the Kimura 2-parameter methods for each comparison.

This difference arises because the SynScan approach uses a larger weighting to calculate the distance between two sequences if a within-purine or within-pyrimidine (section 1.5) substitution has occurred (i.e. a transition). Both the Jukes-Cantor and the Kimura 2-parameter approaches estimate the distance between the two purines or between the two pyrimidines to be zero whereas the SynScan approach estimates the distance for a transition to be 0.5. Similarly, the Kimura 2-parameter approach gives a weight to transversions (i.e. a purine to a pyrimidine substitution or vice-versa), whereas the Jukes-Cantor method only accounts for nucleotide changes that result in an amino acid substitution. In this analysis I consistently found the largest number of nucleotide changes using the SynScan approach and the smallest number using the Jukes-Cantor approach. This indicates that nucleotide changes are occurring in the viral population of these patients, although the changes are likely to be transitions that have not yet resulted in an amino acid substitution. These changes may be important for quantifying resistance

because they may reflect what will happen over the long run, although no study as yet has shown any evidence that they are.

6.5.4. Limitations of the analysis

In these comparisons, the observed increases in nucleotide distances over follow-up are likely to reflect changes to the predominant viral population as a result of drug pressure from the regimen the patient is receiving. In addition, changes may occur as a result of natural evolution of the virus population. We only sequenced the reverse transcriptase and protease genes, and since both genes are under drug selection pressure it is not possible to tell whether the changes identified arise as a result of natural evolution. Further support could come from an analysis of another gene that is not under drug pressure and has a naturally high rate of nucleotide substitutions, such as the envelope gene, in patients in this study. The hypervariable regions of envelope can change significantly even in the absence of cART[400], so analysis of envelope sequences, in addition to protease and reverse transcriptase, would add support to discerning the effect of the M184V mutation.

In this nucleotide distances sub-study I am dealing with consensus sequences rather than clones. Although important information on phylogenetic evolution has been obtained from consensus sequences recently[401;402], use of this method could result in sampling only dominant variants in the quasispecies. As a result, some patients may be misclassified as not having M184I/V in their virus when in fact they had it in less than 20% of a mixed virus population. The best way to address viral diversity over time is by sequencing multiple clones at each time point or using heteroduplex assay systems. Analysis of single consensus sequence at each time point only provides limited information.

These data are derived from a randomised controlled trial but since not all patients are included in the analyses it does not constitute a randomised comparison. Since no randomisation occurred in this sub-group of patients there may not be a comparable prior risk of viral evolution in both treatment arms. As a result unknown factors may have influenced the rate of viral evolution and caused imbalances between treatment groups. However, the rate of suppression was comparable between the two treatment arms in the parent study therefore selecting this group of patients should not induce a selection bias and there should be a comparable risk of viral evolution, in terms of known and unknown factors, in both treatment arms.

Because only a small number of sequences could be included in these analyses, there may be insufficient power to detect a difference in nucleotide distances between treatment arms. If follow-up time had been longer, so that patients in the Off-3TC arm had a longer period in which M184I/V was not present in their dominant viral species, I would have had more power to detect an effect on nucleotide changes associated with discontinuing 3TC.

Our study suggests a marginal benefit of retaining M184V in the virus population because fewer nucleotide changes occur in viruses that show M184I/V. However, patients who retained 3TC in their regimen are not likely to benefit virologically from this strategy in the first 48 weeks of treatment. Larger datasets and a longer follow-up time are needed to see if patients who continue 3TC, in order to maintain M184V, have smaller nucleotide distances to those who discontinue it and are likely to lose the mutation from their dominant virus due to the lack of drug-selection pressure.

6.5.5. Conclusion

Overall, I did not find a difference in nucleotide distances for patients who continued on 3TC compared to those who discontinued it at any stage during 48 weeks of follow-up, but confidence intervals for the difference cannot rule out a moderate effect in favour of continuing 3TC. Post-hoc, exploratory analyses looking at the nucleotide distances according to whether M184I/V was detected or not produced significant results. This could be due to the postulated higher fidelity and lower replication capacity of the mutant reverse transcriptase[272].

Our analyses do not rule out the possibility that M184V could influence virological response alongside less potent cART than is used in our study, or in a minority of patients in whom viral suppression is not a realistic goal. Such an effect, however, has limited clinical relevance given the recommended and prescribed cART regimens in today's management of HIV infection. At the time of the COLATE trial, lopinavir/r was the most recent ARV to enter the market, and since then numerous other ARVs have become available (e.g. tipranavir, darunavir, maraviroc and MK0518) for patients with fairly extensive resistance. If these new ARVs are used appropriately, these data do not suggest that adding 3TC to the regimen will be of any added virological or immunological benefit. However, it could still be a relevant treatment strategy for patients who fail to achieve viral suppression. The results of this chapter are consistent with the theory of an enhanced fidelity of viruses containing the M184V mutation, which should be further investigated as a stratey to preserve future drug options in patients with HIV.

In conclusion, in this randomised open label study no virological or immunological benefit was observed in patients who continued 3TC as part of cART, after failing a 3TC-containing regimen, compared to those who discontinued it. Even though a virological benefit was not observed according to treatment arm, there was a trend for smaller nucleotide distances to be seen in patients whose predominant virus showed the M184I/V mutation, compared to those in whom M184I/V was not detected. These findings, in a heterogeneous study population, support evidence of a beneficial effect of the M184V mutation.

CHAPTER 7: ASSOCIATION BETWEEN RESISTANCE AND CD4$^+$ T CELL COUNT CHANGE IN PATIENTS ON ARVs WITH ONGOING VIREMIA

7. Introduction

In chapter 6, I investigated whether retaining 3TC in a virologically failing 3TC containing regimen could be beneficial because 3TC is associated with the emergence of M184V and there could be a reduction in the fitness of viruses containing the M184V mutation. In the COLATE study there was no significant virological benefit of retaining 3TC throughout follow-up because patients were receiving an active cART regimen simultaneously to the 3TC component. However, there was a trend towards larger nucleotide distances if M184I/V disappeared from the main virus population compared to viruses where M184I/V remained present, which is consistent with the original hypothesis. Current cART regimens are potent and successfully reduce viral load if a patient has a virus that is sensitive to the ARVs in their regimen. The potency of the other drugs in the regimen may have made it difficult to tease out a potential virological benefit of retaining the M184I/V mutation in the COLATE trial.

In the present era, with new ARVs still arriving on the market, it is possible to select an effective viral load reducing regimen for the majority of patients, although there are still some patients at the salvage stage of treatment in whom viral loads cannot be suppressed[403]. The immediate aim of therapy is to suppress viral replication to undetectable levels, thereby limiting the emergence and growth of viruses carrying resistance mutations[109]. However, some mutations may lead to a reduced replication capacity of the virus or an improvement in fidelity (as partly illustrated in the previous chapter for M184I/V)[391]. Patients who require salvage therapy may actually benefit from retaining drugs that preserve these mutations while they wait for other active drugs to become available[172;284].

7.1. Aims of the chapter

Preserving virologically failing drugs in a regimen that keeps beneficial mutations present may improve the clinical prognosis of the patient. If these mutations influence the rates of viral replication, they are likely to be reflected by CD4$^+$ T cell count declines, which in turn, will be related to clinical progression. Further, preserving "failing" drugs could be beneficial because there may still be some residual activity of the ARVs. In this chapter I will assess whether the ARVs used and level of resistance present (i.e. specific mutations

and class of mutations) is associated with $CD4^+$ T cell count changes in EuroSIDA and the UK CHIC study, in combination with data from the UK HIV drug resistance database.

7.2. Literature review

It has been shown in several studies, most clearly in the large PLATO collaboration, that among patients with ongoing viraemia (i.e. two consecutive viral loads > 500 copies/mL) $CD4^+$ T cell count changes are more favourable (or less unfavourable) in patients receiving ARVs (i.e. they experience smaller $CD4^+$ T cell count declines) compared to patients with comparable viral loads who are not on ARVs[152]. Since all patients have high levels of viral replication in these comparisons, it is unclear whether the more favourable $CD4^+$ T cell count changes in patients on ARVs is related to the presence of resistance mutations in the virus and, if so, whether the effect is greater for certain mutations compared to others.

The presence of resistance mutations may cause a virus to have a reduced capacity for inducing $CD4^+$ T cell count declines because it may not be able to infect the T lymphocyte cells as efficiently[281]. A reduction in viral fitness associated with viruses containing some resistance mutations has been shown previously[281-286], but this reduced fitness may act entirely through a reduction in the viral load and not directly impact on the $CD4^+$ T cell counts[287]. In patients on ARVs as well as those off ARVs there is a trend for more favourable (or less unfavourable) $CD4^+$ T cell count changes in patients with a lower viral load.

In this chapter I will identify patients in EuroSIDA and in the UK CHIC study who have linked resistance data in the UK resistance database. All patients who have ongoing viral load > 500 cps/mL were included in these comparisons, in order to assess whether the level of resistance in the virus (i.e. specific mutations and class of mutations) is associated with $CD4^+$ T cell count changes independent of the use of ARVs.

I will also examine the relationship between resistance mutations and $CD4^+$ T cell count declines for a given viral load or extent of viral load suppression from the pre-ART level to see whether the presence of certain mutations provides information on immunological response above-and-beyond what can be derived from the use of viral load testing. Patients in these studies contribute observational data where resistance testing is performed at varying time points depending on the centre. Retrospective resistance testing has also been performed on stored samples for some of the patients.

7.3. Methods

7.3.1. Patient selection

Patients who appeared in both the UK CHIC study and the UK Drug Resistance database were combined with patients from EuroSIDA who had resistance information available. All duplicate entries were removed (i.e. patients who had clinical data in both EuroSIDA and the UK CHIC study). Patients were included in the final analysis if they had:

1. A pair of consecutive HIV RNA measurements (both >500 cps/mL) 8 to 26 weeks apart. If an HIV RNA measurement (>500 cps/mL) was taken within 8 weeks of the first recording, the next HIV RNA was included instead, as long as it was also >500 cps/mL and 8 to 26 weeks after the first measurement.

2. $CD4^+$ T cell counts available at the time each of the above HIV RNA measurements was recorded (+/- 1 week either side)

3. An available resistance test in the above interval (defined by the pair of HIV RNA measurements) or 4 weeks before to 4 weeks after. If more than one resistance test was available in the interval, only the first resistance test was included in the analysis. If more than one resistance test was performed on the same day these test results were merged, so if a mutation had been found using only one test it was still considered to be present overall

4. Patients had to be on the *same* regimen for at least 4 months before the first viral load measurement AND up to the second viral load measurement OR off all ARVs during this period.

Patients could be included in the analysis on more than one occasion: they could have more than one pair of consecutive HIV RNA measurements satisfying the above criteria, each with a separate resistance test, or they could have more than one pair of consecutive HIV RNA measurements satisfying the above criteria with only one resistance test that was used a multiple number of times. No restrictions were made on the time between consecutive HIV RNA pairs; consequently the same HIV RNA measurement could be used repeatedly in consecutive pairs of measurements. This was also the case for $CD4^+$ T cell count pairs. HIV RNA pairs were the unit of analysis in this chapter rather than individual patients.

7.3.2. Genotypic interpretation systems

In both EuroSIDA and the UK Drug Resistance Database, each full nucleotide sequence was aligned to an amino acid reference sequence (i.e. consensus B) and all of the amino

acid substitutions were identified. A genotypic sensitivity score (GSS) was calculated for each patient using the Stanford (version 4.2.6) genotypic interpretation system (GIS).

7.3.3. Statistical analysis

The use of certain ARVs may lead to the emergence of specific mutations in the virus. These will be archived if the selection pressure from the corresponding drug is removed. Since there is a strong relationship between the presence of mutations and the ARVs that are used, it was not possible to enter both variables into a regression model together. Instead, HIV RNA pairs were categorised into groups according to the drugs the patient was receiving at the time and the mutations that were present in the associated resistance test in a composite variable. The groups were as follows:

1. Patients who were receiving a ritonavir boosted PI in whom ≥1 primary PI resistance mutation was detected (On PI/r, PI mutations)
2. Patients who were receiving a ritonavir boosted PI but did not have a virus showing any primary PI resistance mutations (On PI/r, no PI mutations)
3. Patients who were receiving an unboosted PI in whom ≥1 primary PI resistance mutation was detected (On PI, PI mutations)
4. Patients who were receiving an unboosted PI who did not have a virus showing primary PI resistance mutations (On PI, no PI mutations)
5. Patients who were receiving an NNRTI in whom ≥1 NNRTI resistance mutation was detected (On NNRTI, NNRTI mutations)
6. Patients who were receiving an NNRTI and did not have a virus showing NNRTI resistance mutations (On NNRTI, no NNRTI mutations)
7. Patients who were receiving a PI (unboosted or boosted) and an NNRTI in whom ≥1 resistance mutation was detected to both drugs (On PI & NNRTI, mutations to both)
8. Patients who were receiving a PI (unboosted or boosted) and an NNRTI and only had a virus showing resistance to one or neither of the classes (On PI & NNRTI, mutations to 1 or neither)
9. Patients who were receiving NRTIs only in whom ≥1 mutation was detected to NRTIs (On NRTIs only, NRTI mutations)
10. Patients who were receiving NRTIs but did not have a virus showing any mutations to NRTIs (On NRTIs only, no NRTI mutations) and
11. Patients who were not receiving any ARVs (Off-ART)

Patients in all categories except for group 10 could have NRTI mutations present in their virus, those in all categories except group 6 could have a virus showing NNRTI mutations and those in all categories except groups 2 and 4 could have a virus showing PI mutations. The presence of mutations to each of the drug classes was identified by using the 2006 IAS-USA drug resistance mutations lists[233].

I fit standard linear regression models to the $CD4^+$ T cell count slopes over time (i.e. the difference between the pair of $CD4^+$ T cell counts, standardized for the time in between them) for patients satisfying the inclusion criteria. All baseline characteristics (i.e.those measured at the same time as the first HIV RNA measurement) and follow-up variables that were significant ($p<0.2$) in the unadjusted analysis were entered into a backwards selection model. Variables that were still significant ($p<0.2$) were retained in the final model. Variables were assessed for co-linearity prior to any multivariable regression analysis. All of the main variables of interest and the most significant predictors in the cases of co-linearity were retained for inclusion in the final models. The mutation category described above was included in the multivariable models to investigate the relationship between the ARV used, the class of mutation and the $CD4^+$ T cell count slopes. All possible interactions between variables were also investigated.

The relationship between the number of mutations to each class of ARVs and $CD4^+$ T cell count slopes was examined in more detail to see whether there was a linear association, or whether individual mutations need to be weighted differently. Individual mutations were investigated in separate multivariable models, adjusted for all other variables that predicted $CD4^+$ T cell count response in the main analysis.

7.3.4. Sensitivity analysis

I performed a number of different sensitivity analysis on these data. These are described over the next few paragraphs:

Patients who have large HIV RNA changes during the interval are likely to experience a greater number of mutational substitutions in their virus than those with small HIV RNA changes: there is more scope for mutations to emerge in the virus of these patients because their virus is undergoing more changes under drug pressure from the regimen they are receiving. In one sensitivity analysis, I restricted the comparisons to patients who had minimal HIV RNA changes (i.e. ΔHIV RNA <0.25 $\log_{10}$ cps/mL) in order to reduce the amount of noise generated from any mutational changes that occurred to the virus during

the interval between HIV RNA measurements. This means that any difference in $CD4^+$ T cell count slopes is likely to be attributed to the mutations that are present in the virus that was sampled for the resistance test, rather than unidentified mutations that may have evolved over follow-up. Further, if a relationship between resistance mutations and $CD4^+$ T cell count slopes was identified in this subgroup of patients, the analysis would already be partly controlled for HIV RNA changes during the interval between measurements. So, this analysis reflects the association between resistance and immunological response, independently of HIV RNA change, in a more controlled setting.

I also considered the effect of restricting the analysis to patients with high viral loads (i.e. HIV RNA >10000 cps/mL) at the start and at the end of each interval. This analysis was performed to see whether the presence of mutations has a greater impact on $CD4^+$ T cell count changes in patients who have high levels of viral replication. These patients are failing treatment with extremely high levels of viraemia so any potential benefit of retaining certain mutations may be more apparent in this population. These patients reflect patients on salvage therapy who could potentially benefit from new strategies that involve retaining certain drugs in order to retain certain mutations in their predominant virus population.

Since non-adherence (or sub-optimal adherence) can affect the results of these comparisons, in another sensitivity analysis I decided to restrict the population to patients who have a GSS <1. Patients with a GSS <1 are assumed to be adherent because they have a wide range of mutations present in their virus and, as demonstrated in section 1.6.1.1, resistance mutations are not likely to emerge in the predominant virus of non-adherent patients because they will only have limited exposure to treatment. Patients who are selected for this sensitivity analysis also reflect those who are in need of salvage therapy and may benefit from these strategies, because they have very few treatment options available.

In another sensitivity analysis I performed a generalised linear model using generalised estimating equations to account for the repeated measurements on some individuals (i.e. some patients contribute more than one $CD4^+$ T cell count pair to these analyses). This analysis was restricted to patients who had their pairs of $CD4^+$ T cell counts measured at least one year apart. If measurements are taken close together it is likely that the last $CD4^+$ T cell count in an interval will be the first of the pair of $CD4^+$ T cell counts in the next interval, which would result in a negative correlation structure between pairs of $CD4^+$ T cell counts. Restricting pairs of observations to be at least one year apart ensured that there

was a positive correlation structure, because each $CD4^+$ T cell count will only contribute to one interval so consecutive $CD4^+$ T cell count slopes will no longer be negatively correlated.

7.4. Results

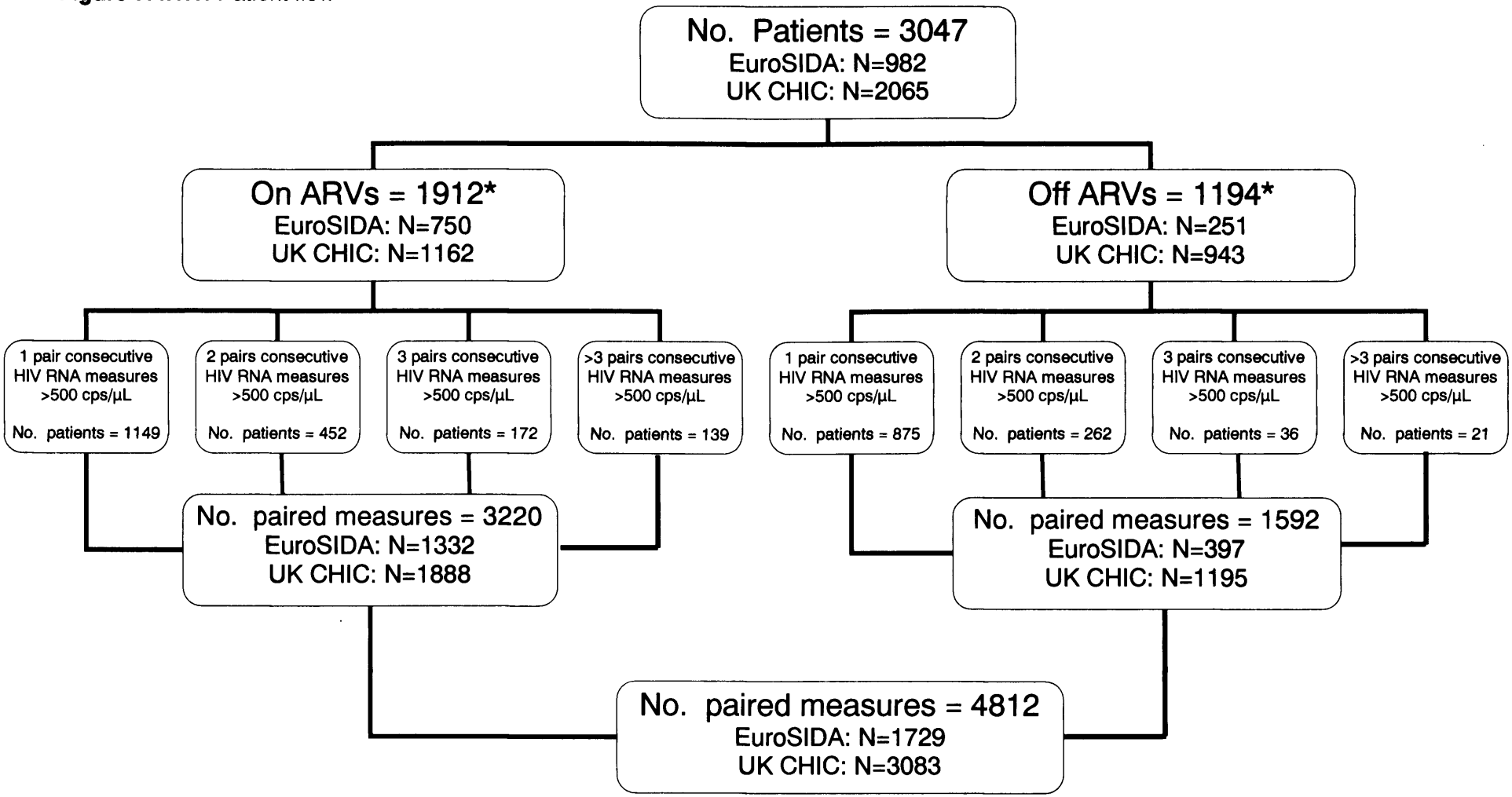
7.4.1. Patient characteristics

Overall there were 3047 patients who satisfied criteria 1 to 4 in section 7.3.1. These patients had 4812 paired HIV RNA measurements, where 3166 resistance tests were used on one occasion and 823 on two occasions (Figure 7.4.1.1).

The characteristics of patients for all time points combined are shown separately for patients who were on ARVs and patients who were off ARVs at the time of their viral load pair (Table 7.4.1.1). Patients who were receiving ARVs contributed a total of 3220 paired HIV RNA measurements to this analysis. In the resistance tests associated with these HIV RNA pairs, there were 1678 (52%) with primary PI mutations, 1480 (46%) with NNRTI mutations and 2700 (84%) with NRTI mutations. The equivalent numbers of patients who were off ARVs and had a virus containing these mutations were 59 (4%), 64 (4%) and 124 (8%), respectively. In patients who were using ARVs there were 742 (23%) isolates with an NNRTI mutation but no primary PI mutation and 940 (29%) isolates with a primary PI mutation but no NNRTI mutation. In contrast, there were only 47 (3%) isolates with an NNRTI mutation but no primary PI mutation and 42 (3%) with a primary PI mutation but no NNRTI mutation in patients who were off ARVs.

The median (IQR) length of time between the first HIV RNA measurement and the second HIV RNA measurement was 13 (10 to 17) weeks; this did not differ according to whether the patient was on or off ARVs. In patients on ARVs the median (IQR) HIV RNA at the start of the episode was 3.93 (3.39 to 4.54) $\log_{10}$ cps/mL with a median increase of 0.08 (-0.19 to 0.40) $\log_{10}$ cps/mL during the interval between HIV RNA measurements. In patients off ARVs, the median (IQR) viral load at the start of the episode was 4.74 (4.24 to 5.15) $\log_{10}$ cps/mL with a median increase of 0.06 (-0.20 to 0.32) $\log_{10}$ cps/mL during the interval between HIV RNA measurements.

Figure 7.4.1.1: Patient flow



* Patients can be classified as being On ARVs AND being Off ARVs if they have paired HIV RNA measurements and an equivalent resistance test while they are receiving ARVs and also at a time when they are off ARVs

Table 7.4.1.1: Characteristics of patients at the start of the HIV RNA pair

		On ARVs N=3220	Off ARVs N=1592
Length of the interval between HIV RNA measurements (weeks):		13 (10, 17)	13 (11, 17)
First $CD4^+$ T cell count of the pair (cells/μL):		274 (168, 410)	303 (217, 410)
Second $CD4^+$ T cell count of the pair (cells/μL):		269 (160, 404)	279 (189, 384)
Change in $CD4^+$ T cell counts between the paired values (cells/μL):		-7 (-50, 38)	-22 (-78, 28)
First HIV RNA of the pair (log_{10} cps/mL):		3.93 (3.39, 4.54)	4.74 (4.24, 5.15)
Second HIV RNA of the pair (log_{10} cps/mL):		4.04 (3.53, 4.63)	4.78 (4.27, 5.23)
Change in HIV RNA levels between paired values (log_{10} cps/mL):		0.08 (-0.19, 0.40)	0.06 (-0.20, 0.32)
First HIV RNA of the pair below the HIV RNA set point[1]: Number (%)		826 (77.8%) (N=1062)	861 (62.1%) (N=1386)
Time between resistance test and first HIV RNA of the pair (weeks):		8.0 (0.3, 13.1) weeks after start	4.0 (0.0, 13.1) weeks after start
Mutation category[2]:	Number (%)		
On PI/r[3], PI mutations*		426 (13.2%)	-
On PI/r[3], no PI mutations*		184 (5.7%)	
On PI, PI mutations*		704 (21.9%)	
On PI, no PI mutations*		292 (9.1%)	
On NNRTI, NNRTI mutations*		698 (21.7%)	
On NNRTI, no NNRTI mutations*		142 (4.4%)	
On PI & NNRTI, mutations to both*		278 (8.6%)	
On PI & NNRTI, mutations to 1 drug or no mutations*		113 (3.5%)	
On NRTIs only, NRTI mutations		326 (10.1%)	
On NRTIs only, no NRTI mutations		57 (1.8%)	
NRTI mutations present:	Number Y (%)	2700 (83.8%)	124 (7.8%)
NNRTI mutations present:	Number Y (%)	1480 (46.0%)	64 (4.0%)
Primary PI mutations present:	Number Y (%)	1678 (52.1%)	59 (3.7%)
Genotypic Sensitivity Score (GSS[4], Stanford):	Median (IQR)	1.0 (0.5, 2.0)	-
Number of ARVs used:	Median (IQR)	3 (3, 4)	-
ARVs used:	Number (%)		
PIs		1606 (49.9%)	-
NNRTIs		840 (26.1%)	
NNRTIs + PIs		391 (12.1%)	
NRTIs only		383 (11.9%)	
AZT used:	Number Y (%)	1016 (31.6%)	-

[1]The viral load set-point is defined as the last HIV RNA recording before commencing cART;
[2]Refers to any major mutation as defined in the IAS-USA 2006 tables[233]; [3]PI/r: ritonavir-boosted PI;
[4]GSS: Calculated using the Stanford 4.2.6 Dec 12 2006 interpretation system; * These groups also contain patients receiving NRTIs and patients with NRTI mutations

7.4.2. $CD4^+$ T cell count changes according to the use of ARVs

Overall, the median (IQR) $CD4^+$ T cell count at the start of the episode was 283 (185 to 410) cells/μL. Patients experienced a median change in $CD4^+$ T cell counts of -10 (-60 to 34) cells/μL over a median of 13 (10 to 17) weeks. This translates into a monthly $CD4^+$ T cell count change of -3.4 (-19.3 to 11.3) cells/μL/month (i.e. the mean (95% CI) of the individual $CD4^+$ T cell count slopes).

In patients who were on ARVs the median (IQR) $CD4^+$ T cell count at the start of the interval was 274 (168 to 410) cells/μL and it was 303 (217 to 410) cells/μL for patients who were off ARVs. During the interval between $CD4^+$ T cell count measurements, there was a median change of -7 (-50 to 38) cells/μL for patients who were on ARVs and -22 (-78 to 28) cells/μL for patients who were off ARVs. As expected, less pronounced declines (i.e. less of a decline in $CD4^+$ T cell counts) were seen in patients who were receiving treatment. From an unadjusted regression model, patients who were off ARVs had a mean (95% CI) 4.48 (2.24 to 6.73) cells/μL greater $CD4^+$ T cell count decline per month compared to those who were on ARVs, $p<0.0001$. After adjustment for the time between the first HIV RNA measurement of the pair and the resistance test, and also for the HIV RNA at the start of the interval, there were still substantial differences in $CD4^+$ T cell count declines between patients who were on ARVs and those who were off ARVs: mean (95% CI) difference: 5.10 (2.66 to 7.55) cells/μL/month greater $CD4^+$ T cell count declines in patients who were off ARVs, $p<0.0001$.

7.4.3. $CD4^+$ T cell count changes, according to the class of mutations

After quantifying the effect of being on ARVs I focussed on patients who were on treatment so that I could examine the relationship between mutations and $CD4^+$ T cell count response in patients who were failing therapy. A test for an interaction between the presence of mutations and the use of ARVs for predicting $CD4^+$ T cell count declines revealed a marginal effect ($p=0.12$). This implies that the relationship between mutations and immunological response may differ in treated patients compared to patients who are off therapy.

When I restricted the analysis to patients who were using ARVs for the duration of the interval, the sample size was reduced to 1912 patients. A total of 3220 paired measurements satisfied the inclusion criteria, 1960 resistance tests were used on one occasion and 630 on two occasions (Figure 7.4.1.1).

Resistance mutations are generally more prevalent in the virus population of patients who are using ARVs. If the presence of certain mutations can be beneficial in patients who are failing a cART regimen, it may be worthwhile trying to keep those mutations in the dominant viral sub-species by using ARVs that select for those mutations. In patients who are off treatment the predominant virus is likely to revert back to wild-type and consequently a beneficial effect of the mutations may not be attained.

The mean monthly change in $CD4^+$ T cell counts according to the class of resistance mutation and according to the ARV that the patient was using is shown in Table 7.4.3.1 for all patients who were receiving ARVs. Patients on NNRTIs had more favourable $CD4^+$ T cell count changes if they did not have a virus containing an NNRTI-specific mutation. Conversely, patients receiving PIs or PI/rs have more favourable $CD4^+$ T cell count slopes if they had a PI-specific mutation present in their virus.

After adjustment for the time between the first HIV RNA measurement of the pair and the resistance test, the genotypic sensitivity score (according to the Stanford interpretation system) and the value of the first HIV RNA measurement of the pair, there was little evidence for a difference in $CD4^+$ T cell count changes according to the IAS-USA mutations that were present (Table 7.4.3.1). The adjusted mean (95% CI) $CD4^+$ T cell count changes were: -0.7 (-4.4 to 3.0); -3.6 (-9.4 to 2.2); -5.0 (-7.9 to -2.1); and -3.2 (-9.9 to 3.5) cells/μL/month for patients on PI/rs with PI-mutations, on PI/rs without PI-mutations, on NNRTIs with NNRTI-mutations and on NNRTIs without NNRTI-mutations, respectively, p=0.21. Although the global p-value was not significant in this case, patients falling into some groups experienced larger $CD4^+$ T cell count changes than patients falling into other groups. However, these results need to be interpreted with caution due to the issue of multiple testing.

Table 7.4.3.1: $CD4^+$ T cell count changes: by class-specific mutations and ARV used

Mutation category (IAS-USA)	Number (%) (N=3220)	Mean CD4 count change cells/μL/month (95% CI)	Mean HIV RNA change Copies/mL/month (95% CI)	ΔCD4+ T cell count cells/μL/month (95% CI) relative to group 1**	Adjusted mean*** CD4+ T cell count change cells/μL/month (95% CI)**
On PI/r, PI mutations* (group 1)	426 (13.2%)	-0.4 (-3.7, 2.9)	0.02 (0.00, 0.04)	Ref	-0.7 (-4.4, 3.0)
On PI/r, no PI mutations*	184 (5.7%)	-3.7 (-8.8, 1.4)	0.03 (-0.01, 0.07)	-2.9 (-10.0, 4.2)	-3.6 (-9.4, 2.2)
On PI, PI mutations*	704 (21.9%)	1.1 (-1.5, 3.6)	0.03 (0.02, 0.05)	1.7 (-2.9, 6.3)	1.0 (-1.9, 3.9)
On PI, no PI mutations*	292 (9.1%)	-1.7 (-6.8, 3.3)	0.04 (0.01, 0.08)	-0.7 (-7.0, 5.5)	-1.4 (-6.2, 3.3)
On NNRTI, NNRTI mutations*	698 (21.7%)	-5.2 (-8.1, 2.3)	0.03 (0.02, 0.05)	-4.3 (-8.9, 0.4)	-5.0 (-7.9, -2.1)
On NNRTI, no NNRTI mutations*	142 (4.4%)	-3.2 (-10.4, 4.0)	-0.03 (-0.08, 0.02)	-2.5 (-10.4, 5.4)	-3.2 (-9.9, 3.5)
On PI & NNRTI, mutations to both*	278 (8.6%)	-3.7 (-7.7, 0.2)	0.03 (0.01, 0.05)	-3.5 (-9.3, 2.2)	-4.2 (-8.9, 0.4)
On PI & NNRTI, muts to 1 class or no muts*	113 (3.5%)	-5.5 (-11.5, 0.4)	0.02 (-0.02, 0.06)	-4.4 (-12.8, 4.0)	-5.1 (-12.4, 2.2)
On NRTIs only, NRTI mutations	326 (10.1%)	-3.4 (-8.7, 1.9)	0.05 (0.03, 0.07)	-2.6 (-8.2, 2.9)	-3.3 (-7.5, 0.8)
On NRTIs only, no NRTI mutations	57 (1.8%)	1.5 (-7.6, 10.5)	-0.06 (-0.15, 0.04)	2.0 (-9.2, 13.3)	1.3 (-9.1, 11.7)

* These can also include NRTI mutations

** Adjusted for the time between the first HIV RNA measurement of the pair and the resistance test, the genotypic sensitivity score (according to the Stanford interpretation system) and the first HIV RNA measurement of the pair

*** Adjusted means are the predicted values, obtained from a multivariable regression model. Adjusted means were estimated by using the mean values of the other covariates in the model (see **)

7.4.3.1. CD4$^+$ T cell count changes, including the first CD4$^+$ T cell count

I also wanted to quantify the effect of the first CD4$^+$ T cell count of the pair on CD4$^+$ T cell count slopes, so I performed a separate multivariable model adjusted for the first CD4$^+$ T cell count (Table 7.4.3.1.1). As expected, for every 100 cells/μL higher CD4$^+$ T cell count there was a 6.02 (5.37 to 6.67) cells/μL/month greater decline in CD4$^+$ T cell counts over the interval, possibly influenced by regression to the mean[404;405]. An alternative explanation for this trend is that there could be greater variability in the slopes at higher CD4$^+$ T cell counts or that the CD4$^+$ T cell count slopes are, in fact, non-linear. Natural history studies have shown that CD4$^+$ T cell counts decline approximately exponentially in patients who are off-ARVs. Adjusting for the first CD4$^+$ T cell count had a negligible impact on the other predictor variables. This variable was excluded from the main model because it is highly correlated to CD4$^+$ T cell count slopes and may bias the results looking at the relationship between CD4$^+$ T cell count slopes and mutation category.

Table 7.4.3.1.1: CD4$^+$ T cell count changes: adjusted for the first CD4$^+$ T cell count

	Unadjusted analysis		Multivariable analysis	
Variable	**ΔCD4$^+$ T cell count cells/μL/month (95% CI)**	**P-value**	**ΔCD4$^+$ T cell count cells/μL/month (95% CI)**	**P-value**
First CD4$^+$ T cell count of the pair (per 100 cells/μL higher)	-5.44 (-6.05, -4.83)	<0.0001	-6.02 (-6.67, -5.37)	<0.0001
First HIV RNA of the pair (per $\log_{10}$ cps/mL increase)	1.43 (-0.31, 3.17)	0.11	-3.99 (-5.80, -2.17)	<0.0001
Time between resistance test and the first HIV RNA of the pair (per week longer)	0.14 (-0.05, 0.32)	0.14	0.16 (-0.02, 0.33)	0.08
Mutation category (IAS-USA):				
On PI/r, PI mutations	Ref	0.17	Ref	0.24
On PI/r, no PI mutations	-3.29 (-9.90, 3.33)		-4.05 (-10.91, 2.82)	
On PI, PI mutations	1.45 (-3.16, 6.05)		0.86 (-3.54, 5.27)	
On PI, no PI mutations	-1.35 (-7.05, 4.34)		-0.71 (-6.71, 5.30)	
On NNRTI, NNRTI mutations	-4.79 (-9.40, -0.18)		-3.65 (-8.09, 0.78)	
On NNRTI, no NNRTI mutations	-2.82 (-10.09, 4.44)		-0.44 (-8.17, 7.29)	
On PI & NNRTI, mutations to both	-3.33 (-9.11, 2.45)		-4.53 (-10.04, 0.98)	
On PI & NNRTI, muts to 1 or no drugs	-5.14 (-13.08, 2.80)		-4.35 (-12.44, 3.74)	
On NRTIs only, NRTI mutations	-3.05 (-8.57, 2.46)		-3.02 (-8.31, 2.26)	
On NRTIs only, no NRTI mutations	1.85 (-8.73, 12.42)		3.69 (-7.30, 14.67)	
GSS score (Stanford v.4.2.6):				
0-0.5	Ref	0.17	Ref	0.04
1-2	1.57 (-1.34, 4.49)		1.39 (-1.53, 4.31)	
>2	-1.97 (-5.77, 1.83)		-4.48 (-9.61, 0.64)	

7.4.3.2. Residual drug activity

I investigated HIV RNA changes within each of the mutation groups to assess whether the trend towards a benefit of harbouring a PI mutation was due to residual activity of the PI(/r) (i.e. despite the presence of mutations the drug is still partially active). If residual activity is an explanation for why these differences are occurring, it is likely to be reflected in the viral load changes before impacting on $CD4^+$ T cell count declines. Or instead, viral load changes could also be a result of the mechanism by which the mutations lead to more favourable $CD4^+$ T cell count declines. Patients in the majority of strata experienced similar HIV RNA increases, but patients who were using NNRTIs and did not have any NNRTI mutations in their virus experienced marginal HIV RNA declines (Table 7.4.3.1).

To further assess the relationship between residual activity of the PI(/r) and $CD4^+$ T cell count changes, I fit a model looking at $CD4^+$ T cell count slopes according to whether the patient was on a PI(/r) (or not) and according to whether a PI mutation was present in the virus (or not). In unadjusted analyses, patients in whom a PI mutation was detected in their virus had a 2.54 (-0.11 to 5.18) cells/μL/month smaller decline in $CD4^+$ T cell counts compared to those who had a virus in which a PI mutation was not detected, p=0.06. Being on a PI(/r) was associated with a 3.04 (0.32 to 5.77) cells/μL/month smaller decline in $CD4^+$ T cell counts compared to those not on a PI(/r), p=0.03. In a model that included both of these variables, the presence of a PI mutation was associated with a 1.41 (-1.65 to 4.47) cells/μL/month smaller decline in $CD4^+$ T cell counts (p=0.37), and being on a PI(/r) was associated with a 2.32 (-0.83 to 5.47) cells/μL/month smaller decline in $CD4^+$ T cell counts, (p=0.15), compared to patients who had a virus in which a PI mutation was not detected and patients who were not on a PI, respectively.

To assess residual activity in patients on PI/rs in more detail, I re-categorised the number of PI mutations into i) no primary PI mutations; ii) one to three primary PI mutations and iii) ≥ four primary PI mutations. If residual drug activity is an explanation for why there is a beneficial effect of retaining PI mutations in patients who are failing a PI containing regimen I would expect to see the smallest $CD4^+$ T cell count declines in patients falling into group ii (i.e. patients with a virus containing one to three primary PI mutations) and the largest declines in patients who have a virus containing ≥ four primary PI mutations.

In patients who were receiving PIs (either in the boosted or unboosted form) there was a 1.39 (-3.23 to 6.01) cells/μL/month smaller $CD4^+$ T cell count decline in patients with one to three primary PI mutations and a 3.83 (-3.17 to 10.82) cells/μL/month greater $CD4^+$ T cell

decline in patients with ≥ four primary PI mutations compared to patients with no primary PI mutations; albeit these differences did not reach statistical significance.

I then examined the relationship between the number of mutations to each drug class and $CD4^+$ T cell count slopes to see whether there was a linear relationship or whether individual mutations need to be accounted for separately. The relationship between $CD4^+$ T cell count slopes and the number of NNRTI mutations was linear: there was a 1.86 (0.31 to 3.41) cells/μL/month, p=0.02, greater $CD4^+$ T cell count decline for each additional NNRTI mutation, after adjustment.

The relationship between the number of PI mutations and $CD4^+$ T cell count slopes was not linear. By examining the data in more detail, patients with a virus containing one or two primary PI mutations experience the smallest $CD4^+$ T cell count declines but once >2 mutations have emerged there appears to be an increasing negative effect of accumulating PI mutations: the more PI mutations that emerge, the greater the decrease in $CD4^+$ T cell counts.

7.4.4. $CD4^+$ T cell count changes according to specific mutations

To investigate whether the presence of any specific mutation has a greater impact on $CD4^+$ T cell count slopes compared to any other mutation I used separate models to examine the IAS-USA mutations that occurred in > 5% of resistance tests overall (i.e. not specifically in patients who received drugs that select for those mutations). These analyses were also adjusted for the time between the first HIV RNA measurement of the pair and the resistance test, the genotypic sensitivity score (according to the Stanford interpretation system) and the value of the first HIV RNA measurement.

The presence of any NNRTI-specific mutation was associated with a greater $CD4^+$ T cell count decline and the presence of most PI-specific mutations were associated with a smaller $CD4^+$ T cell count decline, although most of the comparisons did not reach statistical significance. In separate models, the presence of K103N and Y181C in the RT gene were associated with a mean (95% CI) 2.96 (-0.16 to 6.09), p=0.06, and 4.48 (0.74 to 8.23), p=0.02, cells/μL/month greater $CD4^+$ T cell count decline compared to viruses without either mutation, respectively. No PI or NRTI mutation alone was associated with $CD4^+$ T cell count changes (Table 7.4.4.1). Of note, these could be chance findings due to multiple testing. In patients who were off ARVs, the presence of K103N and Y181C was associated with a mean (95% CI) 0.71 (-11.00 to 12.42), p=0.91, and 9.41 (-9.29 to 28.11),

p=0.32, cells/μL/month smaller $CD4^+$ T cell count reduction compared to viruses without either mutation, respectively.

Table 7.4.4.1: $CD4^+$ T cell count changes: according to specific mutations

		ΔCD4⁺ T cell count for presence vs absence of each mutation cells/μL/month (95% CI)	**P-value**
Specific IAS-USA mutations *			
NRTI mutations:	M41L	-0.32 (-3.33, 2.69)	0.84
	D67N	-0.18 (-3.19, 2.83)	0.91
	K70R	-1.21 (-4.36, 1.94)	0.45
	L74V	-1.45 (-6.05, 3.16)	0.54
	M184V	0.16 (-2.80, 3.11)	0.92
	M184I/V*	**-0.13 (-3.12, 2.85)**	**0.93**
	L210W	0.78 (-2.40, 3.96)	0.63
	T215Y	0.73 (-2.22, 3.68)	0.63
	T215F	-2.08 (-6.13, 1.96)	0.31
	T215Y/F*	**-0.10 (-3.26, 3.06)**	**0.95**
	K219Q	-1.84 (-5.84, 2.16)	0.37
	K219E	-1.08 (-5.65, 3.49)	0.64
	K219E/Q*	**-1.04 (-4.37, 2.28)**	**0.54**
NNRTI mutations:	K103N	-2.96 (-6.09, 0.16)	0.06
	Y181C	-4.48 (-8.23, -0.74)	0.02
	Y181C/I*	**-4.16 (-7.86, -0.46)**	**0.03**
	G190A	-1.93 (-5.98, 2.12)	0.35
	G190A/S*	**-2.00 (-5.84, 1.84)**	**0.31**
PI mutations:	M46I	0.86 (-2.78, 4.51)	0.64
	M46L	3.01 (-1.77, 7.79)	0.22
	M46I/L*	**1.86 (-1.38, 5.10)**	**0.26**
	V82A	1.55 (-2.01, 5.12)	0.39
	V82A/F/L/T/S*	**0.99 (-2.29, 4.28)**	**0.55**
	I84V	-0.43 (-4.49, 3.64)	0.84
	L90M	-0.53 (-3.64, 2.57)	0.74

* Occurring in >5% of the population overall; Mutations in **bold** indicate the presence of one or the other mutation in the virus population. For example, the M184I/V mutation is recorded as being present if either 184I or 184V is indentified in the predominant virus at the time of the test.

7.4.5. Sensitivity analysis

7.4.5.1. The extent of viral load suppression

Data from the PLATO collaboration showed an association between the extent of viral load reduction from the pre-ART value with the $CD4^+$ T cell count slopes. The pre-ART HIV RNA level indicates the maximum level of viral replication for each patient in the absence of ARVs and generally in the absence of resistance mutations (although some patients

may have primary resistance). Therefore, the extent of suppression from this value will enable us to assess the effect that prior treatment had on the overall decline in replication rates and its subsequent effect on $CD4^+$ T cell count slopes.

I repeated the above analysis on the 1062 $CD4^+$ T cell count pairs for which the viral load reduction from the pre-ART value was known. In our comparisons the extent of viral load reduction from the pre-ART value was not a significant predictor of $CD4^+$ T cell count slopes (Table 7.4.5.1.1).

Table 7.4.5.1.1: $CD4^+$ T cell count changes: patients with a pre-ART HIV RNA value

	Unadjusted analysis		**Multivariable analysis**	
Variable	**ΔCD4⁺ T cell count cells/μL/month (95% CI)**	**P-value**	**ΔCD4⁺ T cell count cells/μL/month (95% CI)**	**P-value**
HIV RNA change from pre-ART value (for every log_{10} cps/mL greater reduction)	-1.28 (-3.21, 0.65)	0.19	0.21 (-2.12, 2.55)	0.86
First $CD4^+$ T cell count of the pair (per 100 cells/μL higher)	-5.70 (-6.76, -4.63)	<0.0001	-6.37 (-7.50, -5.24)	<0.0001
First HIV RNA of the pair (per log_{10} cps/mL increase)	2.28 (-0.69, 5.25)	0.13	-1.55 (-5.33, 2.22)	0.42
Time between resistance test and the first HIV RNA measurement of the pair (per week longer)	0.11 (-0.21, 0.44)	0.50	0.10 (-0.21, 0.40)	0.54
Mutation category (IAS-USA):				
On PI/r, PI mutations	Ref	0.62	Ref	0.18
On PI/r, no PI mutations	-2.73 (-14.66, 9.20)		-3.50 (-15.60, 8.61)	
On PI, PI mutations	3.77 (-6.38, 13.92)		2.36 (-7.24, 11.97)	
On PI, no PI mutations	-3.04 (-14.17, 8.09)		0.37 (-10.81, 11.54)	
On NNRTI, NNRTI mutations	-4.86 (-14.11, 4.40)		-8.05 (-16.83, 0.73)	
On NNRTI, no NNRTI mutations	-5.64 (-18.01, 6.74)		-2.99 (-15.53, 9.55)	
On PI & NNRTI, mutations to both	1.59 (-11.64, 14.83)		-3.94 (-16.55, 8.66)	
On PI & NNRTI, mut to 1 or no drugs	-4.31 (-18.53, 9.92)		-1.13 (-15.78, 13.51)	
On NRTIs only, NRTI mutations	-2.69 (-13.89, 8.51)		-8.51 (-19.14, 2.13)	
On NRTIs only, no NRTI mutations	-3.31 (-20.10, 13.47)		-1.98 (-19.36, 15.41)	
GSS score (Stanford v.4.2.6):				
0-0.5	Ref	0.02	Ref	0.001
1-2	4.06 (-1.39, 9.51)		3.50 (-1.91, 8.92)	
>2	-4.13 (-10.66, 2.41)		-10.35 (-19.26, -1.43)	

7.4.5.2. Patients with minimal HIV RNA changes

Viral load changes in the interval between HIV RNA measurements are likely to influence $CD4^+$ T cell count slopes. To quantify the effect of mutations on $CD4^+$ T cell count slopes without interference from patients who experience large HIV RNA changes, I repeated the analysis on patients who experienced minimal viral load changes (i.e. ΔHIV RNA <0.25 $\log_{10}$ cps/mL during the interval between HIV RNA measurements). In the 1374 (42.7%) paired observations satisfying these criteria, the median (IQR) $CD4^+$ T cell count at the start of the episode was 275 (173 to 410) cells/μL and patients experienced a $CD4^+$ T cell count change of -2.0 (-15.6 to 13.0) cells/μL/month. So, overall the $CD4^+$ T cell count changes were marginally more favourable in this subgroup.

In both patients who received PI/rs and those who received NNRTIs, patients with a virus that harboured resistance mutations had greater $CD4^+$ T cell count declines than patients who had a virus that did not. $CD4^+$ T cell count increases were observed in both patients receiving a PI/r or an NNRTI instead of the $CD4^+$ T cell count declines that were seen in section 7.4.3. Since, in the main analysis, the major differences in $CD4^+$ T cell count slopes were seen between patients who had a virus containing a mutation compared to those who had a virus that did not contain a mutation if they were receiving a PI/r or an NNRTI (Table 7.4.3.1 and Table 7.4.3.1.1), I will report the results for patients falling into these groups in these sensitivity analyses. The adjusted mean (95% CI) $CD4^+$ T cell count changes were: 0.28 (-8.49 to 9.05); 0.45 (-10.49 to 11.38); 4.63 (-1.54 to 10.80); and 7.18 (-4.39 to 18.75) cells/μL/month for patients on PI/rs with PI-mutations, on PI/rs without PI-mutations, on NNRTIs with NNRTI-mutations and on NNRTIs without NNRTI-mutations, respectively, for these 1374 patients.

7.4.5.3. Patients with high levels of viral replication

Since the effect of a specific mutation may be stronger in patients who are virologically failing their treatment regimen with high levels of viral replication I restricted the analysis to patients who had a high viral load (i.e. HIV RNA >10000 cps/mL) at both the start and the end of the interval. Even though the sample size was reduced down to 1249 (38.8%), the estimated benefit from retaining PI mutations was similar to the analysis in 7.4.3. In this subgroup analysis, the median (IQR) $CD4^+$ T cell count at the start of the episode was 210 (114 to 322) cells/μL and patients experienced a $CD4^+$ T cell count change of -2.7 (-15.1 to 8.7) cells/μL/month.

The adjusted mean (95% CI) $CD4^+$ T cell count changes were: -2.63 (-7.67, 2.41); -3.71 (-10.95, 3.52); -6.25 (-11.31, -1.18); and -1.88 (-10.52, 6.76) cells/μL/month for patients on PI/rs with PI-mutations, on PI/rs without PI-mutations, on NNRTIs with NNRTI-mutations and on NNRTIs without NNRTI-mutations, respectively.

7.4.5.4. The time between the resistance test and the HIV RNA interval

The entry criteria for all of the patients studied so far (section 7.3.1) includes patients with a resistance test performed in the four weeks after the viral load pair as well as patients with a resistance test in the four weeks prior to the viral load pair. As a result, mutations identified during the test may not have been present during the interval between HIV RNA measurements, but may have appeared afterwards. I restricted the analysis to patients who had a resistance test taken before the end of the viral load interval in order to reduce the possibility of reverse causality between mutation emergence and $CD4^+$ T cell count slopes. In addition, removal of the 602 paired HIV RNA intervals in which the resistance test was performed after the end of the viral load pair excludes all patients who may have started another regimen prior to their resistance test.

The total numbers of paired measurements satisfying these criteria were 2618. The median (IQR) $CD4^+$ T cell count at the start of the episode was 276 (169 to 410) cells/μL and patients experienced a $CD4^+$ T cell count change of -1.7 (-16.2 to 12.6) cells/μL/month. The adjusted mean (95% CI) $CD4^+$ T cell count changes were: 0.86 (-3.24 to 4.97); -3.34 (-9.68 to 3.00); -5.43 (-8.66 to -2.20); and 1.73 (-5.53 to 8.99) cells/μL/month for patients on PI/rs with PI-mutations, on PI/rs without PI-mutations, on NNRTIs with NNRTI-mutations and on NNRTIs without NNRTI-mutations, respectively.

7.4.5.5. Exclusion of non adherent patients

Patients who are not adherent to the ARVs in their regimen will have limited exposure to therapy. These patients are likely to have a virus that is deemed to be susceptible to the ARVs in their regimen because their virus is unlikely to contain many mutations. To exclude these non adherent patients, and to see whether clearer differences can be seen in adherent patients with limited treatment options available, I restricted the analysis to patients with a GSS <1 (N=1267). In these patients, the median (IQR) $CD4^+$ T cell count at the start of the episode was 210 (114 to 322) cells/μL and patients experienced a $CD4^+$ T cell count change of -2.65 (-15.09 to 8.71) cells/μL/month.

Table 7.4.5.5.1 shows there are stronger differences in $CD4^+$ T cell count slopes between patients who are receiving PIs and have a virus containing PI mutations compared to patients who have a virus that does not contain such mutations. In the main analysis on the whole group of patients, there was a 2.9 (-4.2 to 10.0) cells/μL greater decline in $CD4^+$ T cell counts for patients who received a PI/r and did not have a virus containing a PI-resistance mutation compared to those on a PI/r who had a virus containing a PI resistance mutation. In this subgroup analysis, there was an 18.3 (-4.9 to 41.4) cells/μL greater decline in $CD4^+$ T cell counts for patients who received a PI/r and did not have a virus containing a PI-resistance mutation compared to those on a PI/r who had a virus containing a PI resistance mutation. However, numbers are small in this subgroup analysis and no significant differences between mutational categories were observed.

Table 7.4.5.5.1: $CD4^+$ T cell count changes: patients with a GSS <1 (N=1267)

	Unadjusted analysis		**Multivariable analysis**	
Variable	**ΔCD4⁺ T cell count cells/μL/month (95% CI)**	**P-value**	**ΔCD4⁺ T cell count cells/μL/month (95% CI)**	**P-value**
First CD4⁺ T cell count of the pair (per 100 cells/μL higher)	-4.46 (-5.39, -3.53)	<0.0001	-5.40 (-6.40, -4.40)	<0.0001
HIV RNA at start of interval (per log_{10} cps/mL increase)	-1.18 (-4.07, 1.71)	0.42	-7.44 (-10.54, -4.34)	<0.0001
Time between resistance test and first HIV RNA measurement of the pair (per week longer)	0.16 (-0.11, 0.44)	0.24	0.18 (-0.08, 0.45)	0.18
Mutation category (IAS-USA):				
On PI/r, PI mutations	Ref	0.55	Ref	0.48
On PI/r, no PI mutations	-11.15 (-35.13, 12.84)		-18.26 (-41.37, 4.86)	
On PI, PI mutations	-0.12 (-6.02, 5.78)		0.49 (-5.65, 6.63)	
On PI, no PI mutations	27.16 (-43.70, 98.01)		18.48 (-49.54, 86.49)	
On NNRTI, NNRTI mutations	-4.57 (-10.46, 1.32)		-3.08 (-9.55, 3.38)	
On NNRTI, no NNRTI mutations	4.93 (-30.70, 40.57)		-0.89 (-35.18, 33.39)	
On PI & NNRTI, mutations to both	-3.26 (-10.10, 3.58)		-3.51 (-10.53, 3.51)	
On PI & NNRTI, muts to 1 or no drugs	-35.18 (-85.38, 15.02)		-37.78 (-85.99, 10.44)	
On NRTIs only, NRTI mutations	-3.58 (-10.93, 3.77)		-1.99 (-9.42, 5.44)	
On NRTIs only, no NRTI mutations	-		-	

7.4.5.6. Generalised linear models with generalised estimating equations

Some of the patients in these comparisons contribute more than one paired HIV RNA interval to the regression models. To take into account these repeated measurements I examined the data using generalized linear models (GLMs) with generalized estimating

equations. In the initial GLM performed on these data, the resulting scale parameter was unstable because of a negative correlation structure associated with the data. This is because it is possible for successive $CD4^+$ T cell count pairs to use the same $CD4^+$ T cell count (i.e. the last $CD4^+$ T cell count measurement in the pair may be the first value in the subsequent pair). As a result it was not possible to fit generalised estimating equations to the whole dataset. Instead, I re-ran the analysis using generalized estimating equations, but with viral load pairs restricted to be at least a year apart. This ensured there was a positive correlation structure. Patients who were receiving PI(/r)s and had a virus containing PI mutations had more favourable $CD4^+$ T cell count slopes than those who were receiving NNRTIs, irrespective of whether mutations to NNRTIs were present (Table 7.4.5.6.1).

Table 7.4.5.6.1: $CD4^+$ T cell count changes: generalised linear models
Including repeated measures on an individual if they occur >1 year apart, N=2248

Mutation category (IAS-USA)	**ΔCD4⁺ T cell count change cells/μL/month (95% CI) relative to group 1**	**Adjusted mean CD4⁺ T cell count change cells/μL/month (95% CI)**
On PI/r, PI mutations (group 1)	Ref	-0.4 (-4.6, 3.8)
On PI/r, no PI mutations	-3.3 (-10.5, 4.0)	-3.7 (-9.6, 2.2)
On PI, PI mutations	0.5 (-4.8, 5.9)	0.1 (-3.2, 3.4)
On PI, no PI mutations	-2.1 (-8.6, 4.3)	-2.6 (-7.4, 2.3)
On NNRTI, NNRTI mutations	-5.6 (-10.9, -0.3)	-6.0 (-9.2, -2.8)
On NNRTI, no NNRTI mutations	-7.5 (-15.6, 0.6)	-7.9 (-14.9, 1.0)
On PI & NNRTI, mutations to both	-3.9 (-10.8, 3.0)	-4.3 (-9.9, 1.2)
On PI & NNRTI, muts to 1 class or no muts	-6.9 (-15.8, 2.0)	-7.3 (-15.2, 0.5)
On NRTIs only, NRTI mutations	-2.0 (-8.4, 4.5)	2.4 (-7.3, 2.5)
On NRTIs only, no NRTI mutations	-0.4 (-11.9, 11.2)	-0.8 (-11.5, 9.9)

7.5. Discussion

7.5.1. Summary of main results

Although drug resistant HIV RNA can adversely impact on the virological and clinical outcomes of HIV infected individuals[196;348;349], the presence of certain mutations could be beneficial in patients who have no treatment options available to them[279;403;406;407]. In this chapter I investigated the numbers of mutations, the class of mutation, and the prevalence of individual mutations in patients who had continuously high levels of viral replication. I did not identify any clear differences in $CD4^+$ T cell count slopes for a given viral load level

(or for a given viral load change from the pre-ART value) according to the presence of resistance mutations.

There were noticeable differences in $CD4^+$ T cell count declines for patients who were on ARVs compared to patients who were off ARVs: with patients off ARVs experiencing a 5.1 (2.7, 7.6) cells/μL greater $CD4^+$ T cell count decline per month compared to patients who were on ARVs. The association between being off treatment and greater declines in $CD4^+$ T cell counts is in keeping with previous findings[154;408]. To see whether this could be attributed to the presence of resistance mutations I investigated $CD4^+$ T cell count slopes in the subgroup of patients who were on treatment. In this group, patients who were receiving NNRTIs had greater $CD4^+$ T cell count declines if NNRTI mutations were present in their virus and patients who were receiving PI(/r)s had smaller $CD4^+$ T cell count declines if PI mutations were present in their virus; albeit these trends did not reach statistical significance. These patterns are expected based on the reported small impact of common NNRTI resistance mutations such as K103N on viral fitness. On the other hand, PI resistance mutations have been associated with a reduced viral fitness (at least until compensatory mutations emerge), so if they are present in the virus they may benefit the patient immunologically and virologically[284;409;410].

7.5.2. Interpretation of results

Investigation into the relationship between the numbers of PI mutations and the $CD4^+$ T cell count slopes revealed a V-shaped relationship instead of a linear one. When I categorised patients according to the number of PI mutations that were present in their virus, patients had more favourable $CD4^+$ T cell count declines when a single mutation or two PI mutations were present. Patients who had more than two primary PI mutations in their virus experienced the greatest $CD4^+$ T cell count declines, possibly indicating a cut-off level for the benefit of harbouring PI mutations related to the emergence of compensatory mutations.

Alternatively, there could be more residual activity of the PI(/r) when fewer mutations are present, and the high $CD4^+$ T cell count declines that are seen in patients who have no mutations in their virus could possibly be due to poor adherence. The V-shaped relationship between the numbers of PI mutations and the $CD4^+$ T cell count slopes is likely to be due to one or both of the residual activity of the PI and reduced fitness of viruses containing certain mutations, as has been previously demonstrated[411-414].

Since I have not been able to distinguish between residual activity of the PI and reduced fitness of viruses containing certain mutations, studies that investigate partial treatment interruption can help to distinguish between residual drug-activity (i.e. if HIV RNA changes are seen as soon as the drug is interrupted) versus reduced viral fitness as a result of the mutations that are in the virus (i.e. HIV RNA changes are only seen when mutations to the drug disappear). It is likely that residual drug activity is the primary explanation for the trends in this chapter because I have been unable to identify a clear effect of any class of mutation resulting in a reduced fitness of the virus.

In patients on an NNRTI containing regimen, the relationship between the number of NNRTI mutations and $CD4^+$ T cell count changes appears to be linear, with a 1.86 (0.31 to 3.41) cells/μL/month greater decline in $CD4^+$ T cell counts for each additional NNRTI mutation. When I investigated frequently occurring mutations for all patients who were receiving ARVs, the only mutations, from any of the drug classes, associated with $CD4^+$ T cell count slopes were K103N and Y181C. After adjustment for HIV RNA level at the start of the interval, the separate presence of K103N and Y181C was associated with a mean (95% CI) 2.96 (-0.16 to 6.09), p=0.06, and 4.48 (0.74 to 8.23), p=0.02, cells/μL/month greater $CD4^+$ T cell count decline compared to viruses without either mutation, respectively. This is consistent with the high fitness of the K103N and Y181C mutants that has been reported by others[415-417]. No residual activity or beneficial impact on viral fitness has been previously observed with resistance to NNRTIs[416;418]. In patients who were off ARVs, the presence of K103N and Y181C was associated with a mean (95% CI) 0.71 (-11.00 to 12.42), p=0.91, and 9.41 (-9.29 to 28.11), p=0.32, cells/μL/month smaller $CD4^+$ T cell count reduction compared to patients in whom either mutation could not be detected, respectively.

7.5.3. Clinical interpretation

In this study, patients who were receiving an NNRTI, in whom an NNRTI mutation was detected in their predominant virus, experienced larger $CD4^+$ T cell count reductions compared to patients with a virus that did not show any NNRTI mutations. Consequently, these patients may perform clinically worse over the long term. Of note, there were only 142 patients who were on a virologically failing NNRTI-containing regimen who did not have an NNRTI mutation present in their virus. The reasons for this are unknown. Since it could be harmful to keep patients on a virologically failing NNRTI containing regimen it may be better to switch them to a PI containing regimen or reduce to an NRTI containing regimen if they cannot tolerate the toxicities associated with PIs[419-421]. This analysis has

shown that patients who receive NRTIs, without a PI or NNRTI anchor drug, experience similar $CD4^+$ T cell count declines to patients who receive NNRTIs and NRTIs together. Even though all patients are receiving a virologically failing regimen they still benefit from remaining on treatment. As illustrated in this study, and as previously demonstrated, patients off ARVs do immunologically worse than patients who are receiving therapy[154]. Of note, patients who are off ARVs in this analysis have a mean (95% CI) 2.12 (-0.66 to 4.90), p=0.14, and 5.94 (3.48 to 8.41), p<0.0001, cells/μL/month greater $CD4^+$ T cell count declines than patients receiving NNRTIs and patients receiving a non NNRTI-containing regimen, respectively.

It is hard to quantify the sequential relationships between $CD4^+$ T cell count changes, viral load levels and individual mutations due to the timing of these measurements in these studies. The presence of certain mutations may have had an effect on $CD4^+$ T cell counts prior to the interval covered by the pair because some mutations may already be present in the virus before the first $CD4^+$ T cell count was taken. In an attempt to account for the impact of resistance on $CD4^+$ T cell counts just prior to the interval between HIV RNA measurements I adjusted for the first $CD4^+$ T cell count of the pair in a separate model, but not in the main model due to the influence of regression to the mean[404;405]. Similar results were found in this analysis. Exclusion of patients who had their resistance tests taken after the $CD4^+$ T cell count pair served to confirm our findings, but the impact of the mutations on the absolute $CD4^+$ T cell counts prior to treatment is still partially unaccounted for. If the presence of a mutation reduces the $CD4^+$ T cell counts prior to the pair then these patients will start with a lower $CD4^+$ T cell count and will have less scope for decline.

In the current era, with new drugs becoming available, it is unlikely that patients will be maintained on a virologically failing regimen for long. It is more probable that clinicians will switch some, or all, of the drugs in the regimen in the case of falling $CD4^+$ T cell counts. However, patients may still be maintained on the failing regimen if $CD4^+$ T cell counts are stable, or if they start to increase. More data is required to assess whether the presence of mutations in the virus population has a virological or immunological impact in patients who are virologically failing their treatment regimen.

All of the analyses in this chapter have been adjusted for the HIV RNA at the start of the interval in an attempt to account for the initial impact of viral replication on $CD4^+$ T cell count slopes. Even though adjustments have been performed, they do not fully capture

the noise generated through patients experiencing large HIV RNA changes during the interval between HIV RNA measurements. In a subgroup analysis of patients with stable viral loads (i.e. ΔHIV RNA <0.25 $\log_{10}$ cps/mL), minor $CD4^+$ T cell count increases were seen among patients on PIs and also among patients on NNRTIs. Even though these patients had similar HIV RNA levels and $CD4^+$ T cell counts at the start of the interval to patients in the main analysis, they still experienced more favourable $CD4^+$ T cell count declines. Since these patients have more stable viral replication, their $CD4^+$ T cell counts are not likely to decline as much as in patients in the main analysis.

In a sensitivity analysis, I did not find the extent of viral load suppression from the pre-ART value to predict $CD4^+$ T cell count change, which is in contrast to the results published by the PLATO collaboration[154]. Most patients in PLATO started antiretroviral therapy with single or dual therapy, had a higher HIV RNA at the start of the interval, a higher pre-ART HIV RNA and a lower $CD4^+$ T cell count at the start of the interval, so they had less scope for large $CD4^+$ T cell count reductions compared to patients in our study. In fact, $CD4^+$ T cell count increases were seen over time in the PLATO population instead of the $CD4^+$ T cell count declines seen in this analysis because their analysis was also restricted to patients experiencing minimal HIV RNA changes (i.e. ΔHIV RNA <0.3 $\log_{10}$ cps/mL). In PLATO, the level of decline in viral replication from the pre-ART value is a strong indication of further $CD4^+$ T cell count deterioration, but PLATO patients have truly exhausted all of their treatment options and are more severely immunocompromised than patients in our study. Patients in our study are failing their cART regimen, but at a lower HIV RNA and with fairly low levels of mutation emergence. The combination of residual drug activity and a reduced fitness of viruses containing certain mutations might play a stronger role in our study because our patients have greater, albeit still limited, susceptibility to the drugs they are receiving.

In patients with few treatment options available (i.e. a GSS <1) the magnitude of the effect size comparing $CD4^+$ T cell count slopes between patients on a PI(/r) who have a virus containing PI mutations, compared to those on a PI(/r) who do not have a virus containing a primary PI resistance mutations, was greater than that seen in the main analysis (section 7.4.3). This could be partly due to the way GISs ascribe resistance levels. As discussed previously (see chapter 4 and chapter 5) GISs do not capture viral sensitivity precisely; there are differences between systems in how resistant the virus is deemed to be and so these GISs may not reliably judge how effective the whole regimen is. As a result some patients may be classified as having a GSS<1, whereas in fact they had a virus that was

susceptible to more drugs in their regimen. Residual drug activity is likely to be weaker in this population than in the main analysis because these patients will have a virus containing more mutations and so the drugs should have even less activity, but greater differences were observed between patients with and without PI mutations in this subgroup.

The exclusion of patients who had resistance tests taken after the viral load pair and the use of generalised linear models with generalised estimating equations confirmed our findings of a difference in $CD4^+$ T cell count slopes in patients who were using PI(/r)s and had PI-specific mutations compared to patients who did not. Overall, however, there were no remarkable differences in $CD4^+$ T cell count slopes for a given viral load according to the presence of resistance mutations.

7.5.4. Limitations of the analysis

Our analysis has several limitations: we do not have a measure of adherence in our patients and since some mutations disappear fairly quickly in the absence of treatment a good adherence rate is required to keep the drug levels high so that mutations are retained and the virus does not revert back to wild-type[422]. I performed a sensitivity analysis in which was restricted to patients who had several mutations in their virus (i.e. a GSS <1) and found confirmatory results to the analysis in 7.4.3. Since all of these patients have mutations in their virus they must have been exposed to treatment and so can probably be considered to have intermediate to high rates of adherence.

These analyses have not been adjusted for the presence of NRTI mutations or other mutations that are present in the predominant virus. A patient falling into any of the mutational categories (excluding patients on NRTIs with no NRTI mutations) may have had a virus containing NRTI mutations even if they did not have a virus with mutations to the main drug class they were receiving. As a result NRTI mutations may have influenced the relationship between $CD4^+$ T cell count slopes and the mutational category. When I looked at the relationship between the presence of NRTI mutations and $CD4^+$ T cell count slopes overall, there was a 1.97 (-1.62 to 5.56) cells/μL/month smaller decline in patients with NRTI mutations present compared to patients without these mutations, but this did not reach statistical significance.

The $CD4^+$ T cell count slopes in this study were calculated on the basis of two values and so were highly variable. If there was enough data available to include another $CD4^+$ T cell

count measurement in this calculation it would improve the estimate and decrease the variability of the slopes. However, such slopes are of limited use in clinical practice. In addition, use of a third $CD4^+$ T cell count measurement would mean it is possible to calculate the slope over a time-period that is more distant to the time of the resistance test.

One of the main limitations to this analysis is the inability to separate out the pure treatment effect from the effect of mutations. Since these two variables come arm-in-arm (i.e. certain treatments are needed to retain certain mutations) it was not possible to investigate these two variables separately. Instead I used a composite variable to estimate the relationship between the drug, the presence of mutations and $CD4^+$ T cell count slopes. Because of a high correlation between covariates, our current analysis cannot establish definitively whether these differences are attributed to the drugs themselves rather than the mutations present.

7.5.5. Conclusion

In conclusion, there was little evidence of a difference in $CD4^+$ T cell count slopes for a given HIV RNA level (or a given HIV RNA change from the pre-ART value) according to the presence of mutations. Certain NNRTI mutations may be associated with greater $CD4^+$ T cell count declines, but since this analysis has limited power due to the high variability of $CD4^+$ T cell count slopes, further analyses on a larger number of patients is required before any firm conclusions can be drawn.

CHAPTER 8: VIRAL RE-SUPPRESSION AND DETECTION OF DRUG RESISTANCE FOLLOWING INTERRUPTION OF A SUPPRESSIVE NNRTI-CONTAINING REGIMEN

8. Introduction

As I have discussed throughout this thesis, HIV infected individuals may experience resistance evolution in their virus populations as a result of the treatment they are receiving. In chapter 6, I showed that there may be an improvement in fidelity and a reduction in the replication capacity of viruses containing the M184I/V mutation, because presence of this mutation was associated with fewer nucleotide changes in the virus over follow-up.

Although there is a postulated reduction in the replication capacity of viruses containing some of the rarer NNRTI mutations, the highly resistance-conferring NNRTI mutations have little impact on reducing the replication capacity of the virus and are commonly selected in clinical practice[423]. If an NNRTI resistance mutation emerges in the predominant virus of a patient who is receiving either efavirenz or nevirapine, the NNRTI component of the cART regimen will no longer have any activity against viral replication and so there will be no added benefit of retaining the NNRTI in the treatment regimen. This is in accordance with the interpretation of most genotypic interpretation systems (GISs). Most GISs rule out the use of NNRTIs when certain NNRTI mutations are present because of a lack of residual activity of these drugs and the amount of cross-resistance within this drug class (section 1.6.2.1)[33;215;220;221;424;425].

Patients who are on a virologically failing NNRTI containing regimen and have an NNRTI mutation in their predominant virus population experience steeper $CD4^+$ T cell count declines than those who do not show any NNRTI mutations in their predominant virus, as demonstrated in chapter 7. If NNRTI mutations are present, the patient may wish to switch the NNRTI component of the regimen to a PI or an NRTI in order to restore susceptibility to an NNRTI, even if they have a virus containing resistance mutations to these other classes of ARVs. If NNRTI mutations are not present, the patient may still wish to interrupt the NNRTI component of a regimen for reasons such as virological failure (although this is usually indicative of resistance emergence), toxicity, or because they were only using the NNRTI in order to avoid mother-to-child HIV transmission.

Patients with low levels of HIV RNA replication, who wish to switch the NNRTI to an ARV from a different drug class, should not be at an elevated risk of experiencing the emergence of resistance mutations if they remain on therapy at the time of switching, because replication levels should continue to remain relatively low. However, if a patient wishes to interrupt all of the drugs in their regimen, and undergo a treatment interruption, then resistance emergence may be an issue due to the differing half-lives of ARVs from different classes. The best way to interrupt an NNRTI containing regimen and avoid resistance emergence has yet to be identified.

8.1. Aims of the chapter

In this chapter I analyse data from a large trial of treatment interruptions (SMART) and examine the rates of viral re-suppression among patients who interrupt an NNRTI-containing regimen with HIV RNA <400 copies/mL and re-start ARVs in line with the protocol – which mandated re-initiation after experiencing a $CD4^+$ T-cell count drop to <250 cells/μL in the SMART trial.

I also describe drug resistance mutations that were observed in the two months following a treatment interruption, for patients who were virologically suppressed (i.e. HIV RNA< 400 copies/mL) on an NNRTI-containing regimen at entry into SMART and discontinued it in line with the protocol.

8.2. Literature review

Interruption of cART is associated with an increased risk of AIDS and other conditions typically thought to be associated with treatment (i.e. kidney, heart and liver disease) compared to continuous therapy, as recently demonstrated in the SMART study[426]. In SMART, patients who were randomised to the discontinuation arm were advised to stop therapy when their $CD4^+$ T cell count was >350 cells/μL and re-initiate it again when it was <250 cells/μL. Time-guided interruption strategies or strategies using a higher $CD4^+$ T cell count threshold may be a safer way to interrupt treatment than the approach used in SMART[427].

Even though patients are at a higher risk of developing treatment associated events if they discontinue cART[426], there are still occasional circumstances where a patient may want, or need, to interrupt some or all of the ARVs in their regimen. For example, women who take ARVs during pregnancy, in order to prevent mother-to-child HIV transmission, may choose

to discontinue therapy if their $CD4^+$ T cell counts or HIV RNA levels prior to treatment initiation were not sufficient to lead to a recommendation for continued cART after delivery. In addition, in research, trials of interventions such as therapeutic vaccinations have employed short-term interruptions as means of measuring the effect of the intervention on the viral load upon interrupting. Further, in developing countries there may be interruptions in drug supply and this may necessitate an interruption of ARVs.

ARVs have different plasma and intracellular half-lives to each other[428]: the plasma elimination half-lives of nucleoside reverse transcriptase inhibitors (NRTIs) vary from two to six hours, although the half-life of FTC is estimated to be approximately ten hours and the half-life of TDF approximately 17 hours. Protease inhibitors (PIs) also have short plasma elimination half-lives, which are generally less than eight hours, even if they are used with ritonavir boosting. Although the newest PI, darunavir, has a marginally higher half-life of approximately 15 hours. On the other hand, efavirenz and nevirapine have longer elimination half-lives than the other classes of drugs, estimated to be between 30 to 45 hours.

Given the prolonged half-life of current NNRTIs, there is a concern that simultaneous interruption of the drugs in an NNRTI-containing regimen may lead to a period in which the patient carries only a single drug. Monotherapy with an NNRTI can lead to viraemia as it is unlikely to be sufficient to maintain virological suppression, and consequently there will be an elevated risk of drug resistance emergence (section 1.6.1).

The NNRTIs nevirapine and efavirenz have a low genetic barrier, in that only one mutation is required in order to confer high-level resistance to the drug, so the consequences of resistance development for these drugs are particularly serious. Since the key NNRTI mutations are the same for each NNRTI (e.g. the K103N mutation results in resistance to all currently approved NNRTIs), it would compromise future use of all NNRTIs if mutations to these drugs occurred. This has led to the suggestion that the NNRTI component of a regimen could be stopped earlier than other ARVs in the regimen, with or without replacement with a protease inhibitor, to avoid the potential for NNRTI monotherapy[295].

Although the long half-lives of NNRTIs is an advantage in cART (because less frequent dosing is required), these attributes also make them susceptible to the emergence of resistance if an NNRTI-containing regimen is not stopped in a manner that minimizes the

risk of resistance emergence. Limited data are available on the best way to stop an NNRTI in order to avoid monotherapy and the potential emergence of drug resistance.

Stopping the NNRTI component of the regimen 5 to 7 days prior to the nucleoside backbone has been shown to prevent resistance emergence to NNRTIs[427;429]. Other regimen-switching or cycling studies have also shown a greater virological benefit if an alternative method of stopping an NNRTI is used compared to stopping all ARVs simultaneously[430]. Another factor to consider is that the pathway for selecting NNRTI resistance mutations differs according to the drug levels just prior to the treatment interruption; where drug concentrations close to the lower limit of the therapeutic range are associated with resistance emergence of major NNRTI-resistant variants[431].

BHIVA (British HIV Association) guidelines recommend that HIV-infected women who stop an NNRTI-containing regimen that was started in order to prevent mother-to-child transmission, should either continue their nucleoside backbone for another week, or switch the NNRTI to a PI and use three ARVs with similarly short half-lives[432]. Similarly, the U.S. Department of Health and Human Services (DHHS) recommend that women who stop an NNRTI-containing regimen that was started in order to prevent mother-to-child transmission, should continue their nucleoside backbone for a week after interrupting the NNRTI component of the regimen[433].

The SMART study provided clinicians with guidelines to consider utilising one of two different approaches when interrupting a suppressive NNRTI-based regimen as an alternative to interrupting all drugs simultaneously (simultaneous-interruption). They could prescribe a staggered-interruption (i.e. an interruption of the NNRTI while maintaining NRTIs for a longer period); or initiate a PI-substitution for the NNRTI or as a substitution for the entire regimen (switched-interruption). The decision of which approach to adopt was done at the discretion of the prescribing local clinician. Clinicians were encouraged to employ either a staggered- or switched-interruption over a simultaneous-interruption: however, there were still a number of patients who interrupted all ARVs simultaneously. Patients who underwent a simultaneous-interruption were compared to patients who interrupted an NNRTI using either of the other strategies to increase the power of these comparisons.

8.3. Methods

8.3.1. Study population

Details of entry into the SMART study have been described in 2.1.3. In SMART, 5472 patients were randomised to either $CD4^+$ T cell count guided treatment interruptions (N=2720, the drug conservation (DC) arm), or to continuous therapy (N=2752, the viral suppression (VS) arm). The trial was stopped prematurely on 11th January 2006 after the an interim analysis showed that not only were more deaths and AIDS-related events occurring in the DC arm, but that serious non-AIDS events (cardiovascular, hepatic, renal) were also occurring more frequently.

This subgroup analysis investigates patients with HIV RNA <400 copies/mL, who were on an NNRTI-containing regimen at their baseline visit and were randomised to the DC arm of SMART. Resistance test data prior to study entry was not mandatory for entry so information on archived resistance was not available. I assumed that patients with viral suppression on a regimen containing an NNRTI at baseline had no NNRTI resistance mutations in their virus at baseline. However, this assumption may be flawed, because drug-resistant viruses can be selected and replicate during suppressive antiretroviral therapy[434].

The resistance analysis was restricted to patients who were virologically suppressed at baseline, had an elevated HIV RNA (i.e. HIV RNA ≥1000 copies/mL) in the two months following a treatment interruption and had a plasma specimen stored at the the one-month or two-month visit. Where available the plasma sample at the one month visit was used, if this was not available the two month plasma sample was used instead.

The one to two month time point was selected because, in the presence of monotherapy, NNRTI resistance mutations are likely to emerge relatively quickly (section 1.6.5)[435]. I did not choose a longer time point because there would be a greater likelihood that resistance mutations which may have emerged during the 'NNRTI monotherapy period' might have been out-selected by a virus that did not show any resistance mutations. In addition, there is a greater chance that patients will have re-started ARVs if a later time point had been studied, in which case resistance mutations in the dominant viral species would not be directly related to the mutations that emerged during the time of the treatment interruption.

8.3.2. Laboratory methods

Follow-up visits in SMART were scheduled to take place after one month, two months and every two months thereafter for the first year and every four months in the second and subsequent years of follow-up. At each visit, patients had their treatment history recorded and their $CD4^+$ T cell counts and plasma HIV RNA levels measured. Plasma samples were collected for a subset of patients and were used for nucleotide sequencing if HIV RNA ≥1000 copies/mL. Sequence data was translated into amino acid substitutions (full or as part of a mixture) from a reference clade B strain (i.e. HXB2) for both RT and protease.

8.3.3. Statistical analysis

Baseline and pre-baseline characteristics were examined and compared between patients who stopped an NNRTI according to each of the strategies (i.e. stopped all ARVs simultaneously (simultaneous-interruption), experienced a staggered stop (staggered-interruption) or switched the NNRTI to a PI before interrupting all ARVs (switched-interruption)). Baseline covariates include the mode of infection, gender, CDC category, race, age, $CD4^+$ T cell count, $CD4^+$ T cell nadir and HIV RNA level. The four categorical variables were compared between stopping categories using Chi-squared and Fisher's exact tests. To compare the distribution of normally distributed continuous variables between strategies I used ANOVA models, and for skewed continuous variables I used Kruskal-Wallis non-parametric tests.

I used two measures of viral load response in these comparisons: 1) whether the first HIV RNA that was measured four to eight months after re-starting therapy was <400 copies/mL; and 2) the time to first HIV RNA < 400 copies/mL. In the first comparison, I included all patients who had an HIV RNA measurement taken four to eight months after re-starting an NNRTI containing regimen. The proportion of patients with HIV RNA <400 copies/mL four to eight months after the re-start of therapy was investigated through logistic regression analysis.

All patients who re-started an NNRTI containing regimen were included in the second comparison, irrespective of whether they had an HIV RNA measurement in the four to eight month window. Kaplan-Meier plots were produced and Cox proportional hazards analyses were used to investigate the time to viral re-suppression (i.e. HIV RNA <400 copies/mL) after the re-start of therapy, according to the method of interrupting the NNRTI and also according to the NNRTI that was re-started. Patients who did not have an HIV

RNA measurement in the eight months following the re-start of therapy were treated as not having reached an HIV RNA <400 copies/mL.

The number and type of resistance mutation (i.e. specific mutations and class of mutation) that were observed in patients who had resistance data available was then examined. Since the absolute number of specific mutations observed in the predominant virus populations of these patients was small, I used Fisher's exact tests to compare the occurrence of new mutations according to the NNRTI-interruption strategy. Unadjusted logistic regression analysis was also used. This analysis had limited power so I could not perform any multivariable analysis. It should therefore be interpreted as a descriptive analysis of the mutations one can expect to see in patients who stop an NNRTI and should indicate whether certain mutations are more likely to arise if the NNRTI component of their regimen is interrupted in a certain way. All statistical analyses were performed using STATA.

8.4. Results

8.4.1. Patient characteristics

Out of the 2720 patients who were randomised to the DC arm of SMART there were 1131 (41.5%) who discontinued an NNRTI; and 984 (87.0%) of these had an HIV RNA <400 copies/mL at the start of their treatment interruption. The 984 patients were primarily male (76%) and 27% had a CDC category stage C prior to entry in SMART. At baseline, patients had high $CD4^+$ T cell counts (median: 649; IQR: 491 to 835 cells/μL) as required for study entry, and low HIV RNA levels (median: 1.7; IQR: 1.7 to 2.0 $\log_{10}$ copies/mL) (Table 8.4.1.1).

Overall, 719 of the 984 (73.1%) patients re-started an NNRTI-containing regimen during follow-up. From these, there were 688 (95.7%) who had at least one HIV RNA measurement four to eight months after the re-start (Figure 8.4.1.1). Out of the 719 patients who re-started an NNRTI containing regimen after a treatment interruption, 670 (93.2%) re-started a regimen containing the same NNRTI they interrupted: 427 of 434 (98.4%) patients who interrupted efavirenz re-started with efavirenz; 240 of 281 (85.4%) patients who interrupted nevirapine re-started with nevirapine; and three (75.0%) of the four patients who interrupted delavirdine re-started with delavirdine.

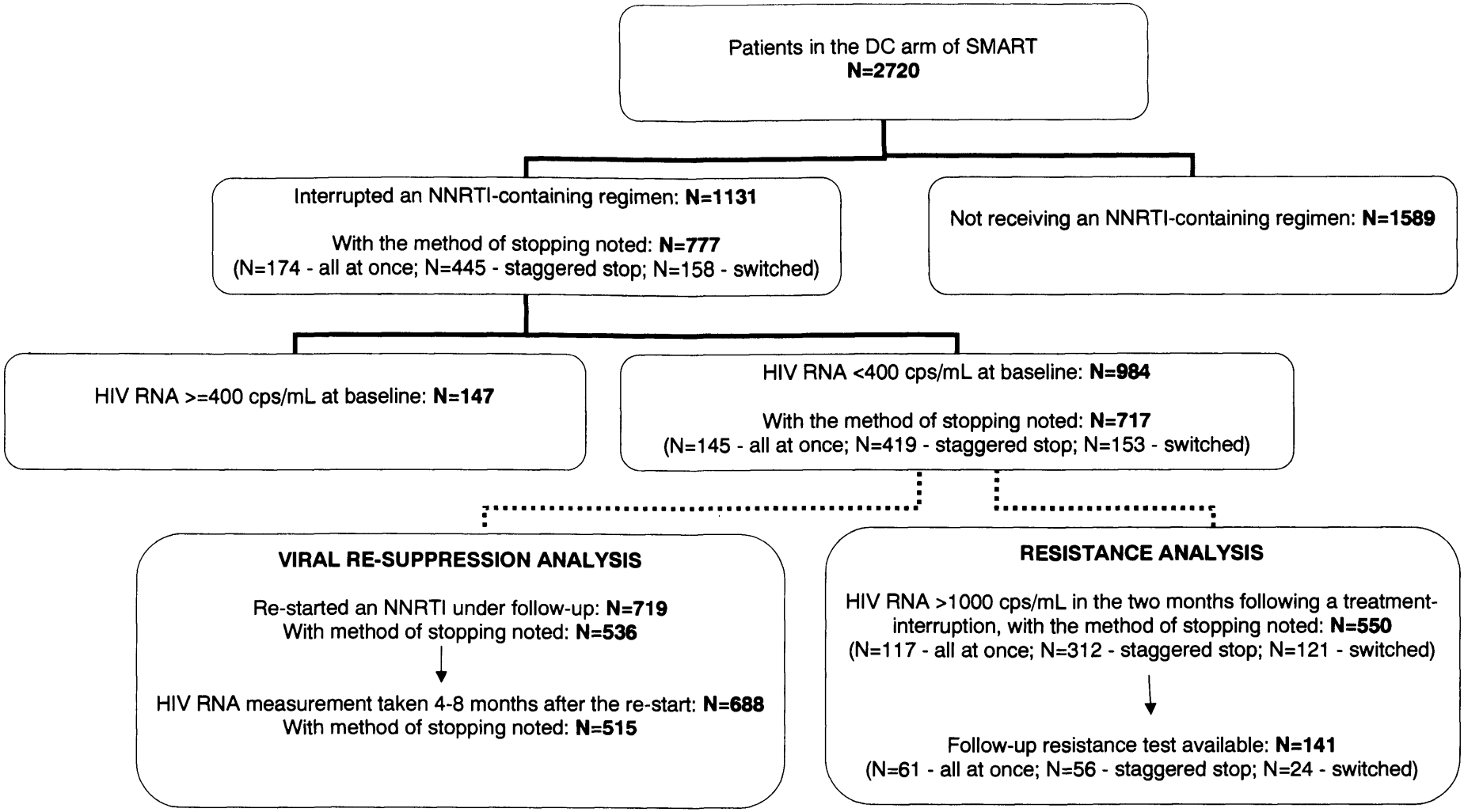
The analysis described in this report mainly focuses on patients who interrupted an NNRTI and have their means of stopping recorded, because I wanted to investigate the virological impact of stopping an NNRTI using different strategies. There were 717 of 984 (72.9%) patients who were virologically suppressed when they interrupted an NNRTI-containing regimen and have their interruption strategy recorded. There were no differences in baseline characteristics between these patients and those who stopped an NNRTI (with an HIV RNA <400 copies/mL) but do not have the method of stopping recorded (data not shown). Out of the 717 patients who have their interruption strategy recorded, 515 (71.8%) re-started an NNRTI after their treatment-interruption and have HIV RNA data available four to eight months after re-starting therapy (Figure 8.4.1.1).

Baseline characteristics for the 717 patients who had their treatment interruption strategy recorded are outlined in Table 8.4.1.1. The only noticeable difference in baseline characteristics between stopping strategies was that patients with simultaneous-interruption tended to have a higher HIV RNA levels immediately prior to the interruption compared to patients in the other interruption arms, p=0.001, even though these comparisons were already restricted to patients with HIV RNA <400 copies/mL at baseline.

Table 8.4.1.1: Baseline characteristics, according to NNRTI interruption strategy

	Patients with HIV RNA<400 N=984	Patients with interruption strategy recorded (N=717)				Patients with resistance data available (N=141)			
		Simultaneous -interruption N=145	Staggered-interruption N=419	Switched-interruption N=153	P-value	Simultaneous -interruption N=61	Staggered-interruption N=56	Switched-interruption N=24	P-value
Risk group									
Same sex	460 (47%)	67 (46%)	182 (43%)	79 (52%)	0.09	31 (51%)	22 (39%)	11 (46%)	0.19
IVDU	34 (3%)	2 (1%)	19 (5%)	1 (1%)		0 (0%)	3 (5%)	0 (0%)	
Opposite sex	339 (34%)	47 (32%)	163 (39%)	56 (37%)		17 (28%)	21 (38%)	4 (17%)	
Blood products	11 (1%)	3 (2%)	7 (2%)	1 (1%)		1 (2%)	1 (2%)	0 (0%)	
>1 risk factor	111 (11%)	20 (14%)	34 (8%)	13 (9%)		10 (16%)	6 (11%)	8 (33%)	
Other/Unknown	29 (3%)	6 (4%)	14 (3%)	3 (2%)		2 (3%)	3 (5%)	1 (4%)	
Gender No. Male, (%)	743 (76%)	110 (76%)	311 (74%)	123 (80%)	0.31	47 (77%)	41 (73%)	21 (89%)	0.38
CDC category No. C, (%)	268 (27%)	32 (22%)	113 (27%)	42 (27%)	0.47	11 (18%)	21 (38%)	7 (29%)	0.06
Race No. (%)									
Black	231 (23%)	38 (26%)	74 (18%)	26 (17%)	0.17	19 (31%)	26 (46%)	9 (38%)	0.22
White	622 (63%)	93 (64%)	293 (70%)	105 (69%)		36 (59%)	21 (38%)	11 (46%)	
Other/unknown	131 (13%)	14 (10%)	52 (12%)	22 (14%)		6 (10%)	9 (16%)	4 (17%)	
Age	45	44	44	44	0.83	45	46	47	0.66
Mean (95% CI)	(44, 45)	(43, 46)	(43, 45)	(42, 45)		(43, 48)	(44, 48)	(43, 51)	
$CD4^+$ (cells/μL)	649	660	648	640	0.43	624	614	643	0.61
Med (IQR)	(491, 835)	(492, 923)	(490, 803)	(481, 823)		(471, 834)	(462, 750)	(522, 757)	
$CD4^+$ nadir (cells/μL)	230	229	229	223	0.84	208	177	209	0.26
Med (IQR)	(140, 342)	(134, 366)	(150, 323)	(140, 340)		(101, 326)	(57, 277)	(71, 290)	
HIV-1 RNA (log_{10} cps/mL)	1.7	1.7	1.7	1.7	0.001	1.9	1.7	1.7	0.25
Med (IQR)	(1.7, 2.0)	(1.7, 2.3)	(1.7, 1.9)	(1.7, 1.8)		(1.7, 2.6)	(1.7, 2.3)	(1.7, 1.9)	

Figure 8.4.1.1: Overview of patients in the DC arm of SMART



8.4.2. Viral re-suppression after re-starting NNRTIs (all patients)

Out of the 688 patients who re-started an NNRTI and have an HIV RNA measurement available four to eight months after resuming therapy, there were 601 (87.4%) who reached an HIV RNA <400 copies/mL. A total of 404 of the 449 (90.0%) patients who re-started on an efavirenz-containing regimen, 194 of the 236 (82.2%) patients who re-started on a nevirapine-containing regimen and all three (100%) of the patients who re-started on a delavirdine-containing regimen, reached viral load re-suppression, p=0.012.

Overall, there were 356 (79.3%), 172 (72.9%) and 3 (100.0%) patients who were still on efavirenz, nevirapine and delavirdine, respectively, at the time of viral load re-suppression. There were 93 (13.5%) who underwent a treatment change between re-starting the NNRTI and the four to eight month HIV RNA measurement (defined as a change to any ARV in the regimen). Among patients who changed ARVs, 70 (75.3%) achieved an HIV RNA <400 copies/mL compared to 531 of 595 (89.2%) patients who continued on the same regimen. The OR of an HIV RNA <400 copies/mL was 0.37 (0.21, 0.63) for patients who underwent a treatment change compared to patients who continued on the same regimen, p<0.0001.

When we focus on the 515 patients who had their initial interruption strategy recorded, there were differences in the proportion of patients with HIV RNA <400 copies/mL in the four to eight months after re-starting therapy. The OR for achieving HIV RNA re-suppression was 2.22 (1.19 to 4.15) for patients with a staggered- or switched-interruption compared to patients with a simultaneous-interruption, p=0.01, indicating a benefit of either or both of these two strategies.

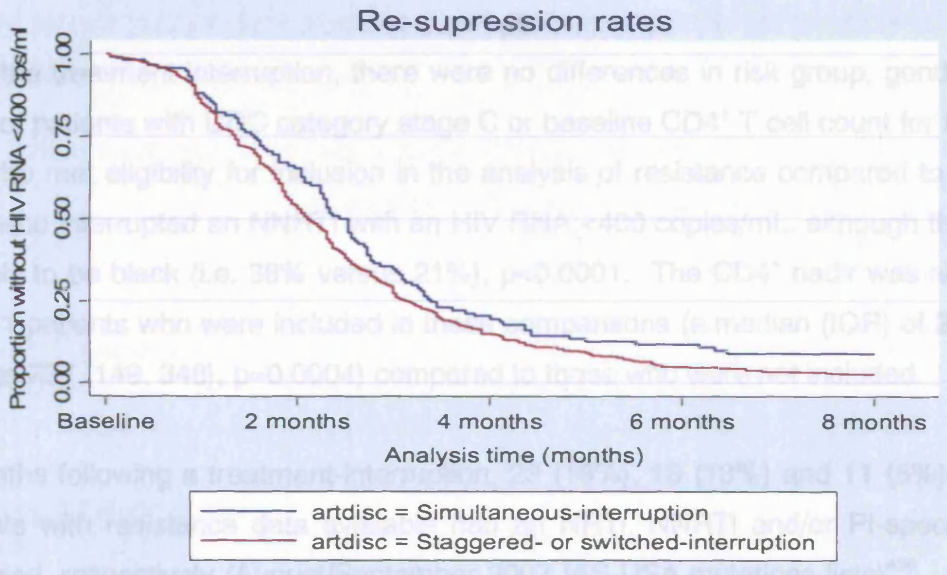
In a multivariable logistic regression model adjusted for the presence of a CDC stage C disease at baseline, the relationship between interruption strategy and the proportion of patients with viral re-suppression was still apparent. The OR for achieving HIV RNA re-suppression was: 2.00 (1.25 to 3.19) for patients with a staggered- or a switched-interruption compared to patients with a simultaneous-interruption, p=0.004; and 0.54 (0.30 to 0.97) for patients with a CDC stage C disease prior to entry compared to patients without, p=0.04.

This was consistent with the results from a Cox proportional hazards analysis exploring the time to viral load re-suppression in the eight months after re-starting therapy according to the initial method of stopping an NNRTI. In this analysis, the hazard ratio (HR) for

achieving HIV RNA re-suppression was 1.27 (1.03 to 1.57) for patients with a staggered- or switched-interruption compared to patients with a simultaneous-interruption, p=0.03 (Figure 8.4.2.1). For 21 of 536 (3.9%) patients, an HIV RNA measurement was not available in the eight months after re-starting therapy, so these patients were assumed not to have an HIV RNA ≤400 copies/mL for the eight months following the re-start of therapy, but were still included in the analysis.

In a multivariable Cox model adjusted for gender, the presence of a CDC stage C disease at baseline and age, the relationship between interruption strategy and the time to viral re-suppression was still apparent. The HRs for achieving HIV RNA re-suppression were: 1.29 (1.04 to 1.59) for patients with a staggered-interruption or a switched-interruption compared to patients with a simultaneous-interruption, p=0.02; 1.29 (1.06 to 1.57) for females compared to males, p=0.01; 0.80 (0.66 to 0.95) for patients with a CDC stage C disease prior to entry compared to patients without, p=0.01; and 1.05 (1.01 to 1.10) for every five years older at baseline, p=0.01.

Figure 8.4.2.1: Kaplan Meier plots illustrating the time to viral re-suppression according to the interruption strategy



Interruption strategy (No. at risk)

Simultaneous:	101	66	19	12	9
Staggered/switched:	435	231	60	18	10

In the multivariable Cox model, an interaction effect existed between the NNRTI re-started and the interruption strategy, suggesting that a relationship between the interruption strategy and the time to viral re-suppression only existed in patients who re-started

efavirenz, p=0.01. From a multivariable model, among patients who re-started efavirenz, the HR for achieving HIV RNA re-suppression was 1.74 (1.28 to 2.39) for patients with a staggered- or switched-interruption compared to patients with a simultaneous-interruption, p<0.0001; whereas among patients who re-started nevirapine, the HR for achieving HIV RNA re-suppression was 0.89 (0.62 to 1.26) for patients with a staggered- or switched-interruption compared to patients with a simultaneous-interruption, p=0.51.

8.4.3. Detection of drug resistance mutations

There were 141 (18.1%) patients, with HIV RNA <400 copies/mL at baseline, who have resistance data available (Figure 8.4.1.1): 61 of these underwent a simultaneous-interruption, 56 had a staggered-interruption and 24 had a switched-interruption (Table 8.4.1.1). This is because plasma was only collected at every visit for a subset of patients from the U.S. The remaining patients in SMART had blood samples collected annually.

When I focussed on these 141 patients, there were no significant differences in baseline characteristics according to the method of interrupting the NNRTI. Marginally more patients with a staggered-interruption had an AIDS defining event prior to entry compared to those using other strategies, but this did not reach statistical significance.

At the time of the treatment interruption, there were no differences in risk group, gender, the proportion of patients with CDC category stage C or baseline $CD4^+$ T cell count for the 141 patients who met eligibility for inclusion in the analysis of resistance compared to all other patients who interrupted an NNRTI with an HIV RNA <400 copies/mL, although they were more likely to be black (i.e. 38% versus 21%), p<0.0001. The $CD4^+$ nadir was also lower in the 141 patients who were included in these comparisons (a median (IQR) of 200 (78, 299) versus 237 (149, 348), p=0.0004) compared to those who were not included.

In the two months following a treatment-interruption, 23 (16%), 18 (13%) and 11 (8%) of the 141 patients with resistance data available had an NRTI, NNRTI and/or PI-specific mutation detected, respectively (August/September 2007 IAS-USA mutations lists)[436]. No significant differences were observed in the proportion of class-specific mutations that were detected according to the method of stopping the NNRTI (Table 8.4.4.1).

NNRTI mutations were marginally more likely to be detected in the dominant virus of patients who had a simultaneous-interruption compared to those who had a staggered- or switched-interruption (Odds ratio for the detection of an NNRTI mutation: OR (95% CI):

0.57 (0.21 to 1.53) for staggered- or switched-interruption versus simultaneous-interruption, p=0.29). In contrast, NRTI mutations were marginally more likely to be detected in the predominant virus of patients who had a staggered- or switched-interruption compared to patients with a simultaneous-interruption, potentially due to the sole use of NRTIs after the NNRTI component of the regimen had been interrupted in patients with a staggered-interruption (OR for the detection of an NRTI mutation (95% CI): 1.23 (0.49 to 3.05) for staggered- or switched-interruption versus simultaneous-interruption, p=0.66). However, neither of these trends reached statistical significance. In contrast to expectations, PI-specific mutations were no more likely to be seen in the predominant virus of patients who used a switched-interruption strategy compared to patients using other approaches (data not shown).

8.4.4. Detection of specific and class-specific mutations

I then explored the detection of specific resistance mutations and investigated how many drugs classes were compromised by the presence of resistance in these patients. No significant differences were seen in these comparisons, but the number of mutations that were detected overall was small, so I only have limited power to illustrate any differences if they truly exist (Table 8.4.4.1). Of note, the 188C/H/L and the 190S/A mutations were more commonly seen in patients who stopped all ARVs simultaneously, yet this difference did not reach statistical significance.

Table 8.4.4.1: Detection of resistance, according to NNRTI interruption strategy

	All patients N=141	**Stopped all ARVs at once N=61**	**Staggered stop N=56**	**Switched before stopping N=24**	**P-value**
Follow-up resistance mutations (N, %):					
NRTI mutations	23 (16.3%)	9 (14.8%)	11 (19.6%)	3 (12.5%)	0.72
NNRTI mutations	18 (12.8%)	10 (16.4%)	7 (12.5%)	1 (4.2%)	0.35
PI mutations	11 (7.8%)	3 (4.9%)	6 (10.7%)	2 (8.3%)	0.49
Follow-up resistance mutations (N, %):					
0 classes	102 (72.3%)	45 (73.8%)	37 (66.1%)	20 (83.3%)	0.57
1 class	28 (19.9%)	11 (18.0%)	15 (26.8%)	2 (8.3%)	
2 classes	9 (6.4%)	4 (6.6%)	3 (5.4%)	2 (8.3%)	
3 classes	2 (1.4%)	1 (1.6%)	1 (1.8%)	0 (0.0%)	
Number of mutations overall (N, %):					
0	102 (72.3%)	45 (73.8%)	37 (66.1%)	20 (83.3%)	0.72
1	18 (12.8%)	7 (11.5%)	10 (17.9%)	1 (4.2%)	
2	6 (4.3%)	3 (4.9%)	2 (3.6%)	1 (4.2%)	
≥3	15 (10.6%)	6 (9.8%)	7 (12.5%)	2 (8.3%)	

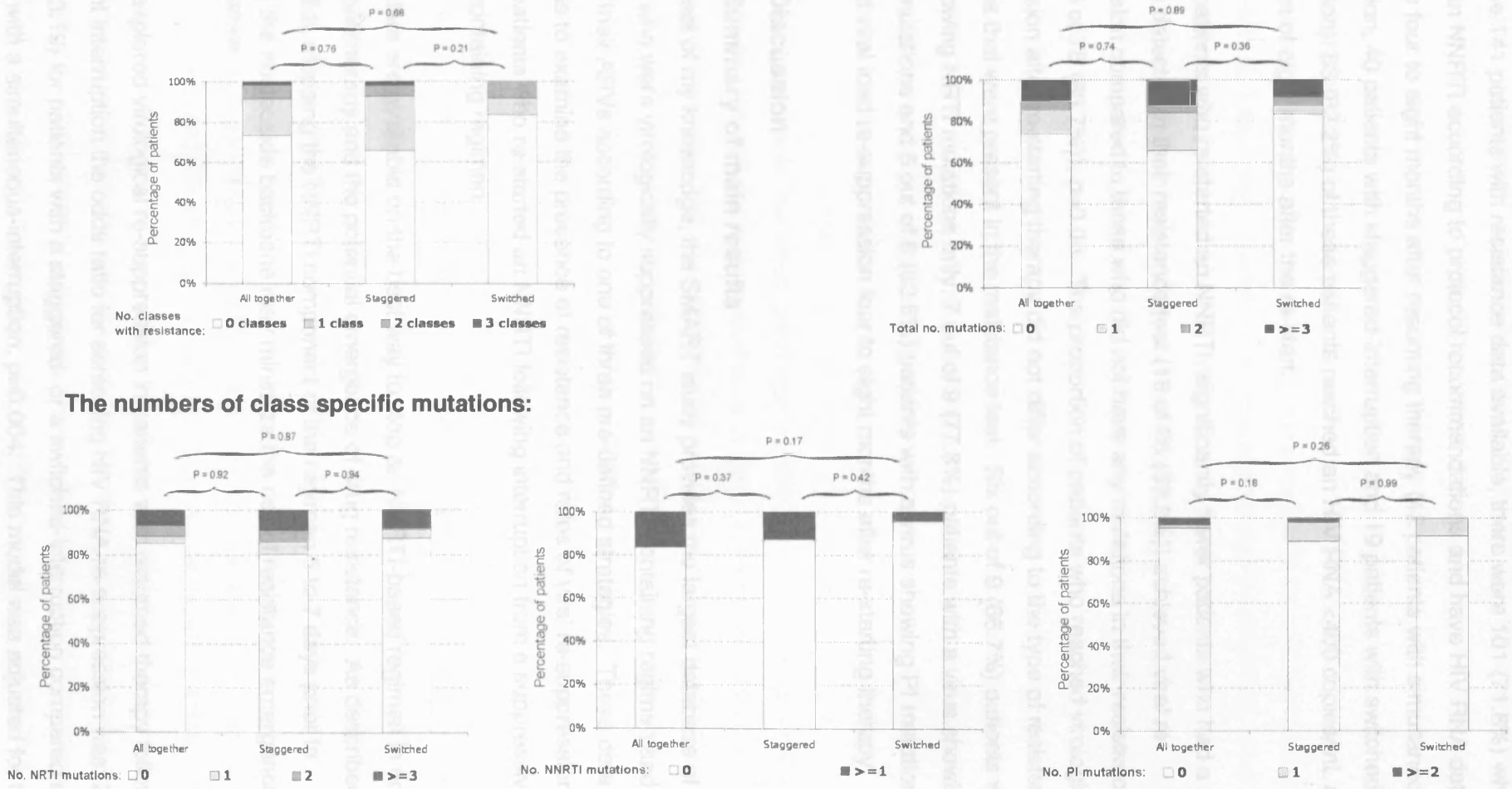
	All patients N=141	Stopped all ARVs at once N=61	Staggered stop N=56	Switched before stopping N=24	P-value
Number of NRTI mutations (N, %):					
0	118 (83.7%)	52 (85.3%)	45 (80.4%)	21 (87.5%)	0.97
1	6 (4.3%)	2 (3.3%)	3 (5.4%)	1 (4.2%)	
2	6 (4.3%)	3 (4.9%)	3 (5.4%)	0 (0.0%)	
≥ 3	11 (8.3%)	4 (6.6%)	5 (8.9%)	2 (8.3%)	
Number of PI mutations (N, %):					
0	130 (92.2%)	58 (95.1%)	50 (89.3%)	22 (91.7%)	0.33
1	8 (5.7%)	1 (1.6%)	5 (8.9%)	2 (8.3%)	
≥2	3 (2.1%)	2 (3.3%)	1 (1.8%)	0 (0.0%)	
NRTI mutations (N, %):					
41L	6 (4.3%)	2 (3.3%)	4 (7.1%)	0 (0.0%)	0.41
62V	2 (1.4%)	2 (3.3%)	0 (0.0%)	0 (0.0%)	0.65
65R	1 (0.7%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0.99
67N	6 (4.3%)	3 (4.9%)	2 (3.6%)	1 (4.2%)	0.99
69 insertion	1 (0.7%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0.99
70R	8 (5.7%)	2 (3.3%)	4 (7.1%)	2 (8.3%)	0.50
74V	2 (1.4%)	2 (3.3%)	0 (0.0%)	0 (0.0%)	0.65
75I	1 (0.7%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0.99
116Y	1 (0.7%)	0 (0.0%)	1 (1.8%)	0 (0.0%)	0.57
151M	1 (0.7%)	0 (0.0%)	1 (1.8%)	0 (0.0%)	0.57
184I/V	13 (9.2%)	5 (8.2%)	5 (8.9%)	3 (12.5%)	0.86
210W	5 (3.6%)	1 (1.6%)	4 (7.1%)	0 (0.0%)	0.20
215Y/F	9 (6.4%)	2 (3.3%)	5 (8.9%)	2 (8.3%)	0.35
219Q/E	5 (3.6%)	2 (3.3%)	2 (3.6%)	1 (4.2%)	0.99
NNRTI mutations (N, %):					
103N	5 (3.6%)	2 (3.3%)	3 (5.4%)	0 (0.0%)	0.72
108I	5 (3.6%)	1 (1.6%)	3 (5.4%)	1 (4.2%)	0.49
181C/I	2 (1.4%)	2 (3.3%)	0 (0.0%)	0 (0.0%)	0.65
188C/H/L	4 (2.8%)	3 (4.9%)	1 (1.8%)	0 (0.0%)	0.52
190S/A	4 (2.8%)	3 (4.9%)	1 (1.8%)	0 (0.0%)	0.52
PI mutations (N, %):					
30N	3 (2.1%)	1 (1.6%)	2 (3.6%)	0 (0.0%)	0.78
46I/L	4 (2.8%)	1 (1.6%)	2 (3.6%)	1 (4.2%)	0.67
50L/V	1 (0.7%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0.99
54M/L	1 (0.7%)	0 (0.0%)	1 (1.8%)	0 (0.0%)	0.57
82A/F/L/T/S	1 (0.7%)	0 (0.0%)	1 (1.8%)	0 (0.0%)	0.57
84V	2 (1.4%)	1 (1.6%)	1 (1.8%)	0 (0.0%)	0.99
90M	3 (2.1%)	2 (3.3%)	0 (0.0%)	1 (4.2%)	0.39

Any IAS-USA mutation that is not listed in the above table was not detected in the predominant virus populations of patients in our comparisons (i.e. NRTI: 77L, 115F; NNRTI: 100I, 106A/M, 225H, 236L; and PI: 32I, 33F, 47V/A, 48V, 76V, 88S).

8.4.5. Pair-wise comparisons

I performed pair-wise comparisons between the three stopping strategies to see whether there were differences between any two stopping methods in the overall numbers of mutations or the numbers of mutations that were detected to each drug class individually. I also investigated the number of drug classes the virus was resistant to using pair-wise comparisons. These comparisons did not highlight any significant differences (Figure 8.4.5.1).

Figure 8.4.5.1: Pair-wise comparisons between NNRTI stopping strategies



8.4.6. Virological re-suppression after re-starting NNRTIs (N=141)

Out of the 141 patients with resistance data available, there were 101 (71.6%) who re-started an NNRTI according to protocol recommendations and have HIV RNA data available four to eight months after resuming therapy (42 patients with simultaneous-interruption, 40 patients with staggered-interruption and 19 patients with switched-interruption): 83 (82.2%) of these patients reached an HIV RNA <400 copies/mL a maximum of eight months after the re-start.

Among patients who re-started an NNRTI, significantly fewer patients who had a mutation detected in their resistance test (18 of 26 (69.2%)) achieved viral re-suppression compared to those who did not have any mutations in their resistance tests (65 of 75 (86.7%)), p=0.05. The proportion of patients who reached virological re-suppression after re-starting therapy did not differ according to the type of resistance mutations that were present in the resistance test. Six out of 9 (66.7%) patients with a virus showing NRTI mutations only, 7 out of 9 (77.8%) patients with a virus showing NNRTI mutations and 5 out of 8 (62.5%) patients with a virus showing PI mutations, achieved viral load re-suppression four to eight months after re-starting therapy.

8.5. Discussion

8.5.1. Summary of main results

To the best of my knowledge, the SMART study provides the largest database of patients who were virologically suppressed on an NNRTI-containing regimen and stopped their ARVs according to one of three pre-defined strategies. These data enable us to examine the presence of resistance and rates of viral re-suppression among patients who re-started an NNRTI following interruption from a suppressive NNRTI-containing regimen.

Limited data are available on the best way to stop an NNRTI-based regimen in order to avoid monotherapy and the potential emergence of drug resistance. As described in section 8.2, stopping the NNRTI component of the regimen 5 to 7 days prior to stopping the nucleoside backbone may minimize the risk of resistance emergence to NNRTIs[427;429].

When I explored virological re-suppression in patients who resumed therapy following a treatment interruption the odds ratio for achieving HIV RNA re-suppression was: 2.00 (1.25 to 3.19) for patients with a staggered- or a switched-interruption compared to patients with a simultaneous-interruption, p=0.004. This model was adjusted for the presence of a CDC stage C disease at baseline. In a survival analysis, there was also

a trend for a difference in the time to viral re-suppression according to the interruption strategy: fewer patients with simultaneous-interruption reached viral re-suppression compared to patients using either a staggered- or switched-interruption, log rank p=0.02.

8.5.2. Interpretation of results

In the vast majority of cases the NNRTI that was re-started was the same as the NNRTI that was interrupted. Therefore, the patterns I observed according to the NNRTI re-started can be considered to reflect those of the NNRTI that was interrupted. In a multivariable Cox proportional hazards analysis, there was an interaction between the NNRTI interruption strategy and the NNRTI that was re-started. Among patients who re-started with an efavirenz-containing regimen, those who used either a staggered- or switched-strategy to interrupt their NNRTI initially had a higher chance of reaching viral re-suppression than patients who used a simultaneous-interruption approach. Of note, fewer patients who re-started on a nevirapine-containing regimen reached HIV RNA re-suppression compared to patients who re-started on efavirenz, irrespective of the interruption strategy.

In general, I have shown that interruption from an NNRTI containing regimen does not always result in HIV RNA re-suppression once therapy is resumed. This study has provided some evidence that, if a patient is interrupting an efavirenz-containing regimen, it is particularly important not interrupt all ARVs simultaneously, but to undergo a staggered- or switched-interruption instead. This could well be the case for nevirapine as well, even though it was not highlighted as strongly in this study.

Drug resistant variants have been shown to emerge during periods of increased HIV-1 replication, when drug concentrations are suboptimal[295;437-440]. Although the absolute number of mutations detected in this chapter was small, NNRTI mutations were observed in a relatively high proportion of patients two months after a treatment-interruption. Even though we cannot exclude the possibility that these mutations were present before therapy interruption, these results emphasise the need for treatment-interruptions to be performed with caution (and only when essential), in order to avoid the emergence of NNRTI-specific mutations and compromise future treatment options.

Among patients who re-started therapy, significantly fewer patients who had a mutation in their resistance test achieved viral re-suppression (18 of 26 (69.2%)) compared to those who did not have any mutations in their resistance test (65 of 75 (86.7%)), p=0.05, re-emphasising the need to interrupt NNRTI-based regimens wisely.

Overall, the absolute number of reverse transcriptase and protease mutations that were detected in this population was small for patients interrupting an NNRTI using each interruption method. NNRTI-mutations were less likely to arise after a staggered- or switched-interruption compared to patients with a simultaneous-interruption, but the number of patients in whom resistance mutations were detected was too small for any of the differences to reach statistical significance. Although the trend was not significant, a staggered or switched-interruption was still associated with nearly half the odds of detecting an NNRTI mutation in the predominant virus population compared to a simultaneous-interruption: OR (95% CI): 0.57 (0.21 to 1.53).

Patients who underwent a staggered- or switched-interruption experienced higher levels of PI mutation detection than patients with a simultaneous-interruption, yet still PI-specific mutations were only seen in the predominant virus of only two patients who switched to a PI-containing regimen. It is not known if this is attributable to the occurrence of new mutations or to the re-appearance of previously existing PI mutations that emerged as a result of re-using a PI-containing regimen.

Patients who withdraw consent to continue in trials or become lost to follow up are likely to remain off treatment until they return to clinical care. These data give us some insight into the risk of mutation-development (and hence, the prevalence of resistance) in patients who drop out of ARV programmes in developing as well as developed countries. They also indicate the likelihood that these patients will experience virological suppression if a similar regimen is re-started.

8.5.3. Limitations of the analysis

NNRTI resistance emergence has been associated with NNRTI drug levels prior to the treatment-interruption, with drug concentrations close to the lower limit of the therapeutic range associated with more resistance emergence[431]. Additional information on drug concentrations as a result of different methods of treatment-interruption in the SMART study would help to identify the impact of these strategies on drug exposure, but these data were not available in this study. In contrast to the observations by Darwich *et al* it is possible that patients with high NNRTI drug concentrations at the time of stopping all of their ARVs simultaneously are at higher risk of NNRTI drug resistance mutations emerging than patients with drug concentrations that are closer to the lower limit of the therapeutic range.

This analysis only looks at the risk of resistance emergence after a first treatment-interruption in SMART. Detailed information on treatment interruptions prior to

enrolment in SMART is not available for these patients. Repeat interruptions of the same regimen could increase the risk of resistance emergence[437]. Some patients show little evidence of resistance emergence in their predominant virus population during a first interruption, but an elevated risk after a second or third interruption of the same regimen[437]. Conversely, Arnedo-Valero *et al* showed that the number of mutations did not increase with the number of interruptions[441]. In SMART, patients could re-initiate the same regimen after their treatment-interruption, but since the study was terminated prematurely, it was not possible to examine the relationship between mutation emergence and further interruption-cycles in more detail.

It is not known whether the mutations observed in this study are attributable to the occurrence of new mutations or to the re-appearance of previously existing mutations that emerged as a result of a previous regimen. Since all of these patients had a virus that was virologically suppressed at baseline it was not possible to perform genotypic resistance testing at entry and so there is no information on what mutations were present prior to enrolment. The use of highly sensitive assays suggests that drug-resistant viruses can be selected and replicate during suppressive antiretroviral therapy[434], so mutations may already be present in the virus at baseline. As a result mutations that emerge during follow-up may already be present in more minor sub-species at baseline; alternatively, they may be newly developed mutations.

In a similar way to all of the comparisons I have performed so far, this study used population sequencing rather than more sensitive methods for identifying mutants that are present as minority viral strains, for assessing drug resistance. If ultra-sensitive genotyping (e.g. real time PCR, clonal or single genome sequencing) analyses had been used, more NNRTI mutations (i.e. low frequency drug resistant variants) may have been detected[442]. Patients may appear to have wild-type strain at the time of their resistance test even if mutations in proviral DNA are present[439]. It is, therefore, possible that our data under-estimate the incidence of resistance mutations as a result of the treatment interruption[439].

Viral re-suppression may be a better indicator of whether resistance has emerged during the treatment interruption compared to directly looking for resistance. While, theoretically, there is a period of time after the interruption when NNRTI resistance can emerge and grow, the wild-type virus is often likely to take over instead. It may take a while for the wild-type virus to be suppressed in order to allow the resistant virus to become dominant after the same regimen is re-started.

8.5.4. Conclusion

To conclude, the method of terminating an NNRTI and the NNRTI re-started may impact on the chances of viral re-suppression on an NNRTI-containing regimen after a treatment-interruption. NNRTI mutations were observed in a relatively high proportion of patients two months after a treatment-interruption. While no significant differences were observed between stopping strategies in the number, type or class of mutation that emerged, there was a consistent trend for more mutations to emerge when a simultaneous-interruption strategy was used.

The simultaneous-interruption of all ARVs in a suppressive NNRTI/NRTI containing regimen may negatively impact on the response to the future use of ARVs due to the emergence of resistance mutations. These data provide further evidence for negative consequences of interrupting an NNRTI-containing cART regimen and further support the recommendation to avoid interruption entirely. Where such interruptions are essential, these data suggest that a staggered- or switched- approach when interrupting an NNRTI may reduce the risk of resistance emergence compared to a simultaneous strategy.

CHAPTER 9: CONCLUDING REMARKS

9. General discussion

Current treatment regimens are highly effective at reducing viral replication, improving the immune function and extending the life-expectancy of individuals infected with HIV. Since 1996 the us\e of combination therapy has led to a dramatic decline in the risk of HIV-related morbidity and mortality. As a result, in areas where this therapy is available, HIV is now considered to be a chronic condition rather than a fatal disease. Yet, over 25 years since the discovery of HIV, we have still not found a cure for it. This is because HIV cannot be expunged from latently infected cells - there are continual low levels of viral replication and HIV remains present in the pro-viral DNA of resting $CD4^+$ T cells. So, HIV infected patients are likely to remain infected for the rest of their lives

Even though UNAIDS reduced its estimates of global infections by over six million (from 39.5 million to 33.2 million) between November 2006 and November 2007, the latest figures reflect improvements in country data collection and analysis, as well as a better understanding of the natural history and distribution of HIV infection, rather than an actual decrease in the global prevalence rates of HIV infection. The total number of people living with HIV is still increasing because the number of new HIV infections is rising and individuals who are infected with HIV are surviving for longer. Since access to treatment is continuing to improve in many areas of the world, resistance emergence to available drugs and the transmission of resistance-containing viruses are two key public health concerns related to how best to manage HIV-infected populations globally in the future.

9.1.1. Summary of main findings

In chapter 3 I examined the emergence of new PR mutations in the virus population of patients who were failing a PI/r containing regimen. Regimens that included a ritonavir boosted PI were increasing in popularity when this thesis was started. At the time, it was thought that ritonavir boosting would ensure successful viral suppression so large amounts of resistance emergence would be prevented. New PI mutations associated with reduced viral efficacy emerged in the predominant virus of patients who failed a regimen containing any of the PI/rs studied in the MaxCmin and COLATE trials (i.e. IDV/r, SQV/r and LPV/r), but this analysis was substantially under-powered to identify any clinically meaningful differences in the rates of mutation emergence if they truly existed. Although only nine patients had a virus that acquired a new primary PI mutation, the odds of acquiring a new mutation was nearly four-fold higher if a patient already had a primary PI mutation present in their virus at baseline.

In this analysis 59% of the patients were PI-experienced prior to study entry and so may have had PI mutations present in their baseline virus, even if they were not identified in their baseline resistance test (i.e. they may have been present in more minor species). In addition, PI-naïve patients with HIV RNA <500 copies/mL at baseline may have also had unidentified resistance mutations in their protease gene because the gene could not be sequenced due to the low viral load. The mutations that were identified in the follow-up resistance tests may be new mutations that occur due to sub-optimal use of their treatment regimen, or they may reflect the re-emergence of a previously existing mutation. This chapter highlighted some of the problems of dealing with viral sub-species that are present in low levels (including virus circulating in plasma), which is a problem with the resistance testing methods used in all of the chapters.

In chapters 4 and 5 I examined baseline resistance mutations for predicting viral load response in patients who were recruited to the MaxCmin and COLATE trials and EuroSIDA. I assumed that mutations that were sampled in the majority virus at baseline reflected the virus over the first 12 weeks of follow-up. In chapter 3, I investigated resistance emergence in the predominant viral population of patients who were failing a PI/r containing regimen. Since these patients are patients who have the highest risk of experiencing resistance emergence and showed little resistance emergence between baseline and the time of failure, the assumption that baseline resistance mutations reflect mutations over the short-term (in chapters 4 and 5 where patients are at a lower risk of mutation emergence) is likely to be valid.

GISs are used to translate genotypic data into a resistance score that can be applied in clinical practice. Many GISs have been created, and there are disagreements between them on what mutations confer resistance to certain ARVs, especially for PI/rs[443]. In these trials I found moderate concordance between the baseline PI/r resistance levels ascribed by four GISs. By comparing different GISs it is possible to improve our knowledge on the relationship between specific mutations and viral load response under drug pressure from a particular ARV. This analysis highlights that there are some mutations which are incorporated into certain GISs but omitted by others. However, although there were differences in the interpretation of the genotypic data by each of the GISs, there were negligible differences in their abilities to predict HIV RNA response after four and 12 weeks follow-up. GISs need to be continually updated with the latest developments on resistance emergence to existing ARVs and to new ARVs that enter clinical practice.

In chapter 6, I investigated nucleotide distances over follow-up in the COLATE study. Nucleotide distances were compared: between patients who received 3TC and those who stopped 3TC; and also according to the presence of the M184V mutation. The presence of this mutation was associated with a decrease in the number of nucleotide changes that occurred in the major virus population over follow-up, which was not likely to be explained by a shift to an archived sub-population. These findings add support to other studies that have found a decrease in the replication capacity and an increase in the fidelity of viruses containing the M184V mutation. However, it is not possible to say with certainty that the results in this chapter reflect the same virus evolving over time. This is partly due to the sampling methods that were used in this chapter and the changes that occurred in the viral populations over follow-up (such as a shift to an archived sub-species). Even though slight differences in nucleotide distances were observed between viruses containing M184V compared to viruses in which this mutation was not present, the short-term virological benefit of retaining this mutation was not apparent in these patients, possibly because they were also receiving several active antiretroviral drugs during follow-up. Current treatment guidelines recognise that continued use of lamivudine in patients harbouring viruses containing 184V may be of benefit, and this is usually done in clinical practice.

In chapter 7 I investigated the relationship between resistance mutations (specific mutations and the class of mutation) and immunological response in patients with ongoing viraemia, after adjusting for HIV RNA levels and HIV RNA changes. In this analysis there was a trend towards more favourable $CD4^+$ T cell count responses if a PI mutation was present in the virus and less favourable $CD4^+$ T cell count responses if an NNRTI mutation was present. One important limitation of this analysis is that it was not possible to differentiate between the residual effects of the antiretroviral drug that may have selected for the specific mutation. However, since NNRTIs have a lower genetic barrier to resistance emergence than PIs, the emergence of a single NNRTI mutation is likely to be more virologically and immunologically damaging than the emergence of a PI mutation in patients who are using the corresponding drugs.

Patients who cannot tolerate their current cART regimen well may wish to switch one or more components or interrupt treatment completely before they experience virological failure. Patients who fail a first-line NNRTI containing regimen are significantly more likely to experience drug resistance emergence than patients who fail a first-line regimen that contains a PI/r[318]. In patients who wish to interrupt their treatment regimen, interrupting in a way that minimises the risk of prolonged exposure to one drug alone, and hence a raised risk of resistance emergence, is important for

preserving future treatment options. Interrupting NNRTIs using different strategies (i.e. a simultaneous interruption strategy, a staggered interruption strategy or by switching the NNRTI to a PI before interrupting treatment) was related to resistance emergence and viral re-suppression rates in patients who re-started therapy, in chapter 8. No significant differences were observed between stopping strategies in the number, type or class of mutation that emerged, although there was a trend for more NNRTI mutations to appear in the virus of patients who stopped all ARVs simultaneously compared to all other strategies.

Overall, treatment interruptions are not recommended, even if they are only used in patients who are failing their treatment regimen in order to allow the majority virus to revert back to wild-type. In HIV-infected patients who have had multiple virological failures and have no therapeutic options available to them, it has been shown that significant reversion of resistance mutations after a treatment interruption fails to restore antiviral efficacy of a salvage regimen, and is actually clinically harmful[173]. In a study conducted on heavily pre-treated patients, all baseline resistance mutations that disappeared during the interruption re-occurred after treatment resumption[444].

Although this thesis has highlighted some of the benefits of analysing sequence data, resistance data needs to be interpreted with caution. I have already discussed the presence of archived resistance species and how erroneous sampling methods can lead to incorrect interpretation of the data. An additional problem with interpreting resistance data concerns patients who are eligible for resistance testing. Since resistance testing is generally only possible if a patient has an HIV RNA >500 copies/mL (preferably 1000 copies/mL), the results of resistance testing cannot be extended to all patients infected with HIV. Resistance tests are usually performed on a sub-group of individuals who are starting a new treatment regimen and so will only be generalisable to a select group of individuals.

9.1.2. Resistance emergence in relation to new therapy

This thesis evaluates resistance data from observational studies and randomised trials in order to increase our understanding about the occurrence and role of drug resistance in HIV infection. With the recent introduction of fusion inhibitors, CCR5 inhibitors, integrase inhibitors and maturation inhibitors there are likely to be new drugs available for the foreseeable future. These can be used in patients who have high levels of resistance to the three main classes of ARVs because they have different resistance pathways and act on the virus at different stages of its replication cycle.

Raltegravir is the latest ARV to have been approved by the FDA. It was approved by the FDA in October 2007 and is the first integrase inhibitors to receive such approval.

In the last two years two new PI/rs have been approved – tipranavir (TPV) was approved for use in HIV infected individuals in June 2005, which was followed by the approval of darunavir (DRV) exactly a year later. Patients with high levels of resistance emergence and no treatment options available still have good virological and immunological responses on regimens containing these new drugs[323;445-448].

A recent study, performed on HIV-1-infected individuals who had no treatment options available, showed positive virological and immunological responses over 24 weeks of follow-up on a regimen containing both etravirine (the newest NNRTI to enter the market) and DRV[449]. In another study, patients with up to three mutations conferring resistance to DRV still had a 50% chance of achieving an undetectable viral load after six months of treatment with DRV[450], indicating that these newer drugs may be more resilient against mutations that occur along the *pol* gene than the older ARVs. Further, these newer drugs also appear to be well tolerated[451].

A question that still needs to be addressed is whether PI/rs, especially with the introduction of DRV, should be used in first-line therapy or reserved for later regimens. The virological impact of incorporating these new ARVs into regimens containing older ARVs or into regimens containing other new ARVs still needs to be evaluated. Resistance patterns also need to be documented.

The pursuit of new and novel treatment regimens is ongoing, in particular there is a drive to reduce the toxicity and simplify regimens, in order to reduce poor adherence and consequent resistance emergence. Single-tablet, fixed dose regimens are likely to become the treatment of choice for patients who start therapy[452].

9.1.3. Plans for future work

Although only a few mutations emerged in the predominant viral population of patients who contributed resistance data to chapter 3, there could be other areas of the HIV genome that reflect resistance to these drugs and compromise response to therapy. There is growing evidence that gag cleavage site (CS) mutations emerge under PI pressure. Already several mutations have been well characterized, and among these, the S373P mutation and the I437V mutation have been proposed as independent predictors of PI failure. Currently analyses are being performed on the combined MaxCmin and COLATE data to assess the emergence of CS mutations and whether

they have an impact on virological response in these patients. Early analysis suggests that CS mutations emerged in 25% of patients who experienced virological failure on PI/r, including 7% who did not experience the emergence of a PR mutation. In line with other reports[453], these results support the idea of introducing some mutations in the gag CS into the GISs used for the interpretation of genotypic data, although the relationship between CS mutations and virological failure still needs to be explored in more detail in this study.

At the moment there is only marginal concordance between GISs for predicting the degree of resistance to the newly approved ARVs. In a recent analysis I investigated concordance between GISs in their ascribed resistance levels to both TPV and DRV. In these analyses, concordance between GISs was fair-to-moderate for determining resistance to TPV, but it was poor for DRV; possibly reflecting the longer use of TPV and the converging of opinions on resistance mutations for this drug. The level of agreement between GISs was also shown to vary by subtype for TPV. The resistance levels ascribed by each GIS for TPV and DRV still need to be related to viral load changes in patients receiving these drugs. Longer follow-up time is required in order to provide enough power for these comparisons.

There has been little change in the frequency of resistant viral strains over time; however, there has been an increasing prevalence of non-B subtypes overall and among Caucasian individuals[454]. Because of differences in natural polymorphisms, PI resistance could be enhanced in particular subtypes once the relevant major substitutions are selected[455].

9.1.4. Summary and concluding remarks

Resistance emergence to HIV drugs is a vast area that has only been touched upon in this thesis. Although this thesis is not a comprehensive analysis of all aspects of resistance emergence, it has highlighted some of the benefits and some of the major problems associated with resistance emergence. I hope this thesis will contribute to research investigating resistance emergence to new ARVs, and help to understand the relationship between different mutations and viral replication further.

APPENDICES

Appendix I: The International AIDS Society (IAS-USA) mutation lists

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Update of the Drug Resistance Mutations in HIV-1: 2007

Victoria A. Johnson, MD, Françoise Brun-Vézinet, MD, PhD, Bonaventura Clotet, MD, PhD, Huldrych F. Günthard, MD, Daniel R. Kuritzkes, MD, Deenan Pillay, MD, PhD, Jonathan M. Schapiro, MD, and Douglas D. Richman, MD

Katholieke Universiteit Leuven
Rega Institute for Medical Research and University Hospitals Leuven
Microbiology and Immunology
Clinical and Epidemiological Virology

Kristel Van Laethem[1,2], Koen Deforche[1], Anna Maria Geretti[4], Ricardo Camacho[3] and Anne-Mieke Vandamme[1]

[1] Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium
[2] AIDS Reference Laboratory, University Hospitals Leuven, Leuven, Belgium
[3] Molecular Biology Laboratory, Centro Hospitalar de Lisboa Ocidental, Lisbon, Portugal
[4] Royal Free Hospital and RF&UC Medical School, London, United Kingdom

www.kuleuven.ac.be/rega/cev/links
kristel.vanlaethem@uz.kuleuven.ac.be

Algorithm for the use of genotypic HIV-1 resistance data
Rega v7.1.1 ©, Leuven, 5 July 2007

Appendix III: The MaxCmin Study CRFs

The MaxCmin Trial – CRF v. 1.0

Appendix IV: Statistical Methods

Regression models

Linear regression:

Linear regression models are used when we wish to predict the value of a continuous variable, y, known as the *dependent* or *outcome* variable, from a set of *explanatory* or *independent* variables; $x_1, x_2, x_3, \ldots, x_n$. We estimate the magnitude of the effect of the independent variables on the dependent variable using a sample of observations from the population of interest. We assume that the association takes the following form:

$$y = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ldots + \beta_n x_n + \varepsilon$$

The random error term, ε, for each observation is assumed to be independent of all other error terms and comes from a normal distribution with expected value (mean) of zero, and a variance of σ^2; i.e. ε are mutually independent and are all $\sim N(0, \sigma^2)$.

Estimates of the parameters $\alpha, \beta_1, \beta_2, \ldots, \beta_n$, are usually obtained using the method of least squares or the maximum likelihood methods[456]. The parameter estimate of α predicts the value of y for an observation where all independent variables $x_1, x_2, \ldots, x_n$ are equal to zero. The parameter estimate for β_i gives the impact of a unit increase in x_i on y when all other independent variables are kept constant.

The impact of the explanatory factor x_i on the outcome variable y can be formally tested. Here, the null hypothesis is that there is no association between x_i and y, i.e. β_i is equal to 0. A Wald test statistic is calculated by dividing the estimate of β_i by its standard error. We compare this test statistic to the standard normal distribution to obtain a p-value. Furthermore, a 95% CI for β_i can be calculated by adding and subtracting 1.96 multiplied by the estimate's standard error to the estimate of β_i.

Censored regression analysis:

A censored variable has a large proportion of its observations at the minimum or maximum. In this thesis I have modelled HIV RNA reductions and since, in most cases, the HIV RNA value cannot be measured below the lower limit of quantification of the assay (i.e. 50 copies/mL), patients with a follow up HIV RNA measurement at the lower limit of quantification may have a greater HIV RNA decline than can be calculated.

So, to estimate the magnitude of the effect of the independent variables on the (censored) dependent variable using a sample of observations from the population of interest, we assume that the association takes the following form:

$$y^* = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ldots + \beta_n x_n + \varepsilon$$

This is the same model that is used in simple linear regression analysis, but now y^* can represent censored observations. So, in the case of HIV RNA reductions, y^* is not completely observed when $y^* < 50$ copies/mL, yet we still know that $y^* < 50$ copies/mL.

Because the censored variable is not measured over its entire range, the mean and variance of a censored variable will be biased. In addition, ordinary least squares estimates of y on x will be biased. Instead maximum likelihood estimates of the parameters $\alpha, \beta_1, \beta_2, \ldots, \beta_n$, can be obtained using other methods. Tobit models allow estimation of linear regression models when y has been subject to left censoring, right censoring or both[457].

Generalised Linear Models

Generalised linear models are a family of regression models that include linear regression and logistic regression. Similarly to linear regression, we wish to investigate the impact of a set of independent variables on a dependent variable y. However, y no longer has to be a continuously distributed variable. Generalised linear models take the following form:

$$\mu = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ldots + \beta_n x_n + \varepsilon$$

where

μ = linear predictor

$\mu = f(\theta)$

$f(\theta)$ = link function

The random error term, ε, for each individual observation is assumed to be independent of all other error terms, each has an expected value of zero and the same variance.

In the linear regression model, described in the previous section, the link function is simply equal to the value of the dependent variable, y, and the error terms are normally distributed.

Logistic regression:

The logistic regression model has been used frequently in the analyses presented in this thesis. This section will give a short summary of the statistical ideas that form the basis of the model.

Logistic regression models are used when the dependent variable, *y*, is binary (i.e. it can only take the values 0 or 1). Here, the link function is logit(θ), where θ is the probability that *y* is equal to one and logit(θ) is the log odds of θ given by the formula $\log_e(\theta/[1-\theta])$. The estimates of β_i obtained from the model give the log odds of the impact of a unit increase in x_i on the probability that y=1, and the exponential of this gives the odds ratio. So, the final model would take the form

$\text{logit}(\theta) = \log_e(\theta/1-\theta) = \alpha + \beta_1 x_1 + \beta_2 x_2 + \ldots \beta_n x_n$ where

θ is the proportion of patients with the outcome

$\exp(\alpha)$ is the odds of the outcome in patients without any of the predictors

$\exp(\beta_j)$ is the odds ratio of virological failure in patients with the predictor x_j compared to those without the predictor x_j

The reason for modelling the log odds of the outcome rather than just the proportion of patients with the outcome is that log odds can take any value, positive or negative, whereas proportions are constrained to lie between 0 and 1. When using statistical models it is better to model a quantity that is unconstrained to avoid the possibility of predicting impossible values (like proportions that are <0 or >1).

Wald tests and 95% CIs can be calculated in the same way as the linear regression case. A Wald test is simply constructed by dividing β_j by the standard error of β_j. The value of the test is then compared with a standard normal distribution to obtain a p-value. If the p-value is ≤ 0.05, it is conventionally accepted that there is enough evidence to reject the null hypothesis of no association between x_j and the outcome of interest.

The standard error of β_j can also be used to construct a 95% CI around the log odds ratio as follows:

95% C.I. for log odds of outcome = $\beta_j \pm 1.96$ x standard error of β_j

Cox proportional hazards regression models:

We are often interested in calculating the time to the occurrence of an event. When some individuals do not experience the event (and perhaps have different lengths of follow-up) then we have censored data, as individuals are still at risk of experiencing the event in the future. We use survival methods to account for these censored data[458].

For each individual, we can calculate a hazard, *h(t)*, which is the instantaneous rate of having an event at any point in time, *t*, given that the individual has not experienced the event of interest up until this time point. However, we may not be interested in the value of the hazard itself, we are often interested in the hazard ratio – the multiplicative effect that a unit increase in a factor of interest has on the hazard. Cox proportional hazard regression models take the following form:

$$h(t) = h_0(t)\exp(\beta_1 x_1 + \beta_1 x_1 + ...\beta_n x_n) \qquad t > 0$$

Here, $h_0(t)$ is the baseline or underlying hazard. Cox proportional hazard regression models make no assumptions on the form of this hazard (and thus the models are semi-parametric). However, the models make the *proportional hazards* assumption; the multiplicative impact of factor x_1 on the hazard remains constant, regardless of the current time point.

The parameters $\beta_1, \beta_2, ... \beta_n$, are estimated by maximising the partial likelihood. The estimate of $exp(\beta_i)$ gives the hazard ratio for factor x_i. We can construct hypothesis tests to assess the impact of factor x_i on the hazard and 95% confidence intervals similarly to that described for linear regression models.

Generalised Estimation Equations

Generalized Estimation Equations (GEE) are methods of parameter estimation for correlated data. In chapter 6, each patient could have more than one measurement of their nucleotide distances from baseline because a baseline resistance test, and more than one follow-up resistance test, may have been available. When data are collected on the same patient across successive points in time, these repeated observations are correlated over time. If this correlation is not taken into account then the standard errors of the parameter estimates will not be valid and hypothesis testing results will not be reproducable.

GEE was introduced by Zeger and Liang[459] as a method of estimating regression model parameters when dealing with correlated data. In addition to specifying the distribution of the dependent variable (which must be a member of the exponential family), the link function and the independent variables, to define a regression model using the GEE methodology one needs to specify the covariance structure of the repeated measurements.

In GEEs, the random error terms, ε, for each observation are no longer normally distributed with an expected value (mean) of zero, and a variance of σ^2. Instead ε are correlated using one of the following matrices[460]:

The ability of four genotypic interpretation systems to predict virological response to ritonavir-boosted protease inhibitors

Zoe V. Fox[a,b], Anna Maria Geretti[c], Jesper Kjær[a], Ulrik Bak Dragsted[a], Andrew N. Phillips[b], Jan Gerstoft[d], Schlomo Staszewski[e], Bonaventura Clotet[f], Viktor von Wyl[g] and Jens D. Lundgren[a]

Background: Limited information exists on the prognostic value of genotypic interpretation systems (GISs) for ritonavir-boosted protease inhibitors (PI/rs). We compared PI/r resistance levels ascribed by four GIS and examined their abilities to predict HIV-RNA reductions after starting a PI/r-based regimen (baseline).

Methods: Data on viraemic (HIV-RNA > 500 copies/ml) patients starting a PI/r with a baseline resistance test were combined from an observational cohort study (EuroSIDA) and three randomized trials (MaxCmin1; MaxCmin2 and COLATE). The GIS surveyed were ANRS, DMC, REGA and Stanford. Factors associated with HIV-RNA change were identified through censored regression analysis.

Results: We included 744 patients, of whom 67% were PI experienced. At baseline 12–28% (depending on the GIS) patients had a virus with predicted resistance/intermediate resistance to the PI/r initiated. Concordance between GISs on ascribed PI/r resistance levels was moderate: kappa values ranged from 0.01 to 1.00, with the lowest kappas seen for amprenavir. The median (interquartile range) baseline HIV-RNA was 4.4 (3.5–5.1) $\log_{10}$ and was reduced by 2.2 (2.1–2.3) $\log_{10}$ 12 (9–13) weeks after baseline. GIS consistently showed greater HIV-RNA reductions as the ascribed level of sensitivity to the PI/r increased. Conversely, the number of other active drugs in the rest of the regimen, according to each GIS did not predict HIV-RNA reductions consistently.

Conclusion: Despite large variations in how GIS classify HIV susceptibility to PI/r, all GIS predicted HIV-RNA reductions of a similar magnitude. The ascribed level of susceptibility to other drugs in the regimen did not predict HIV-RNA decline.

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Keywords: Concordance, genotypic data, interpretation systems, ritonavir-boosted protease inhibitors, virological response

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A randomized trial to evaluate continuation versus discontinuation of lamivudine in individuals failing a lamivudine-containing regimen: The COLATE trial

Zoe Fox[1], Ulrik B Dragsted[1], Jan Gerstoft[2], Andrew N Phillips[3], Jesper Kjaer[1], Lars Mathiesen[4], Mike Youle[5], Christine Katlama[6], Andrew Hill[7], Johan N Bruun[8], Nathan Clumeck[9], Pierre Dellamonica[10] and Jens D Lundgren[1] on behalf of the The COLATE study group*

[1]Copenhagen HIV Programme (CHIP), Hvidovre University Hospital, Copenhagen, Denmark
[2]Rigshospitalet, Copenhagen, Denmark
[3]Royal Free and University College Medical School, London, UK
[4]Hvidovre University Hospital, Copenhagen, Denmark
[5]Royal Free Centre for HIV Medicine, London, UK
[6]Hôpital de la Pitié-Salpêtrière, Paris, France
[7]Pharmacology Research Laboratories, Liverpool University, Liverpool, UK
[8]Ullevål Hospital, Oslo, Norway
[9]C.H.U. Saint-Pierre, Brussels, Belgium
[10]Hôpital de l'Archet, Nice, France
[†]See additional web file for a full list of The COLATE study group

Background: Lamivudine (3TC) therapy can cause the emergence of M184I/V. Previous studies suggest a higher fidelity of the mutant reverse transcriptase and lower replication capacity of the mutant virus. No data exist from clinical comparative studies evaluating the benefit of M184I/V in patients receiving combination antiretroviral therapy (cART).

Methods: HIV-1-infected adults failing a 3TC-containing regimen were randomized to continue (On-3TC) or discontinue 3TC (Off-3TC) whilst receiving cART. The primary efficacy measure was the $\log_{10}$ average-area-under-the-curve-minus-baseline reduction in HIV RNA over 48 weeks. Cryopreserved plasma samples from patients with baseline and ≥1 follow-up sample with HIV RNA >500 copies/ml were sequenced for a nucleotide distances substudy. Evolutionary distances were compared between treatment arms and between viruses with and without M184I/V.

Results: The overall 48-week $\log_{10}$ HIV RNA change was -1.4 (95% CI: -1.6, -1.1) for On-3TC (n=65) and -1.5 (95% CI: -1.7, -1.2) for Off-3TC (n=66; P=0.51). No difference was seen in the magnitude of the $CD4^+$ T-cell count increases (median increase: 87 vs 76 cells/ml for 3TC vs Off-3TC, respectively). Thirty-seven patients had baseline and follow-up sequencing. Overall, there were 1.2 (95% CI: -2.2, 4.6) more nucleotide substitutions from baseline for Off-3TC patients (P=0.50), and 10.7 (95% CI: 7.5, 14.0) fewer nucleotide changes in viruses containing M184I/V (P<0.0001).

Conclusion: This study found no added virological or immunological benefit of continuing 3TC in patients on cART harbouring M184I/V. Evolutionary distances from baseline were larger in viruses that did not contain M184I/V. More discernable benefits may be seen in patients with fewer drug options as potent cART may eclipse a benefit of M184I/V in COLATE.

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