UNIVERSITY OF WEST LONDON
The Career University

UWL REPOSITORY

repository.uwl.ac.uk

The impact of a UK HIV-1 resistance database for the management and improvement of the clinical care of people living with HIV-1

MacRae, Eilidh (2015) The impact of a UK HIV-1 resistance database for the management and improvement of the clinical care of people living with HIV-1. Doctoral thesis, University of West London.

This is the Accepted Version of the final output.

UWL repository link: https://repository.uwl.ac.uk/id/eprint/2859/

Alternative formats: If you require this document in an alternative format, please contact: open.research@uwl.ac.uk

Copyright:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy: If you believe that this document breaches copyright, please contact us at open.research@uwl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

THE IMPACT OF A UK HIV-1 RESISTANCE DATABASE FOR THE MANAGEMENT AND IMPROVEMENT OF THE CLINICAL CARE OF PEOPLE LIVING WITH HIV-1

EILIDH MACRAE

A thesis submitted in partial fulfilment of the requirements of the University of West London for the degree of Doctor of Philosophy

December 2015

Abstract

Background: The introduction of highly active antiretroviral therapies (HAART) in 1996 to treat patients living with the human immunodeficiency virus type 1 (HIV-1), led to dramatic improvements in their mortality and morbidity. However, high levels of adherence to HAART regimens are required and due to the very nature of HIV-1: its high replicative capacity and lack of a proof reading mechanism, drug resistance mutations emerge, which impact on the ability of the drugs to suppress the patient's circulating viruses. Genotypic resistance testing can determine whether mutations have developed which confer resistance to specific antiretrovirals (ARV) and thus enhance clinical care.

Methods: A clinical cohort database was developed to host the demographic, treatment and resistance mutation data for patients living with HIV-1 across the United Kingdom (UK) who had a genotypic resistance test (tests) conducted as part of their clinical care. These data were pooled and interrogated to determine the evolution and dynamics of resistance in targeted sub-groups of patients including treatment-naïve patients; treatment-experienced patients and their potential susceptibility to new ARV drugs; and the evolution of new subtype profiles within the clinical cohort and the impact of this on clinical outcomes. The over-riding aim of each of the studies was to improve the clinical care of patients with HIV-1 infection in the UK.

Results: In the treatment-naïve patient cohort (n=380), a resistance prevalence rate of 16.5% was determined. In the treatment-experienced cohort (n=1,786), the resistance prevalence rate was 68.1%. Of those treatment-experienced, 91.3% would be susceptible to the new ARV Etravirine (ETV) and 89.7% to Darunavir (DRV). In the subtype patient cohort (n=1,642), an increase in the prevalence of pure and recombinant non-B subtypes over time was demonstrated and characterised, as well as the identification of polymorphisms specific to non-B subtypes compared to subtype B.

Conclusions: The resistance prevalence rate of >10.0% in the treatment-naïve patient cohort supported the need to conduct genotypic resistance tests for all treatment-naïve patients with HIV-1 infection before commencement of HAART in order to ensure the patient was starting on the optimal first-line treatment regimen to control their virus. National and European guidelines were subsequently amended to reflect this requirement. The treatment-experienced patient cohort analyses confirmed the resistance mutations circulating within the treated HIV-1

community which are the source of transmitted resistance to the treatment-naïve patients. Further analyses of the treatment-experienced cohort suggested two new ARVs which were due to be licenced for use with HIV-1 patients would be "theoretically susceptible", providing further treatment options for these patients with resistance mutations. The subtype patient cohort work determined that subtype characterisation should be introduced as part of clinical care due to the impact of non-B subtypes on the success of genotypic resistance testing, and the different mutational pathways which might occur, leading to resistance in different subtypes.

All these studies provided data and evidence of current issues which impacted on the clinical care of patients living with HIV-1 in the UK and influenced changes in guidelines on how best to manage and improve patient care.

.

Acknowledgements

I would like to express my immense gratitude to Professor Clive Loveday for introducing me to the field of HIV-1 and resistance to ART. His knowledge and passion for the field and ability to explain complicated concepts to me has been a wonderful experience – I will miss our meetings and discussions very much. Thank you Clive for everything.

A huge thank you to all my family and friends who have supported me over the many years I have been undertaking this thesis! I'm not sure everyone understands the topic area and what I have been trying to achieve over the years but hopefully now on completion of my thesis it will make sense to people! Particular thanks must be extended to my beautiful Mum and Dad for their continued love and support; to the lovely Meggie Morse who was my saving grace at TVU/UWL when I needed something, and to my Kate who kindly read through my thesis and made some excellent comments and observations.

I must thank my boy, my cariad, Anthony Thomas for his support and patience and for looking after our baby girls these last few months so I could concentrate! I hope my babies Aeronwen Isabella, Gwennan Haf and Betsan Efa are proud of their Mummy and one day understand what I have achieved! I love that Gwennan has lately been referring to my PhD as my 'BFG'!

And finally, I would like to dedicate this thesis to my Granny Dingwall xXx.

Table of Contents

List of figures, tables and graphs

Figures

the sequence of sense and antisense to determine the true sequence of the clinical sample.

Figure 2.2d: Step 4_ELECTROPHORESIS: The heating of the sequencing reactions to denature the double-stranded deoxyribonucleic acid (DNA) fragments into two single-strands was undertaken on a thermocycler at 95°C.

Figure 2.2e: Step 5_DATA ANALYSIS: Bi-directional CLIP™ sequences were produced (Protease (PR), PR II, Reverse transcriptase (RT) beginning and RT middle) and interpreted by the laboratory technician to ensure consistency to produce a consensus sequence and ultimately a resistance report.

Figure 2.3: An example of a consensus sequence of a patient's protease (PR) and reverse transcriptase (RT) genotype.

Figure 2.4: A patient's mutational profile. To exemplify, the Resistance Mutation highlighted in yellow indicates: the protease base position (PR 28), the wild-type (WT) codon (CTC), the amino acid position (L10I, with 'L' indicating the amino acid WT and 'I' the amino acid substitution conferring resistance), the change in the codon from the WT with the small letters indicating a manual change in the sequence by the laboratory technician (aTc).

Figure 2.5a: Front cover of the TRUGENE® HIV-1 resistance report providing a colour co-ordinated, at-a-glance resistance summary of mutations present and the interpreted resistance level (Resistance, *Possible Resistance*, No Evidence of Resistance) per drug class.

Figure 2.5b: List of specific TRUGENE® HIV-1 resistance report rules and Evidence Basis for the interpretation of the mutations seen and the resistance level assigned, per drug (nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs)).

Figure 2.5c: List of specific TRUGENE® HIV-1 resistance report rules and Evidence Basis for the interpretation of the mutations seen and the resistance level assigned, per drug (non-nucleoside reverse transcriptase inhibitors (NNRTIs) contd. and protease inhibitors (PIs).

Figure 2.5d: Definitions of the TRUGENE® HIV-1 resistance report Resistance Effect levels and Evidence Basis levels.

Figure 2.6: An example of a patient's FASTA sequence as generated by the TRUGENE® genotype system. The first line of the sequence indicated the hospital number of the patient (M94-0099), the unique sample identifier (RV number) assigned by the ICVC's laboratory (65165), the date and time the report was generated (200401261109) and the nucleotides which were analysed: PR (protease) (10-297), RT (reverse transcriptase) (112-741). After this, the first part of the sequence represented the PR nucleotides with the long list of 'N's' confirming the transition between the PR and RT sequence.

Figure 3.1: Map showing the distribution of the clinical centres across the United Kingdom (UK) that formed the International Clinical Virology Centre (ICVC) Collaborative Research Group.

Figure 5.1: The mutational profile of one of our patients who had five resistance entries available on the ICVC Clinical Cohort Resistance Database. The boxes

highlighted in red indicate the identification of mutations that year and showed that at some time points, mutations seen previously had been archived. For example, the PI mutation M46I/L was identified in 2000, was undetected in 2001, detected again in 2002 and 2004 and undetected in 2005. The final column shows the accumulated mutations that can be found in the patient's plasma or memory cell archive since the patient started his first treatment.

Figure 6.1: Genotypic 'weighted score' of individual etravirine (ETV) mutations on virological response. In practice, a patient with an ETV weighted score of '0 to 2', would have the highest response to the drug; if a patient had an ETV score of '2.5 to 3.5' they would have an intermediate response to the drug and if a patient had a score of '≥4', they would have a reduced response (Vingerhoets et al 2008; Vingerhoets et al 2010).

Figure 7.1: Attempts to define the 'genetic fingerprint' of the non-B subtype treatment-naïve patients with >20.0% prevalence of protease (PR) mutations/polymorphisms compared with the subtype B treatment-naïve patients.

Tables

Table 1.1: Target areas in the HIV-1 lifecycle where drug classes are available to suppress viral replication.

Table 1.2: Viral load (VL) replication and number of days before mutation arises: the higher the VL, the shorter the timeframe for the development of resistance mutations.

Table 3.1: Breakdown of the International Clinical Virology Centre (ICVC) Collaborative Research Group by geographical region and city/town/borough where the clinical centre was based. The majority of the clinical centres were from the Greater London area (64.3%).

Table 3.2: Number of resistance entries per patient in the International Clinical Virology Centre (ICVC) Clinical Cohort Resistance Database showed that the majority only had one resistance data entry in the database.

Table 3.3: Completion of the resistance request form showed great variability in data provided by the clinicians requesting a genotypic resistance test for their patients.

Table 3.4: Crosstabulation showing Ethnic origin by Origin of infection by Risk exposure group. The majority of men who have sex with men (MSM) cases originated in Europe (90.0%) and were of White non-Hispanic ethnicity (92.3%). The majority of heterosexual cases were of African origin (81.2%) and classed as Black African ethnicity (77.5%). All injecting drug users (IDU) cases (n=19) originated in Europe and were of White non-Hispanic/White Hispanic ethnic origin.

Table 3.5: Year of diagnosis, by sex, for patients in the International Clinical Virology Centre (ICVC) Clinical Cohort Resistance Database showed frequency of female infection was delayed, compared to males, but showed expansion since 1998.

Table 3.6: Major reasons for requesting a resistance test.

Table 3.7: Sample and/or most recent viral load (VL) data.

Table 3.8: Most recent CD4 cell count data showed that 38.3% of patients had CD4 counts below 200 cells/µL with a total of 67.8% of patients with a CD4 count ≤350 cells/µL; as per the British HIV Association (BHIVA) treatment guidelines (Gazzard on behalf of the BHIVA Treatment Guidelines Writing Group 2008), these patients should be taking ART.

Table 3.9: Adherence comment: of those patients currently taking antiretroviral therapy (ART), it was indicated the majority of patients (78.0%) had excellent/reasonable adherence to their ART regimens.

Table 3.10: Frequencies of each of the antiretroviral drugs by current antiretroviral therapy (ART) usage and previous drug experience; each of the antiretroviral drugs showed wide use of nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) and less use of the protease inhibitors (PIs).

Table 3.11: Number of nucleoside reverse transcriptase inhibitors (NRTIs) in a patient's regimen: including those currently taking and with previous experience of taking NRTIs.

Table 3.12: Number of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in a patient's regimen: including those currently taking and with previous experience of taking NNRTIs.

Table 3.13: Number of protease inhibitors (PIs) in a patient's regimen: including those currently taking and with previous experience of taking PIs.

Table 4.1: Treatment-naïve patients derived from 26 of 39 denominator hospitals with the majority from clinical centres within the Greater London geographical region (64.2%).

Table 4.2: Prevalence of primary HIV-1 infection (PHI), chronic-naïve (CN), pregnant and treatment-naive patients with resistance, by year.

Table 4.3a: Viral load and evidence of resistance.

Table 4.3b: Viral load (VL), evidence of resistance (R) to the non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs); and presentation as primary HIV-1 infection (PHI), chronic-naive (CN) or pregnant and treatment-naïve.

Table 4.4: The persistence of resistance associated mutations circulating in a patient's virus population (sample date minus date of diagnosis) by drug class (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs)). The table illustrates some of these related mutations can persist for >12 months.

Table 4.5: Preferred first-line antiretroviral therapy (ART) regimens from the British HIV Association (BHIVA) Guidelines for the treatment of HIV-infected adults with antiretroviral therapy: a combination of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitors (NNRTI) was preferred (Gazzard on behalf of the BHIVA Writing Committee 2005).

Table 5.1: Frequencies of antiretroviral drugs by current usage and previous experience; showed wide use of nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) and less use of protease inhibitors (PIs).

Table 5.2: Comparison of the prevalence of major mutations in the early cohort (EC, 1996-2000) and the late cohort (LC, 2001-2006) by drug classes affected. There was a decrease in the frequency of major mutations impacting all three drugs classes between the EC (14.9%) and LC (9.2%); and an overall decrease in major mutations affecting two drugs classes (EC: 43.0%, LC 27.5%), particularly the nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs) drugs classes between the EC (22.5%) and LC (4.6%).

Table 5.3: Major mutations and drug class affected as per the Geographical regions where the 39 clinical centres were based. Major mutations were identified across all regions, impacting all three drugs classes.

Table 5.4: Prevalence of specific nucleoside reverse transcriptase inhibitors (NRTI) “lifetime mutations” in the treatment-experienced clinical cohort. M184V was the most common mutation, followed by T215Y and M41L.

Table 5.5: Prevalence of specific non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations in the treatment-experienced clinical cohort. K103N was the most common mutation, followed by Y181C.

Table 5.6: Prevalence of specific protease inhibitor (PI) mutations in the treatment-experienced clinical cohort. L90M was the most common major mutation, followed by M46I and V82A. L63P was the most prevalent minor mutation, followed by M36I and L10I.

Table 5.7: Comparison of the prevalence of major mutations by the type of analysis: year by year or accumulative; by drug classes affected. Year by year analyses show the number of patients with major mutations in that year. Cumulative analyses reflect the accumulation of major resistance mutations over the years.

Table 6.1: Demographics of patients currently failing (CF) or with previous experience (PE) of non-nucleoside reverse transcriptase inhibitors (NNRTIs) containing regimens.

Table 6.2: The number of non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations that were identified in the cohort and the breakdown of these by the number of etravirine (ETV) specific mutations that were seen in all patients; those who were currently failing (CF) on a NNRTI regimen (n=550) and those with previous experience (PE) of NNRTI usage (n=417).

Table 6.3: The prevalence of specific etravirine (ETV) related mutations seen in all non-nucleoside reverse transcriptase inhibitors (NNRTI) experienced patients; currently failing (CF) and with previous experience (PE).

Table 6.4: The weighted score of 31 patients (22 were currently failing (CF) on a non-nucleoside reverse transcriptase inhibitor (NNRTI) containing regimen, nine had previous experience (PE) with a NNRTI-containing regimen) identified with ≥3 etravirine (ETV) related mutations, and their likely clinical response to ETV

(Vingerhoets et al 2008; Vingerhoets et al 2010). Thirty out of the 31 patients (96.8%) would have a reduced response to ETV.

Table 6.5: Demographics of patients currently failing (CF) or with previous experience (PE) of protease inhibitors (PIs) containing regimens.

Table 6.6: The number of protease inhibitors (PI) mutations that were identified in the cohort and the breakdown of these by the number of darunavir (DRV) specific mutations that were seen in all patients; those who were currently failing (CF) on a PI regimen (n=532) and those with previous experience (PE) of PI usage (n=353).

Table 6.7: Prevalence of specific darunavir (DRV) related mutations and the combinations of mutations in those patients with more than one related mutation.

Table 6.8: Background prevalence of protease mutations in the case and control groups. The case group on average had more protease inhibitor (PI) mutations (6.45 PI mutations) than the control group (2.07 PI mutations), ($p<0.001$).

Table 6.9: Virtual phenotype analyses of the case group - the 'maximal response' finding of 86.6% was comparable with the genotype finding of 88.7% susceptibility (not significant (NS)).

Table 6.10: Review of the 12 case group patients with a 'reduced response' as per the virtual phenotype analyses; in relation to the darunavir (DRV) related mutations and combinations, and genotype interpretation.

Table 6.11: Comparison of the demographics, treatment history and mutation profiles of the minimal response patient and the reduced response patient who both presented with five darunavir (DRV) related mutations: V32I+L33F+I47V+I54L+I84V.

Table 6.12: Virtual phenotype analyses of the control group: the maximal response finding of 100.0% supported the genotype analyses findings of no darunavir (DRV) related mutations.

Table 7.1: Prevalence of specific pure subtypes and circulating recombinant forms (CRFs) within the treatment-naïve and treatment-experienced clinical cohort populations. Overall, 52.6% of patients were subtype B, 38.0% of patients had a pure non-B subtype and 9.2% of patients had a CRF.

Table 7.2: Overall, the majority of patients in the North West, Wales and East Midlands geographical regions were characterised with subtype B viruses; a greater number of patients in the South West and Eastern regions were characterised with non-B subtypes/circulating recombinant forms (CRFs); whilst the prevalence of subtype B/non-B/CRFs were in total, comparable across the South East and Greater London regions.

Table 7.3: Significant protease (PR) mutations associated with non-B subtypes compared to subtype B in the treatment-naïve and treatment-experienced patients, $p<0.05$.

Table 7.4: Significant protease (PR) mutations associated with subtype B compared to subtype non-B/CRF in the treatment-naïve and treatment-experienced patients, $p<0.05$.

Graphs

Graph 4.1a: Non-nucleoside reverse transcriptase inhibitors (NNRTIs), associated mutations and prevalence of resistance over the study (and presentation as primary HIV-1 infection (PHI), chronic-naive (CN) or pregnant and treatment-naïve). K103N was the most prevalent of the NNRTI mutations impacting use of the whole of the NNRTI drug class. V179D mutation was the next most frequent mutation which translated as possible resistance to all of the NNRTIs.

Graph 4.1b: Nucleoside reverse transcriptase inhibitors (NRTIs), associated mutations and prevalence of resistance over the years (and presentation as primary HIV-1 infection (PHI), chronic-naive (CN) or pregnant and treatment-naïve). The graph depicts low frequencies of NRTI mutations with treatment options within this drug class available to the majority of the patients with these mutations. However, for the patient with four NRTI resistance mutations (K70R+M184V+T215Y+K219E) the accumulation of these mutations impacts a number of the specific drugs available within this class.

Graph 4.1c: Protease inhibitors (PIs), associated mutations and prevalence of resistance over the years (and presentation as primary HIV-1 infection (PHI), chronic-naive (CN) or pregnant and treatment-naïve). The graph depicts low frequencies of PI mutations with other PI treatment options within this drug class available to the majority of the patients with these mutations.

Graph 5.1: Percentage of patients, per year, identified with major mutations (muts) which conferred resistance to the antiretroviral therapies within one drug class, two drugs classes or all three drugs classes. The graph also shows the frequency of minor protease inhibitor (PI) mutations over the years with a clear increase from 1997 to 2006.

Graph 5.2a: Prevalence of thymidine analogue mutations (TAMs) and M184V (major nucleoside reverse transcriptase inhibitors (NRTI) mutations) from 1996 to 2006. Pattern shows suboptimal treatment in 1996-1997 (stavudine (d4T)/zidovudine (ZDV)). From 1998 onwards, the prevalence of the NRTI mutations decreased as highly active antiretroviral therapy (HAART) was successful in suppressing replicating virus.

Graph 5.2b: Prevalence of other major nucleoside reverse transcriptase inhibitors (NRTI) mutations (contd.) from 1996 to 2006. The frequency of these NRTI mutations was generally lower (<10.0%) compared with the prevalence of the thymidine analogue mutations (TAMs) and M184V illustrated above.

Graph 5.3a: Prevalence of major non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations from 1996 to 2006. K103N was the most prevalent major NNRTI mutation overall, followed by Y181C and G190A.

Graph 5.3b: Prevalence of other major non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations (contd.) from 1996 to 2006. Compared with Graph 5.3a these major NNRTI mutations were prevalent at much lower rates (<7.0%).

Graph 5.4a: Prevalence of major protease inhibitor (PI) mutations from 1996 to 2006. All mutations excluding M46I had a prevalence of <10.0%, with a clear decline to approximately 2001 and stability afterwards reflecting the use of more potent PIs.

Graph 5.4b: Prevalence of major protease inhibitor (PI) mutations (contd.) from 1996 to 2006. Prevalence of these PI mutations peaked in 1997/1998 and declined to <10.0% (as per the PI mutations in the graph above) from 2001 onwards.

Graph 5.5a: For 298 treatment-experienced patients (pt's) with more than one genotypic result available (n=725 results), the graph shows the percentage of pt's per year, identified with major mutations (muts) which conferred resistance to the antiretroviral therapies within one drug class, two drugs classes or all three drugs classes. The graph also shows the frequency of minor protease inhibitor (PI) mutations over the years.

Graph 5.5b: For 298 treatment-experienced patients (pt's) with more than genotypic result available (n=725), the graph shows the accumulation of major mutations (muts), year on year, which conferred resistance to the antiretroviral therapies within one drug class, two drugs classes or all three drugs classes; alongside minor protease inhibitor (PI) mutations over the years.

Graph 6.1: Shows the effect of an increased number of etravirine (ETV) mutations on the virological suppression of a patient's virus: the greater the number of baseline ETV mutations, the fewer the patients with undetectable viral load (VL) (Katlama et al 2007a).

Graph 7.1: Non-B subtypes and circulating recombinant forms (CRFs) characterised in the early cohort (1996-2000): subtype C was the predominant non-B subtype (50.0%).

Graph 7.2: Non-B subtypes and circulating recombinant forms (CRFs) characterised in the late cohort (2001-2006): there was a significant expansion in non-B subtypes and CRFs compared with the early cohort.

Abbreviations

A	
A	adenine
ABC	Abacavir
AIDS	acquired immune deficiency syndrome
APV	Amprenavir
ART	antiretroviral therapy
ARV	antiretrovirals
ATV	Atazanavir
AZT	Azidothymidine
B	
BHIVA	British HIV Association
BVM	Bevirimat
C	
C	cytosine
CA	capsid
CCR5	C-C chemokine receptor type 5
CDC	Centers for Disease Control and Prevention in America
cDNA	complementary deoxyribonucleic acid
CF	currently failing
c/mL	copies per millilitre
CN	chronic-naive
CPA	Clinical Pathology Accredited
CRF	circulating recombinant forms
cRNA	carrier ribonucleic acid
CXCR4	C-X-C chemokine receptor type 4

D

d4T	Stavudine
ddC	Zalcitabine
ddI	Didanosine
DGH	district general hospital
DLV	Delavirdine
DNA	deoxyribonucleic acid
DNTPs	deoxyribonucleotides
DRV	Darunavir
DTG	Dolutegravir
DTT	Dithiothreitol

E

EC	early cohort
EFV	Efavirenz
EMEA	European Medicines Agency
env	*envelope*
ER	endoplasmic reticulum
ETV	Etravirine
EVG	Elvitegravir

F

FDA	The Food and Drug Administration
FPV	Fosamprenavir
FTC	Emtricitabine

G

G	guanine
gag	*group-specific antigen*
gp	glycoprotein
GRID	gay-related immune deficiency

GT	Genotype
H	
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HPA	Health Protection Agency
HTLV-III	human T-cell lymphotropic virus type 3
I	
IAS-USA	International Antiviral Society-United States of America (formerly International AIDS Society-United States of America)
ICO	Information Commissioner's Office
ICVC	International Clinical Virology Centre
IDU	injecting drug user
IDV	Indinavir
IN	Integrase
K	
KS	Kaposi's sarcoma
L	
LA	Los Angeles
LAV	lymphadenopathy-associated virus
LC	late cohort
LPV/r	Lopinavir/boosted with ritonavir
LTR	long terminal repeat sequence
M	
MA	matrix

mL	millilitre
MRC	Medical Research Council
mRNA	messenger ribonucleic acid
MSM	men who have sex with men
MVC	Maraviroc
N	
NAM	National AIDS Manual
NC	nucleocapsid
NCI	National Cancer Institute in America
nef	negative regulatory factor
NFV	Nelfinavir
NHS	National Health Service
NRTI	nucleoside reverse transcriptase inhibitor
NNRTI	non-nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
NYC	New York City
O	
OI	opportunistic infection
P	
PCP	*Pneumocystis jiroveci* (*carinii*) pneumonia
PCR	polymerase chain reaction
PE	previous experience
PEP	post-exposure prophylaxis
PHE	Public Health England
PHI	primary HIV infection
PI	protease inhibitor
pol	*polymerase*
PR	protease

PT	Phenotype
R	
R	resistance
RAL	Raltegravir
rev	regulator of expression of virion particles
RNA	ribonucleic acid
RNase H	ribonuclease H
RPV	Rilpivirine
RT	reverse transcriptase
RT-PCR	reverse transcription and polymerase chain reaction
RTT	Research Think Tank
RTV	Ritonavir
S	
SPSS	Statistical Package for the Social Sciences
SQV	Saquinavir
T	
T	thymine
TAMs	thymidine analogue mutations
tat	trans-activator of transcription
TDF	Tenofovir
TPV	Tipranavir
T-20	Enfuvirtide
U	
UK	United Kingdom
UNAIDS	Joint United Nations Programme on HIV/AIDS
USA	United States of America

V	
vif	viral infectivity factor
VL	viral load
vpr	viral protein R
vPT	virtual phenotype
vpu	viral protein unique
W	
WHO	World Health Organization
WT	wild-type
Z	
ZDV	Zidovudine
Other	
3TC	Lamivudine
µL	microlitre
~	approximately

Chapter 1: Introduction

Emergence of a new virus

In 1981, a new syndrome was reported in previously healthy, young, homosexual men in Los Angeles (LA) and New York City (NYC) with cases of *Pneumocystis jiroveci* (*carinii*) pneumonia (PCP) and/or Kaposi's sarcoma (KS), (Centers for Disease Control and Prevention in America (CDC) 1981a; CDC 1981b; CDC 1982; Masur et al 1981). Previously, such infections were uncommon and only seen in people whose immune systems were immunocompromised. The first cases of PCP reported in LA were accompanied by an editorial surmising 'the fact that these patients were all homosexuals suggests an association between some aspect of a homosexual lifestyle or disease acquired through sexual contact…in this population' (CDC 1981a).

On realisation of an increase in reporting of such cases to the CDC, as well as an increase in the incidence of requests for controlled pentamidine to treat PCP, the CDC formed a Task Force on KS and opportunistic infections (KS/OI) to determine what was happening in the community (CDC Task Force on KS/OI 1982). Initially, members of the Task Force (Epidemic Intelligence Service Officers) interviewed the men with these syndromes and theorised these likely occurred as a result of immune suppression due to sexually transmitted diseases, immunosuppression associated with poppers or a sexually transmitted agent, or immune overload. The Task Force conducted a case-control study to try and determine possible causes of the KS/OI syndromes. The case participants were all men who had sex with men (MSM) with evidence of PCP and/or KS, and the control participants were healthy MSM, matched by age, race and city of residence (Jaffe et al 1983; Rogers et al 1983). Analysis of the data suggested increased sexual activity, with multiple partners, was a mitigating factor.

This new syndrome was generally referred to by the CDC as KS/OI, with others calling it 'gay compromise syndrome' (Brennan et al 1981) or 'gay-related immune deficiency' (GRID, CDC 1982). It quickly became evident however that these uncommon infections were not just related to a 'homosexual lifestyle', with

cases identified in injecting drug users ((IDUs), Masur et al 1981), haemophiliacs (CDC 1982a), those who had received blood transfusions and blood products (CDC 1982c), mother-to-child transmission (CDC 1982d) and likely heterosexual transmission (CDC 1983). The term acquired immune deficiency syndrome (AIDS) was universally adopted, with the CDC publishing an official definition in September 1982 (CDC 1982b).

In 1983, the Institute Pasteur in France reported they had isolated a new virus, which might be the cause of AIDS, which they termed lymphadenopathy-associated virus (LAV), (Barre-Sinoussi et al 1983). Meanwhile, a group of scientists from the National Cancer Institute in America (NCI) announced they had isolated a virus which caused AIDS, and named it 'human T-cell lymphotropic virus type 3' (HTLV-III), (Gallo et al 1984). Another group from San Francisco reported on AIDS-associated retroviruses as the cause of AIDS (Levy et al 1984). It became evident from detailed sequencing that LAV, HTLV-III and AIDS-associated retroviruses were the same virus; these multiple nomenclatures were rationalised in 1986 by the International Committee on the Taxonomy of Viruses who appointed the unifying name human immunodeficiency virus (HIV), (Coffin et al 1986).

A scientific dispute ensued between the Institute Pasteur research group in France (led by Professor Luc Montagnier) and the American research group at the NCI (led by Dr Robert Gallo) as to which group was the first to isolate HIV (LAV/HTLV-III) as the virus which caused AIDS, as well as to design and develop a commercial blood test that screened for antibodies to HIV. An agreement of the 'chronology of AIDS research' was published in 1987 with a supporting statement from both the Montagnier and Gallo research groups and it was deemed that the blood test would be regarded as a 'joint invention' (Gallo and Montagnier 1987; Palca 1987).

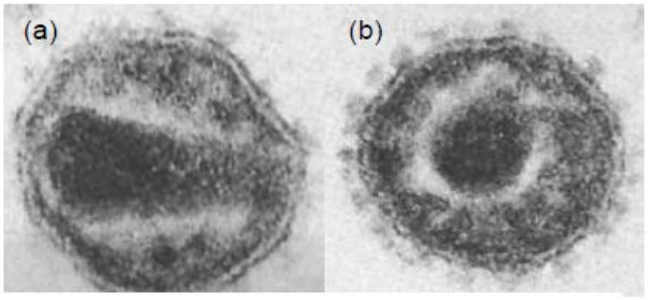
In 1986, a second HIV virus was identified and characterised by the Institute Pasteur with the original virus named HIV type 1 (HIV-1) and this new virus HIV

type 2 (HIV-2), (Clavel et al 1986). HIV-1 is the most common and prolific of the viruses and is more prevalent worldwide. HIV-2, although genetically similar but distinct from HIV-1, is associated with West Africa and countries which have colonial links with West Africa (Clavel et al 1986). HIV-1 will be the focus of this research thesis.

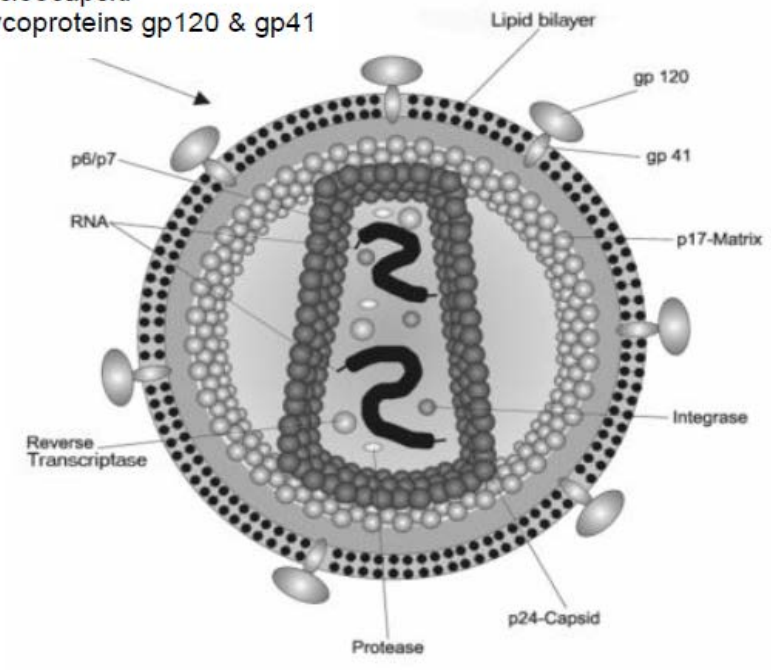
The human immunodeficiency virus type 1 (HIV-1)

HIV-1 was classified as a member of the *Lentivirus* genus which is part of the family of *Retroviridae* (retroviruses).

Figure 1.1: HIV-1 virions (Figure used with kind permission from Prof Clive Loveday and adapted by myself).



(a) clearly shows nucleocapsid
(b) clearly shows glycoproteins gp120 & gp41



A HIV-1 virion (as depicted in Figure 1.1) has an outer bilipid envelope with the glycoproteins (gp) gp120 and gp41 implanted. Within the envelope, the viruses'

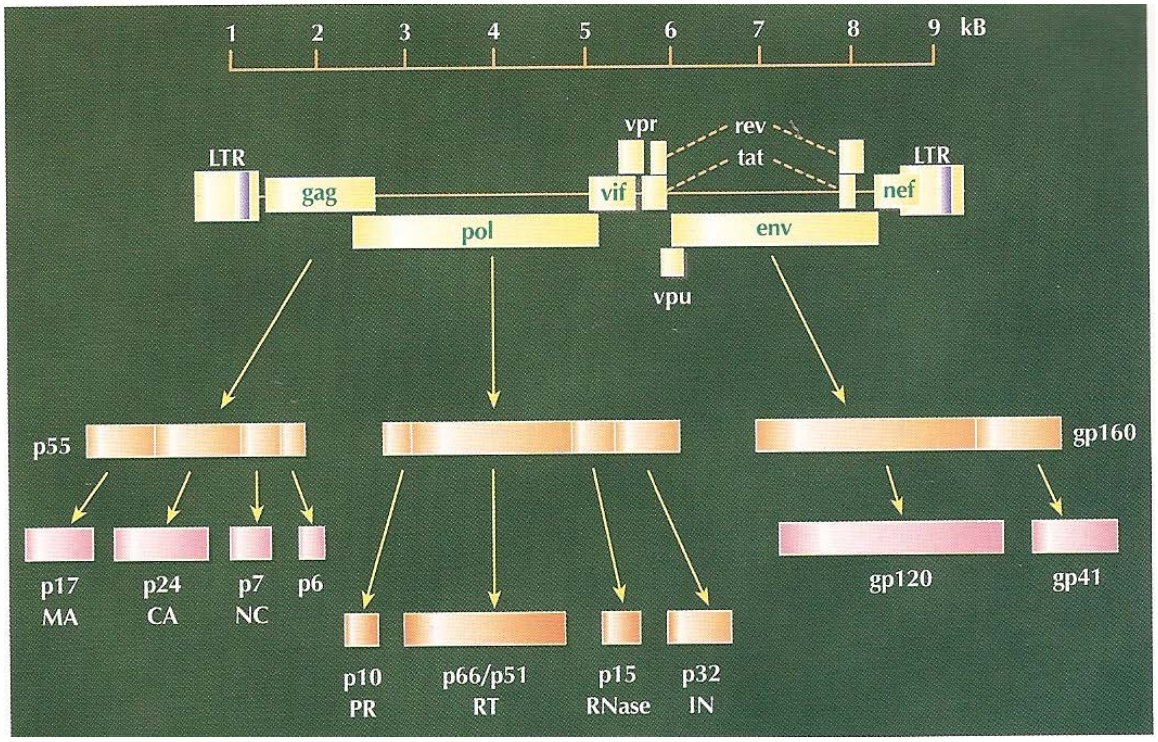
genetic message is carried within a protective nucleocapsid protein (p24) as two identical copies of positive single stranded ribonucleic acid (RNA), and the nucleocapsid also contains enzymes which assist the replication process, including reverse transcriptase (RT), protease (PR) and integrase (IN). All species that are potential hosts for retroviruses store their genetic information as double stranded deoxyribonucleic acid (DNA). For HIV-1 to successfully parasitize the host it needs to generate a DNA copy of its genetic message: the RT enzyme, found within the nucleocapsid, reverse transcribes the retroviral positive RNA to double stranded proviral DNA in the host cell.

The HIV-1 genome (the central core of a virus which contains the genetic message) i.e. viral RNA, is composed of at least nine different genes (as illustrated in Figure 1.2). The three structural genes: group-specific antigen *(gag),* polymerase *(pol)* and envelope (*env)*, are the three open reading frames for HIV-1 and contain the information that is needed, and has the potential to make proteins, for building new virions.

The *gag* gene encodes the proteins which form the viral core (p24 the capsid (CA), p17 the matrix (MA), p7 the nucleocapsid (NC), and p6). All four proteins are found in mature viral core particles (Erickson 2001). The *pol* gene encodes the essential enzymes RT, PR, IN and ribonuclease H (RNase H) which are vital proteins/enzymes central to the HIV-1 replication process. The *env* gene encodes the glycoprotein gp160 and this is further cleaved into gp120 and gp41.

The other six genes: viral protein R (*vpr),* viral infectivity factor *(vif),* viral protein unique (*vpu),* regulator of expression of virion particles (*rev),* trans-activator of transcription (*tat)* and negative regulatory factor *(nef),* (Gallo et al 1988); are accessory genes which encode for proteins that control the ability of HIV-1 to infect a cell, produce new copies of virus, or cause disease. At each end of the viral genome are long terminal repeat sequences (LTRs) which contain binding sites which promote and enhance viral expression.

Figure 1.2: The genetic structure of HIV-1 (Erickson 2001). The genome consists of three open reading frames: group-specific antigen *(gag),* polymerase *(pol)* and envelope (*env)* genes with long terminal repeat sequences (LTRs) at each end and six further accessory genes: viral protein R (*vpr),* viral infectivity factor *(vif),* viral protein unique (*vpu),* regulator of expression of virion particles (*rev),* trans-activator of transcription (*tat)* and negative regulatory factor *(nef).*



The *pol* gene, in particular the PR and RT enzymes which are targets of antiretroviral drugs, are of importance in this thesis.

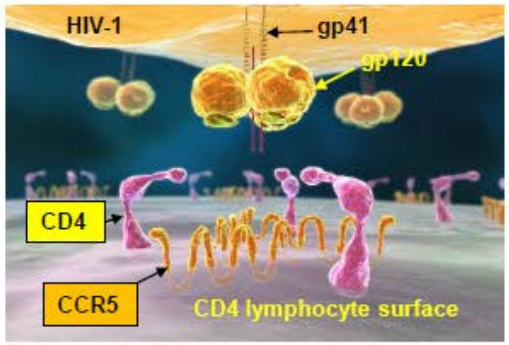
Replication of HIV-1

The primary target of HIV-1 infection are CD4 (helper) lymphocyte cells. To infect the CD4 lymphocyte, HIV-1 binds primarily to the CD4 receptor via the *env* protein gp120 (Figure 1.3a-c). This primary binding initiates the involvement of one of two co-receptors (Berger 1999), either C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) (Figure 1.3d), which initiates unfolding of the gp41 protein for membrane binding (Figure 1.3e). There then follows fusion of viral and CD4 lymphocyte envelopes (Figure 1.3f). The membranes fuse and the nucleocapsid enters the host cytoplasm: radio microimaging indicates that the viral components move in the cytoplasm through

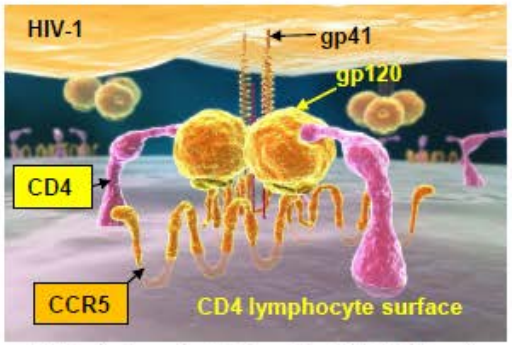
the endoplasmic reticulum (ER), (Kadiu and Gendelman 2011), and the two single stranded RNA are released. The RT enzyme transcribes the single positive viral RNA into a single negative DNA copy and finally into double stranded proviral DNA. The DNA circularises and enters the host CD4 lymphocyte nucleus and is integrated into the genome of the host cell by the IN enzyme. New virus production uses existing cellular transcription mechanism; the proviral DNA is transcribed into multiple messenger RNA (mRNA), leaves the nucleus to the ribosomal system and is translated into viral polypeptides. The viral components and proteins coalesce at the host cell surface, PR enzymes 'cleave' polypeptides into functional units and complete new virions are assembled and bud from the surface of the host cell into extracellular space.

Whilst CD4 lymphocyte cells are the primary target for HIV-1 infection, the virus also has secondary targets for HIV-1 to continue replicating. These include macrophages, CD8 lymphocyte cells and specific cells in the lungs, brain, gastrointestinal tract and kidneys (Wiley 1986; Erickson 2001).

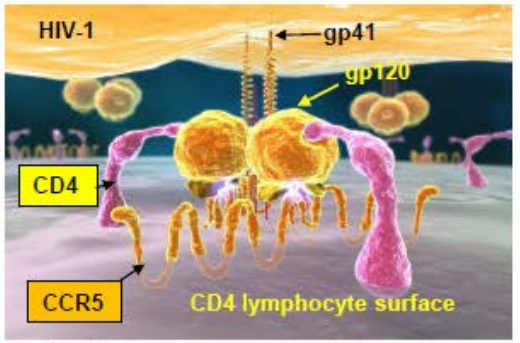
Figure 1.3: Binding of the HIV-1 envelope glycoproteins gp41 and gp120, to the CD4 lymphocyte, allowing fusion of the viral and cell membranes (Figure used with kind permission from Prof Clive Loveday and adapted by myself).



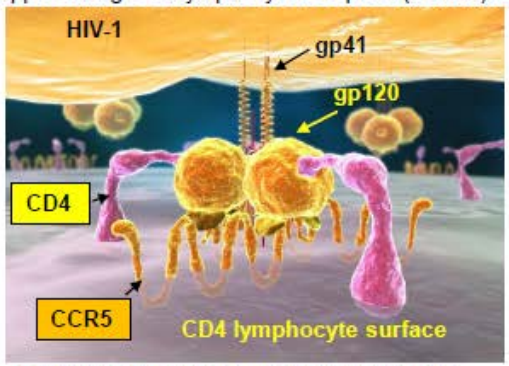
(a) Schematic of the structure of the HIV-1 envelope glycoprotein, composed of gp41 and gp120 (top). Approaching CD4 lymphocyte receptors (bottom).



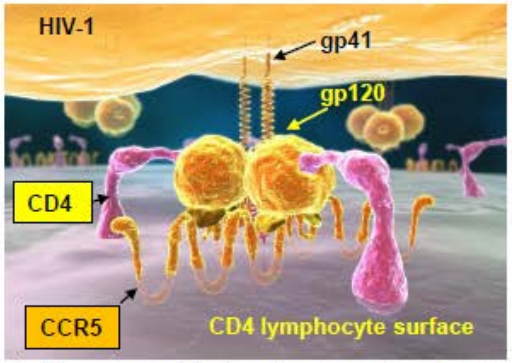
(b) Firstly there is binding of gp120 (V3 loop) to the CD4 receptor on the lymphocyte.



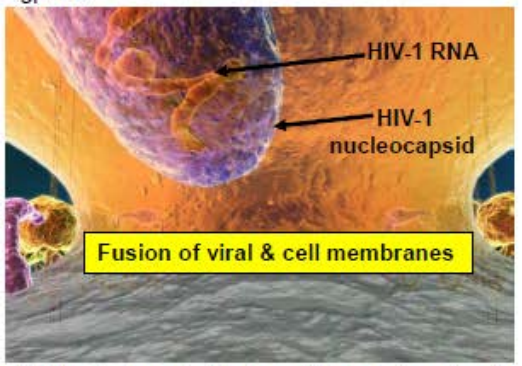
(c) This induces conformational change that exposes the CCR5 co-receptor binding site in gp120.



(d) This change allows gp120 to bind to the CCR5 co-receptor.

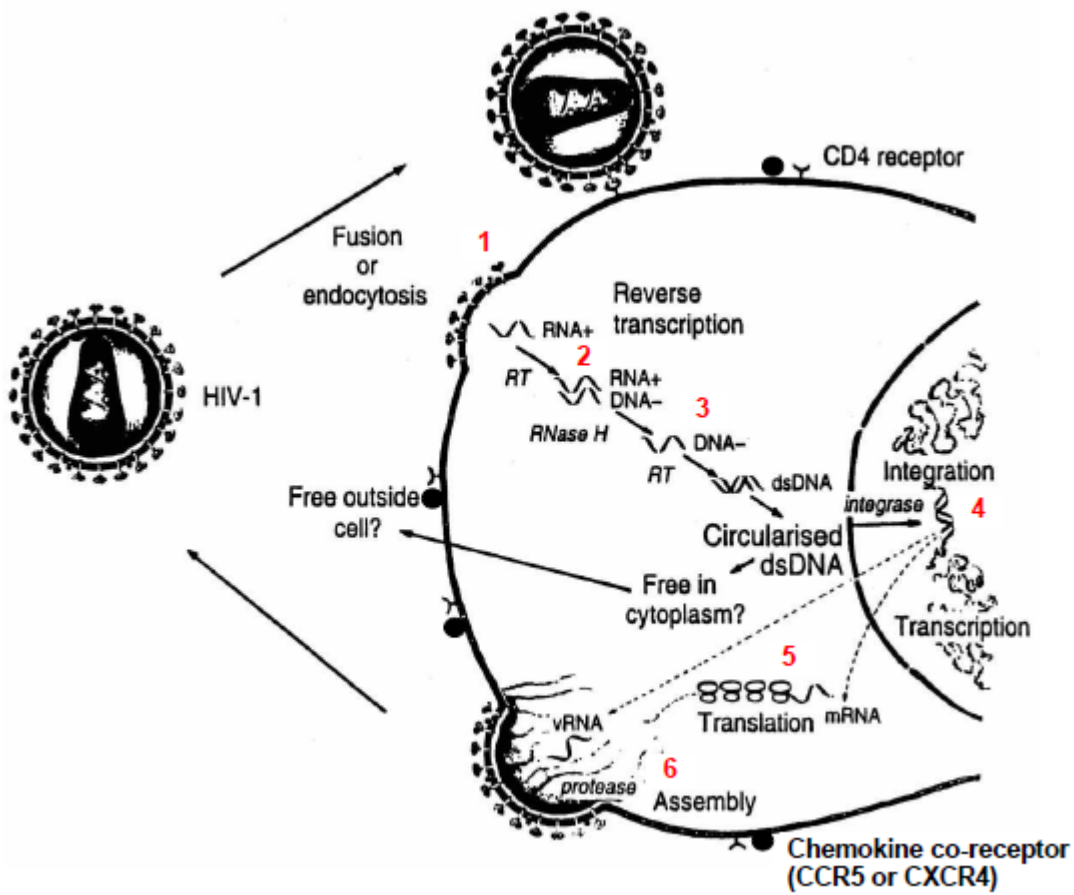


(e) Co-receptor binding triggers conformational changes in the gp41 subunit, leading to insertion of its N terminal fusion peptide into the host cell's membrane.



(f) Physio-chemical fusion of the viral and cell membranes occurs, resulting in the entry of the viral nucleocapsid (including genetic information) into the cytoplasm.

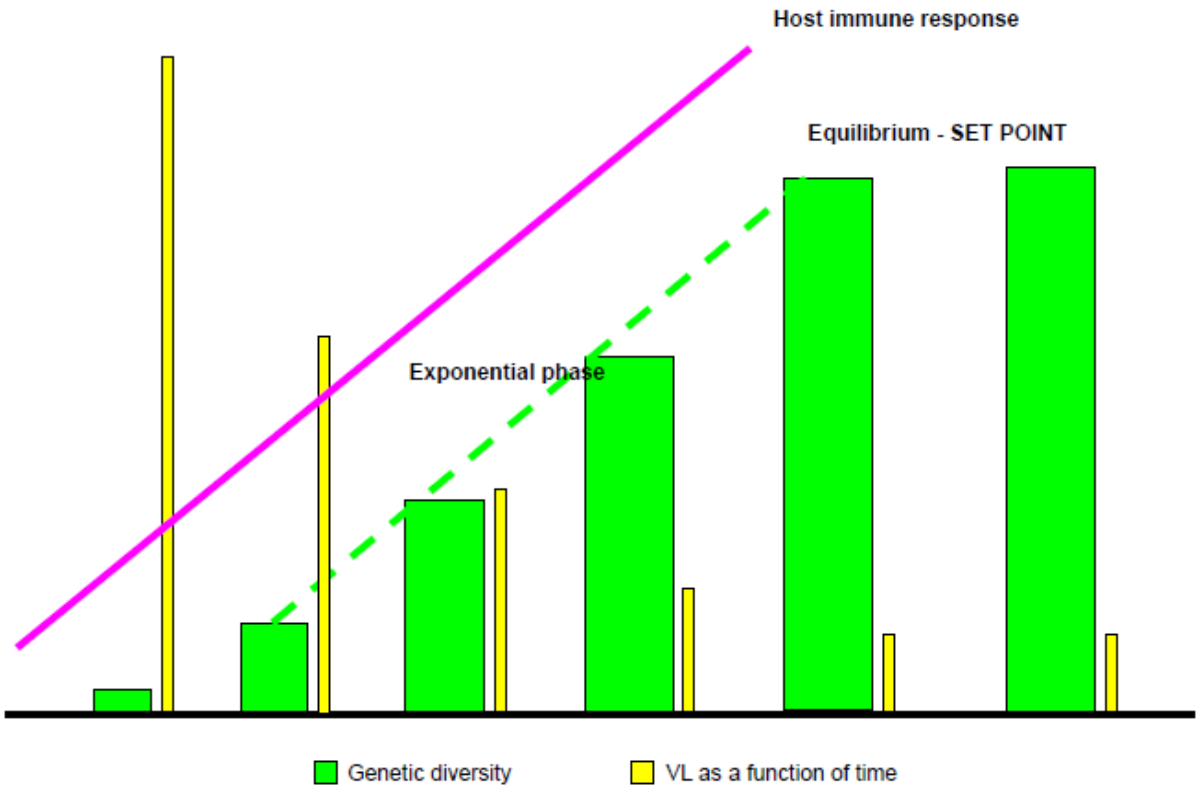
Figure 1.4: Replication of HIV-1 virions (Figure used with kind permission from Prof Clive Loveday and adapted by myself).



Key:
The red numbers on the figure above illustrate the target areas in the lifecycle where drug classes are available to treat HIV-1 and are discussed in detail (see pg 13-14).

The viral generation time of a HIV-1 virion is approximately 2.5 days, with ten billion virus particles produced each day (Perelson et al 1996). The RT process is error prone with at least one base error per replicative cycle. This high replication rate, in addition to error prone replication, gives HIV-1 the potential for real time evolution. Following primary HIV-1 infection (PHI) with only one or two genetically distinct viruses, the above criteria result in a genetic expansion of viruses within one host and a year after infection (chronic infection) the individual will have thousands of genetically distinct, though closely related, virions circulating in their body (quasispecies).

Figure 1.5: Primary HIV-1 infection (PHI) and viral quasispecies: at PHI, the viral load (VL) is very high but as the host's immune system responds, the VL decreases over time, with the genetic diversity within the host expanding (Figure used with kind permission from Prof Clive Loveday).



Following PHI, the amount of HIV-1 circulating, that is the viral load (VL), is very high. As the specific host immune system responds, the VL will drop and

stabilise. The 'set point', that is, the level at which the VL stabilises, is predictive of how the HIV-1 infection will progress (Henrard 1995; Mellors 1996).

Transmission and stages of HIV-1 infection

The predominant transmission pathway for HIV-1 is through unprotected sexual activity whereby the virus can be transmitted across the mucosal barrier of the vagina, vulva, penis or rectum (Mortimer and Loveday 2001).

A number of factors increase the likelihood of sexual transmission:

- the pathogenicity of the virus itself,
- the VL of the person infected with HIV-1: the higher the VL the increased risk of 'successful' transmission,
- concurrent sexually transmitted diseases, including genital herpes; syphilis; gonorrhoea,
- the number of sexual partners: the greater the number of sexual partners the greater the risk of acquiring HIV-1,
- the condition of the host's immune system.

HIV-1 can also be transmitted via sharing injecting drugs equipment; vertically from mother to-child; through exposure to infected blood or blood products; or occupational exposure from, for example, needle-stick injuries. Transmission opportunities via these routes have been limited in the United Kingdom (UK) due to prevention systems that have been put into place including needle exchange schemes (introduced in 1986); the introduction of routine testing of all pregnant women for HIV-1 (introduced in England in 2000 and across the UK by 2003 (Townsend et al 2006)) and screening of the blood supply (which was introduced as early as 1985).

Worldwide epidemiology of HIV-1 infection

There are currently 35.3 million (32.2-38.8 million) people living with HIV-1 worldwide (Joint United Nations Programme on HIV/AIDS (UNAIDS) 2013). The majority of the infected (25 million, 70.8%) reside in sub-Saharan Africa (23.5-26.6 million). There were 2.3 million (1.9-2.7 million) new infections diagnosed

globally in 2012, with 1.6 million (1.4-1.8 million) of these new diagnoses occurring in sub-Saharan Africa.

In the UK, it was estimated that 107,800 people (101,600-115,800) were living with HIV-1 in 2013, with 24.0% (26,100) unaware of their positive status (Yin et al 2014). There were 6,000 new HIV-1 infections diagnosed in the UK in 2013.

Patients with HIV-1 included in the studies presented in this thesis were diagnosed as early as 1981 up to 2006. To set the context as per the above, there were an estimated 73,000 people living with HIV-1 in the UK in 2006 with approximately 21,600 (29.6%) unaware of their infection (The UK Collaborative Group for HIV and STI Surveillance 2007). An estimated 7,800 new infections were diagnosed in the UK in 2006. Of those currently diagnosed as HIV-1 positive in the UK (n=47,800): the predominant exposure category was MSM (43.0%), followed by heterosexual women (31.0%), heterosexual men (21.0%) and a small minority were infected as a result of being IDU (4.0%). Of those in the heterosexual exposure category, 12,100/16,200 (74.7%) of women and 5,700/9,100 (62.6%) of men were born in Africa and had since moved to the UK.

HIV-1 group and subtype nomenclature

HIV-1 is a genetically diverse virus and through phylogenetic analysis of sequence data, HIV-1 has been classified into four distinct virus groups: Group M (major group, De Leys et al 1990), Group O (outlier group, Charneau et al 1994), Group N (non-M and non-O group, Simon et al 1998) and Group P (putative group, Plantier et al 2009). Group M is the most predominant, with the majority of HIV-1 infections belonging to this group. At the time of writing, at least nine genetically distinct subtypes (also termed clades) had been determined within Group M including: A (further divided into sub-subtypes A1 and A2), B, C, D, F (further divided into sub-subtypes F1 and F2), G, H, J and K.

In 1995, Robertson et al identified the potential of the Group M subtypes to recombine, that is, they identified different sequence subtypes within the *gag* and *env* genes they phylogenetically analysed, indicating individuals could become infected with HIV-1 strains from different sequence subtypes. These mixtures of different subtypes (A to K) combining their genetic material to form a hybrid virus became known as circulating recombinant forms (CRFs) and the first CRF identified, CRF01_AE, represented a putative subtype A and subtype E recombinant (Carr et al 1996; Gao et al 1996).

Guidelines issued stated that new CRFs/subtypes should be designated only when there were three full-length genome sequences available from three epidemiologically unlinked patients (Robertson et al 1999). In 1999, Robertson et al reported four CRFs including CRF01_AE, CRF02_AG, CRF03_AB and CRF04_cpx (which was a combination of three or more subtypes). By 2015, sixty-eight circulating CRFs have currently been characterised (Los Alamos National Laboratory, 2015). Due to the high genetic variability of HIV-1, new CRFs/subtypes will evolve and be detected in the future.

Worldwide distribution of HIV-1 subtypes

Analyses of global HIV-1 sequences (Hemelaar et al 2011) determined subtype C as the most predominant type (48.0%), followed by subtype A (12.0%), subtype B (11.0%), subtype G (5.0%), subtype D (2.0%) and any of subtypes F, H, J or K (<1.0%). The CRF02_AG was prevalent in 8.0% of the global population with CRF01_AE in 5.0% of the population, with other recombinants accounting for 4.0% (Hemelaar et al 2011).

In the first wave of the epidemic, subtype B was the most prevalent subtype in Western Europe and the UK and was associated with the MSM and IDU risk groups (Buonaguro et al 2007). Non-B subtypes identified in Western Europe populations were associated with the heterosexual risk group, in persons endogenous to the local geographical area and those who had

immigrated from endemic regions, such as Africa and Asia (Buonaguro et al 2007).

Treatment of HIV-1 infection: antiretroviral therapy (ART)

With increasing understanding of the structure of HIV-1 and its lifecycle, therapies were - and still are - developed to try and manage the circulating virus. Currently, there are six classes of antiretroviral drugs available which target different phases of the HIV-1 lifecycle and interfere with the replication process.

Table 1.1: Target areas in the HIV-1 lifecycle where drug classes are available to suppress viral replication (see the red numbers on Figure 1.4, pg 8 which highlight the areas where the drug classes target).

Drug class	Target area of drug in HIV-1 lifecycle	Name of antiretroviral drug (abbreviation)
1 Entry/fusion inhibitors	HIV-1 entry into CD4 lymphocyte cells	• Maraviroc (MVC)[*] • Enfuvirtide (T-20)
2 Nucleoside reverse transcriptase inhibitors (NRTIs)	RT enzyme	• Zidovudine (ZDV)[*] • Didanosine (ddI) • Zalcitabine (ddC) • Stavudine (d4T) • Lamivudine (3TC)[*$] • Abacavir (ABC)[*$] • Tenofovir (TDF)[*$] • Emtricitabine (FTC)[*$]
3 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	RT enzyme (but in a different way to NRTIs)	• Nevirapine (NVP)[*$] • Delavirdine (DLV) • Efavirenz (EFV)[*$] • Etravirine (ETV)[*] • Rilpivirine (RPV)[*$]
4 Integrase inhibitors	IN enzyme	• Raltegravir (RAL)[*$] • Elvitegravir (EVG)[*$] • Dolutegravir (DTG)[*]
5 HIV-1 maturation inhibitors	*gag* protein	• Bevirimat (BVM)[#]

Table 1.1 (contd.): Target areas in the HIV-1 lifecycle where drug classes are available to suppress viral replication (see the red numbers on Figure 1.4, pg 8 which highlight the areas where the drug classes target).

Drug class	Target area of drug in HIV-1 lifecycle	Name of antiretroviral drug (abbreviation)
6 Protease inhibitors (PIs)	PR enzyme	• Saquinavir (SQV) • Indinavir (IDV) • Ritonavir (RTV)* • Nelfinavir (NFV) • Amprenavir (APV) • Lopinavir/boosted with RTV (LPV/r)*$ • Atazanavir (ATV) *$ • Fosamprenavir (FPV)*$ • Tipranavir (TPV)* • Darunavir (DRV)*$

Key:
* currently approved for use in the UK and European Union (National AIDS Manual (NAM) 2014)
$ recommended for use by the British HIV Association (BHIVA, Williams et al 2014)
# drug in development

1 Entry/fusion inhibitors

The CCR5 entry inhibitor MVC was designed to target the HIV-1 lifecycle at the first phase by blocking viral entry by binding to the transmembrane protein CCR5 and preventing it from interacting with the V3 loop of gp120 (Figure 1.3b). The fusion inhibitor T-20 was designed to bind to gp41 and prevent the shape changes that enable HIV-1 virions and CD4 lymphocyte cells to fuse (Figure 1.3e+f).

2 NRTIs

The NRTI drug class includes nucleoside and nucleotide RT inhibitors. These inhibitors were designed to target the RT enzyme and mimic the naturally occurring building blocks of DNA, deoxynucleosides. Nucleoside inhibitors require more metabolic steps to become active against the RT than nucleotide inhibitors which are already monophosphorylated (Pratt 2003). Nucleoside inhibitors require conversion (phosphorylation) to their triphosphate form, then

they can compete with the natural deoxynucleoside triphosphates for binding to RT. These NRTIs compete with the natural deoxynucleoside triphosphates for incorporation into new viral DNA chains and as the NRTIs have a different structure, the naturally occurring nucleotides cannot be added on to continue the transcription, resulting in chain termination and thereby halting HIV-1 replication (Erickson 2001).

3 NNRTIs

The NNRTIs are structurally diverse from NRTIs: they do not compete with the naturally occurring nucleotides but were designed to inhibit RT by binding directly to a pocket of the enzyme (p66) and inhibiting the catalytic step of RT polymerisation (Spence et al 1995).

4 Integrase inhibitors

Integrase inhibitors were designed to block the IN enzyme from inserting the HIV-1 viral genome into the DNA of the host cell.

5 HIV-1 maturation inhibitors

HIV-1 maturation inhibitors were designed to disrupt and bind to the *gag* protein, preventing the cleavage of the *gag* protein and therefore interfering with the maturation of the virus, forming non-infectious, immature virions, which were incapable of infecting other cells (Salzwedel et al 2007).

6 PIs

The PIs were designed to inhibit HIV-1 replication at a later stage of the lifecycle: they target the PR enzyme whose role is to cleave large polyproteins into smaller proteins (p17, p24, p2, p7, p1 and p6) prior to virus assembly at the cell membrane and the PIs ‘block’ the PR enzyme from completing assembly of functional new virions (Calvez 2001).

Measuring the success of ART

The success of these drugs are measured by virological outcomes: the drugs should suppress the replication of HIV-1 virions and therefore the number of viruses circulating. The aim of ART is to suppress the VL of a patient so that they are deemed as biologically 'undetectable': VL tests can determine the number of 'copies' of RNA per millilitre in a patient's plasma sample and when monitored, this ideally should be <50 copies per millilitre (c/mL). Successful therapy therefore 'controls' viral replication in the host, decreasing the number of circulating virions and allowing the immune system to recover, resulting in an increase in the CD4 lymphocyte cell count.

The history of ART (please see Figure 1.8 on pg 25 for a timeline of ART)

The first ART was introduced in 1987 after a double-blind, placebo-controlled clinical trial found that for those patients living with AIDS who were treated with the NRTI ZDV, (commonly known at the time as Azidothymodine (AZT)); at 24 weeks of follow-up, ZDV prolonged the life of those living with AIDS (Fischl et al 1987). There was general optimism in the field: before the introduction of ZDV, the prognosis for patients with HIV-1 was poor, with opportunistic infections and eventual progression to AIDS and death. This optimism was soon shattered however when results from the Concorde Trial showed that symptom free individuals with HIV-1 who were randomised to take ZDV monotherapy immediately or had treatment deferred until the onset of symptoms and were followed-up over a three-year period; ZDV did not slow down progression to AIDS or improve survival (Concorde Coordinating Committee 1994). Even though other NRTIs became available to treat patients with HIV-1/AIDS, including ddI (1991), ddC (1992), and d4T (1994), monotherapy treatment was sub-optimal and morbidity and mortality rates remained high and drug-resistant strains emerged and evolved (Larder and Kemp 1989; Larder et al 1995).

Dual therapy with a combination of two NRTIs: ZDV+3TC (Schlomo et al 1996; Katlama et al 1996) or ZDV+ddI or ddC (Delta Coordinating Committee 1996; Hammer et al 1996) were employed to try and control HIV-1 replication and delay

the onset of drug resistance. Results from these studies found that taking a combination of two NRTIs compared to NRTI monotherapy, significantly prolonged life and delayed progression to AIDS but drug resistant mutations were still identified at week 24 of treatment for those on dual therapy (Katlama et al 1996).

In 1996, the field of treating patients with HIV-1/AIDS took a quantal leap forward. New classes of ART were introduced with the PIs SQV, RTV and IDV introduced to the field as well as the first NNRTI NVP. Further studies indicated that combining the different drugs classes, e.g. NRTIs with PIs, reduced the frequency of AIDS and death (Hammer et al 1997, Gulick et al 1997, Cameron et al 1998). Dramatic improvements in the morbidity and mortality of patients with HIV-1/AIDS were witnessed, with a decrease in the incidence of opportunistic infections, tumours and deaths (Rubbert and Ostrowski 2003; The Antiretroviral Therapy Cohort Collaboration 2008). This became known as the era of highly active antiretroviral therapy (HAART).

In spite of the dramatic improvements in VL suppression, the complexity of HAART regimens and associated adverse effects raised new challenges in the clinical management of patients with HIV-1. These HAART regimens often required the patient to take up to 30 tablets a day, with food, drink and time constraints (Hoffman 2003). This high pill burden resulted in problems with adherence to the therapies and patients often experienced severe side-effects to the regimens. Again, the development of drug resistance mutations for patients taking HAART were observed.

Resistance to ART

As suggested above, the development of resistance mutations to ART have a major impact on successful treatment outcomes. The primary goal of ART is to suppress HIV-1 replication so the VL remains undetectable. If this does not occur and HIV-1 is able to continue replicating: alongside the RT enzyme's error-prone replication with at least one base error per replicative cycle, and with the

absence of 'proof-reading' mechanisms, mutations can evolve on a daily basis (Perelson et al 1996). The higher the VL, the less time it takes before resistance mutations/viruses evolve and continue replicating (as depicted in Table 1.2).

Table 1.2: Viral load (VL) replication and number of days before mutation arises: the higher the VL, the shorter the timeframe for the development of resistance mutations (Table used with kind permission from Prof Clive Loveday).

VL (c/mL)	Days before mutation arises
300,000	0.1
30,000	1
3,000	10
300	100
30	1,000

Therefore, suppressing VL is essential to decrease the opportunities for a mutant viral population to develop and to ensure successful treatment with ART.

Other factors which can impact on the development of resistance mutations include pharmacological issues: poor absorption and poor adherence to the drug regimen can lead to subinhibitory drug levels, as well as the failure of the host's immune system; and combined with the biological factors described above, lead to persistent viral replication and therefore the continued evolution of drug resistance and ultimately, drug failure.

Measuring resistance to ART

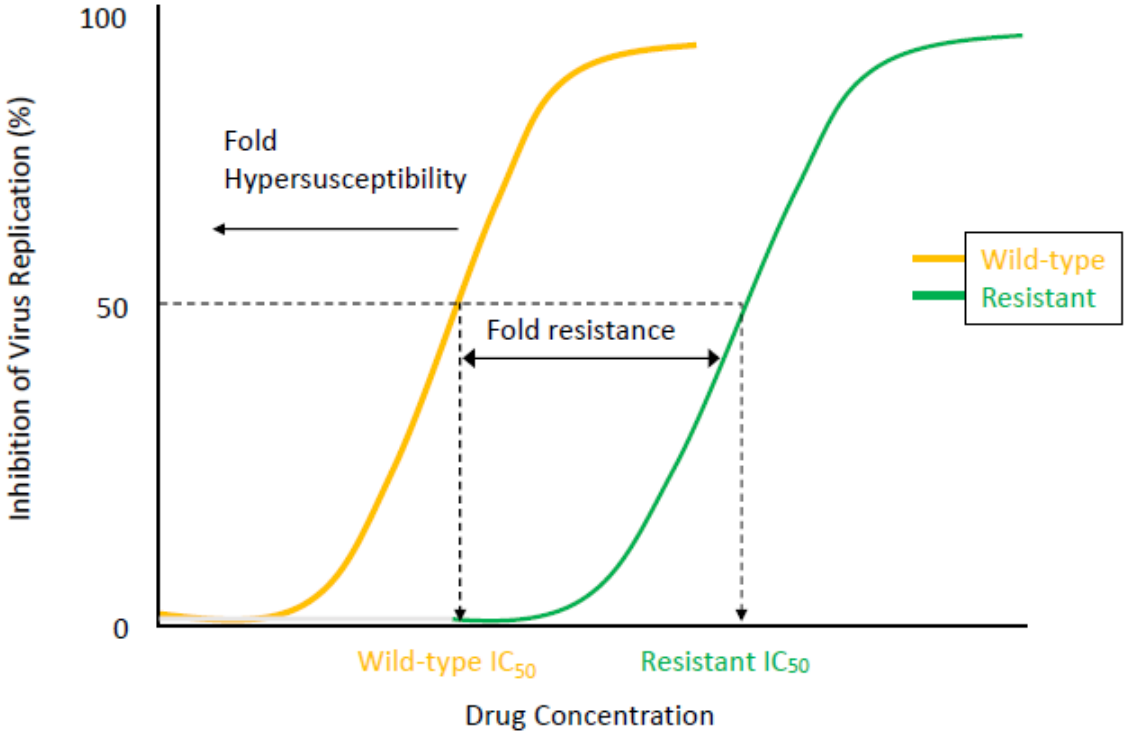
Drug resistance tests are available to measure the emergence of resistance mutations including genotype tests and phenotype tests.

Genotype tests (Molecular assay) define the nucleotide sequence from which the PR and RT enzymes amino acid sequence can be deduced. The sequence shows the genetic code of the host's virus. The genetic code is composed of four bases: adenine (A), cytosine (C), guanine (G) and thymine (T); with A binding with T and C with G. These bases, combined in different sequences in groups of three, known as codons, encode for a specific amino acid. The genotypic resistance test compares this sequence to a reference wild-type (WT),

detects any base changes in the codons of the PR and RT genomes and interprets the consequent change in amino acid translation, and hence the protein (enzyme) structure and function.

Phenotypic tests (Biological assay) measure the sensitivity of a patient's circulating viruses to a drug in a replicating virus system, compared with a reference WT virus. When resistance mutations occur, viruses require higher concentrations of drug to suppress their replication: results are expressed as a fold change in sensitivity between the patient's and the reference WT virus replication rate (Figure 1.6).

Figure 1.6: Interpretation of phenotypic susceptibility: drug concentration able to inhibit virus growth in vitro to 50.0% (50.0% inhibitory concentration, IC_{50}) relative to a wild-type (WT) reference virus (Hirsch et al 2008).



Genotypic and phenotypic tests have been clinically validated for use in patient care but genotypic tests are generally used to determine the resistance profile of patients with HIV-1 as they involve a quicker turnaround than phenotype tests and are cheaper to conduct (Torre and Tambini 2002).

Interpretation of genotype resistance mutations

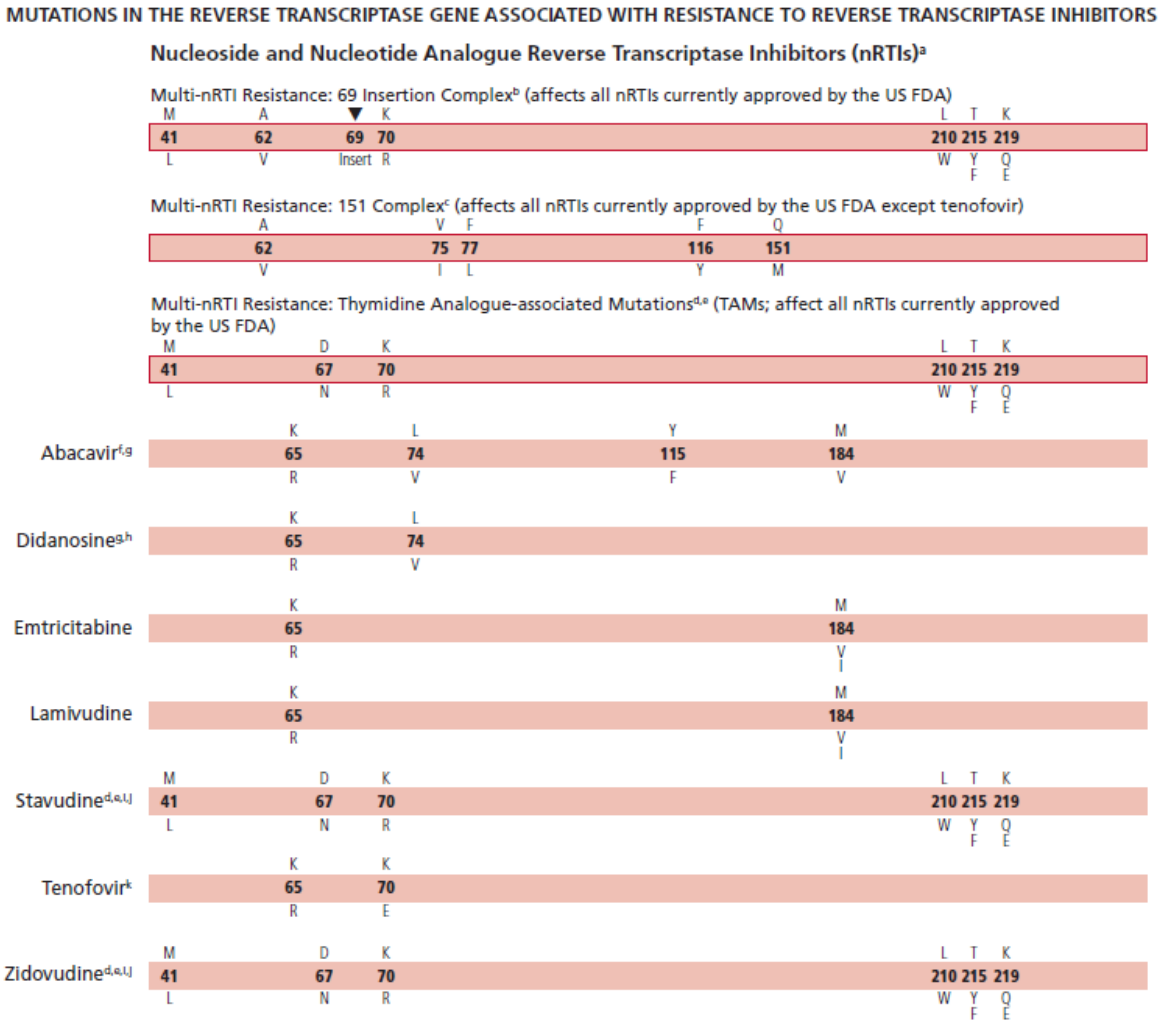
Interpretation of genotypic resistance mutations is complex: there are over 100 RT and PR mutations identified that are involved in the development of HIV drug resistance (Calvez 2001). These mutations interact and emerge in complex patterns to suggest a person is resistant to their therapy. They therefore need to be analysed using computerised tools which may exist within the given genotyping system or may be available as a separate entity: these tools have the capacity to define the importance of combinations of mutations and the impact for clinical care.

In addition, genotype results can be interpreted using a 'virtual phenotype database' such that the genotypic result can be compared to an extensive database containing a large number of genotypic and corresponding phenotypic results; a 'virtual phenotype' can then be inferred based on a known genotypic profile with the result given as a fold change in sensitivity to the drug.

An independent group of clinical experts in the HIV-1 field produce a reference list of known mutations which are associated with clinical resistance to the specific drugs and drug classes (the International Antiviral Society-USA (IAS-USA) Drug Resistance Mutations Group (formerly known as the International AIDS Society-USA (IAS-USA) Drug Resistance Mutations Group)). These charts are a useful reference tool to determine resistance mutations and are updated annually by a group of World experts, but caution must be exercised when using them due to the complexity of interpreting resistance mutations and patterns.

Illustrated below are the IAS-USA list of mutations associated with the NRTIs (Figure 1.7a), the NNRTIs (Figure 1.7b) and the PIs (Figure 1.7c), (Johnson et al 2009).

Figure 1.7a: Mutations associated with resistance to the nucleoside reverse transcriptase inhibitors (NRTIs) as per the International Antiviral Society-USA (IAS-USA) Drug Resistance Mutations Group (Johnson et al 2009).



Key:
Amino acid abbreviations: A alanine; C cysteine; D aspartate; E glutamate; F phenylalanine; G glycine; H histidine; I isoleucine; K lysine; L leucine; M methionine; N asparagine; P proline; Q glutamine; R arginine; S serine; T threonine; V valine; W tryptophan; Y tyrosine.

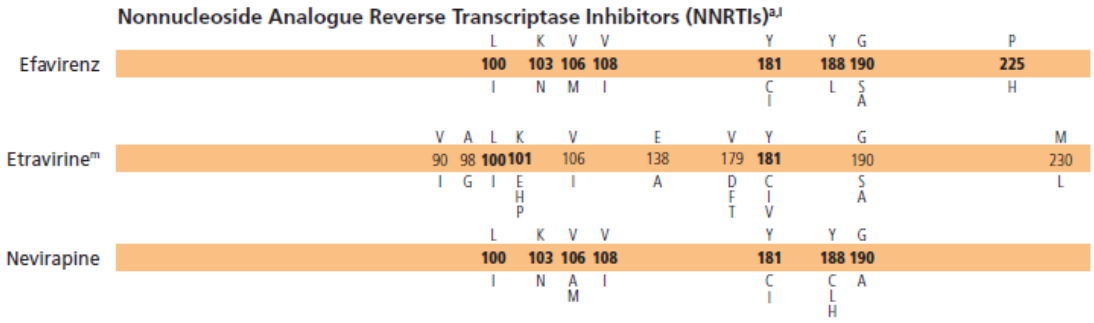
Using the first ART, ZDV to exemplify: the pink panel represents the RT gene and the resistance mutations M41L, D67N, K70R, L210W, T215Y/F and K219Q/E have been defined as conferring resistance to ZDV.

The number in the panel (41) represents the codon position at which a mutation has occurred, the upper case letter above the number (M: methionine)

represents the WT amino acid expressed and the letter below (L: leucine) represents the resistant mutation associated amino acid change. If the codon number is in bold, it indicates a major mutation.

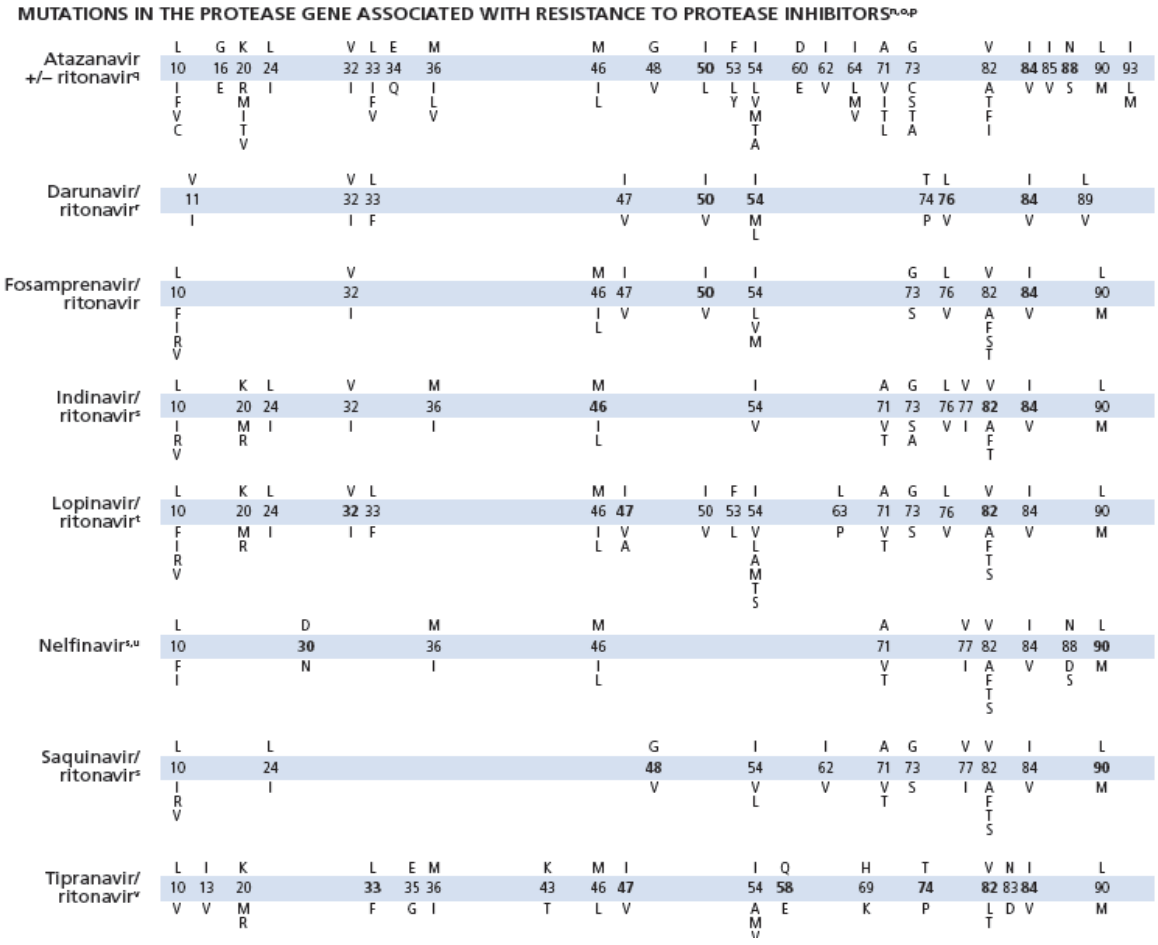
These mutations to ZDV (M41L, D67N, K70R, L210W, T215Y/F and K219Q/E) are known as thymidine analogue mutations (TAMs) and are associated with both ZDV and d4T resistance and specific TAMs impact the antiviral activity of the other NRTIs also. The mutations M41L and T215F/Y are associated with higher levels of resistance and two patterns of TAMs that tend to cluster together have been identified: TAM pattern 1, M41L, L210W and T215Y are associated with d4T resistance; TAM pattern 2, D67N, K70R, T215F and K219E/Q are associated with AZT resistance (Cozzi-Lepri et al 2005).

Figure 1.7b: Mutations associated with resistance to the non-nucleoside reverse transcriptase inhibitors (NNRTIs) as per the International Antiviral Society-USA (IAS-USA) Drug Resistance Mutations Group (Johnson et al 2009).



Key:
Amino acid abbreviations: A alanine; C cysteine; D aspartate; E glutamate; F phenylalanine; G glycine; H histidine; I isoleucine; K lysine; L leucine; M methionine; N asparagine; P proline; Q glutamine; R arginine; S serine; T threonine; V valine; W tryptophan; Y tyrosine.

Figure 1.7c: Mutations associated with resistance to the protease inhibitors (PIs) as per the International Antiviral Society-USA (IAS-USA) Drug Resistance Mutations Group (Johnson et al 2009).



Key:
Amino acid abbreviations: A alanine; C cysteine; D aspartate; E glutamate; F phenylalanine; G glycine; H histidine; I isoleucine; K lysine; L leucine; M methionine; N asparagine; P proline; Q glutamine; R arginine; S serine; T threonine; V valine; W tryptophan; Y tyrosine.

Each ARV drug has a different genetic barrier, that is, the number of resistance mutations required, to attain resistance (Paredes and Clotet 2009). Some ART have a low genetic barrier to resistance, that is, a single mutation confers resistance. For example, the K103N mutation alone confers resistance to EFV with the Y181C mutation conferring resistance to NVP (Figure 1.7b). Other ART have a high genetic barrier to resistance, that is, a number of mutations need to accumulate to confer resistance e.g. resistance to the PIs (Figure 1.7c).

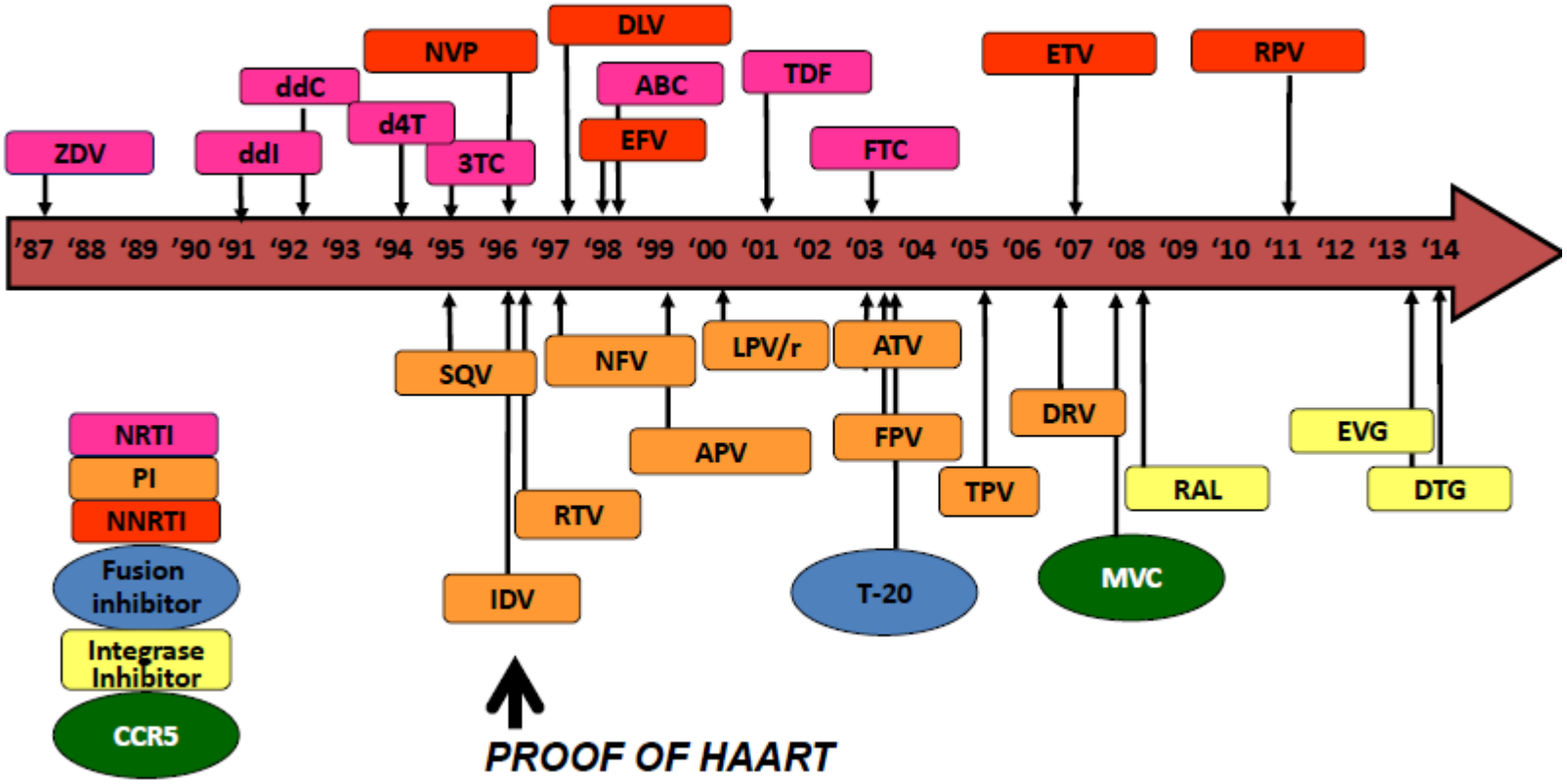
Objectives of this thesis

Using a clinical cohort resistance database of patients infected with HIV-1 who had genotypic tests conducted during 1996 to 2006, a series of studies were devised and conducted to determine whether the clinical care of targeted sub-groups of patients with HIV-1 could be improved with a better understanding of:

- the evolution and dynamics of resistance mutations in treatment-naïve patients (Chapter 4)
- the evolution and dynamics of resistance mutations in treatment-experienced patients (Chapter 5)
- the potential susceptibility of treatment-experienced patients to new ARV drugs (Chapter 6)
- the evolution of subtype profiles within the clinical cohort, including treatment-naïve and treatment-experienced patients (Chapter 7).

The main objective for conducting each of these studies was to try and improve our knowledge and ensure that patients with HIV-1 were receiving the best possible clinical care with regards to resistance and established, successful treatment.

Figure 1.8: Timeline of the introduction of specific antiretroviral therapies (ART), by drug class **(Figure adapted and used with kind permission from Prof Clive Loveday).**



NRTIs: ZDV Zidovudine, ddI Didanosine, ddC Zalcitabine, d4T Stavudine, 3TC Lamivudine, ABC Abacavir, TDF Tenofovir, FTC Emtricitabine
PIs: SQV Saquinavir, IDV Indinavir, RTV Ritonavir, NFV Nelfinavir, APV Amprenavir, LPV/r Lopinavir (boosted with RTV), ATV Atazanavir, FPV Fosamprenavir, TPV Tipranavir, DRV Darunavir
NNRTIs: NVP Nevirapine, DLV Delavirdine, EFV Efavirenz, ETV Etravirine, RPV Rilpivirine
Fusion inhibitor: T-20 Enfuvirtide **Integrase inhibitor:** RAL Raltegravir, EVG Elvitegravir, DTG Dolutegravir **CCR5 entry inhibitor:** MVC Maraviroc

Chapter 2: Materials and Methods

The International Clinical Virology Centre (ICVC) was a small, not for profit charity, specialising in the development and application of molecular (and related) laboratory tests, to support the clinical management of patients with HIV-1/AIDS. The ICVC evolved from the team that developed the first VL and resistance assays for HIV-1 in the World (Semple et al 1991, Kaye et al 1992, Semple et al 1993) and applied these tests in Medical Research Council (MRC), EuroSida and other international clinical trials, and in direct patient care. With this experience, 39 clinical centres (District General Hospitals (DGH)) across the UK asked to collaborate with the ICVC, to support the clinical management of their patients. It was agreed the ICVC would conduct the VL and genotypic resistance testing for these 39 clinical centres and that the ICVC's Clinical Director would interpret each resistance report and be available to discuss any patients' case management, if the clinician required any advice.

The philosophy of the ICVC was to provide quality assured molecular technologies to enhance patient care and the understanding of viral infections. The ICVC Collaborative Research Group was formed and I was recruited in 2002 as a research assistant to establish and coordinate the ICVC Clinical Cohort Resistance Database. The main objective of the ICVC Clinical Cohort Resistance Database was to pool the resistance data generated from the patients at all clinical centres. Often, the numbers of patients seen within these centres were relatively small and all 39 members of the ICVC Collaborative Research Group agreed the data should be unified to provide a powerful database for audit and analysis, to answer contemporary scientific questions related to future patient clinical care.

Ethical approval

All clinical centres had to attain local ethical approval from their hospital before they could become members of the ICVC Collaborative Research Group: permission had to be granted from the hospital that the patients' samples and data could be used by the ICVC. All 39 clinical centres signed an agreement

with the ICVC that their patients' clinical and resistance data could be included in the ICVC Clinical Cohort Resistance Database, and these data pooled and used anonymously, to conduct cohort audits for the overall improvement of clinical care for patients with HIV-1.

Each clinical centre was responsible for attaining their patients' written consent that after a VL/resistance test was conducted at the ICVC, any residual sample could be used in future research; as well as their clinical and resistance data included in the ICVC Clinical Cohort Resistance Database.

The ICVC did not share any data nor results for commercial purposes. Each of the clinical centres retained overall ownership of their data and on request, the ICVC provided access to their data as well as transferred their data to other clinical databases e.g. the Brighton clinical centre requested their data was forwarded to the MRC.

On completion and final submission of this thesis, the ICVC Clinical Cohort Resistance Database and all data will be returned to the ICVC Charitable Trust Headquarters which is covered by the Data Protection Registration, Information Commissioner's Office (ICO): 00043047707 (current registration date until 06/06/2017).

The ICVC resistance request form

For each patients' sample sent to the ICVC requesting a genotypic resistance test was conducted, the clinicians were asked to complete a corresponding resistance request form (Figure 2.1). I designed and developed the ICVC resistance request form after a consensus meeting with the ICVC's Clinical Director and the ICVC's Laboratory Manager, as to the clinical data that would be most appropriate to collect. These data would be used to assist the laboratory with conducting the resistance test: an indication of the VL of the sample to confirm a resistance test was feasible and epidemiological data to identify the origin of infection and hence potential genetic diversity. These

data assisted the Clinical Director with the interpretation of the resistance report and allowed clinical cohort research to be conducted.

Generic clinical data was requested on the ICVC resistance request form including the sex and date of birth of the patient; an indication of the origin of the infection; as well as the risk exposure group of the patient with HIV-1 infection. Other clinical data was request specific i.e. why the clinician required that a patients' sample was tested; was the patient currently taking ART and if not, were they naïve to ART or in a phase of treatment interruption. The clinicians were asked to comment on the patients' adherence to their ART regimen and also indicate the VL and CD4 counts of the sample (or alternatively, the most recent values).

Figure 2.1: The ICVC resistance request form to be completed alongside each patients' sample submitted for genotypic resistance testing, to assist the ICVC laboratory with conducting the test, the ICVC's Clinical Director in interpreting the genotype report and to allow clinical cohort research to be conducted.

ICVC international clinical virology centre

Resistance Request Form

PLEASE FILL IN ALL SECTIONS CLEARLY

HOSPITAL No: _____ HOSPITAL / CLINIC: _____

SURNAME: _____ CONSULTANT / DOCTOR: _____

FORENAME: _____ PHONE: _____

DATE OF BIRTH: _____ FAX: _____

SEX: ☐ M ☐ F

SAMPLE DETAILS: DATE: _____ TIME: _____ ☐ BASELINE ☐ URGENT

Sample / recent viral load (date): _____(___/___/___) Most recent CD4: _____

Origin of Infection: ☐ Europe ☐ Africa ☐ Asia ☐ N. America ☐ S. America ☐ Australia ☐ Other _____

Current drug therapy: _____

Major reason for test: _____

Adherence comment: _____

Drug	Start Date	Stop Date
Zidovudine (ZDV)		
Didanosine (ddI)		
Zalcitabine (ddC)		
Lamivudine (3TC)		
Stavudine (d4T)		
Abacavir (ABC)		
Tenofovir (TDF)		
Emtricitabine (FTC)		
Enfuvirtide (T-20)		
Nevirapine (NVP)		
Delavirdine (DLV)		
Efavirenz (EFV)		
Saquinavir (SQV)		
Indinavir (IDV)		
Ritonavir (RTV)		
Nelfinavir (NFV)		
Amprenavir (APV)		
Lopinavir/ Ritonavir (LPV/r)		
Tipranavir (TPV)		
Atazanavir (ATV)		
Other:.....		

Please, complete the following information to assist us with testing and clinical reporting:

Ethnic Origin: White ☐ Non Hispanic ☐ Hispanic Black ☐ African ☐ Afro-Caribbean

Asian ☐ Middle Eastern ☐ Sub-continent ☐ Oriental Other _____

Date of Diagnosis: _____

Undergoing Acute Infection: ☐ Yes ☐ No

Risk Exposure Group:

☐ Men who have sex with Men (MSM)
☐ Injecting drug user (IDU)
☐ MSM/ IDU
☐ Heterosexual contact
☐ Blood/ tissue Transfer
☐ Mother to Child
☐ Other _____

For Lab Use only Date: _____ RV Number: _____

Q18 Issue: v2.0 (EM: me) Page 1 of 1

ICVC Charitable Trust - Apollo Centre - Desborough Road – High Wycombe - HP11 2QW
Tel: (44) 01494 836 503 - Fax: (44) 01494 836 556

To ensure I understood the processes and procedures required to produce a resistance report, I was afforded the opportunity to shadow the laboratory technicians. These processes and procedures are briefly described below (Figures 2.2a-d: Steps 1-4, which were conducted by the trained laboratory technicians). I was trained by the Laboratory Manager to conduct data analyses and assist the laboratory technicians with interpreting the bi-directional and consensus sequences that were generated (see Figure 2.2e: Step 5 and Figure 2.3).

Sample collection, separation and storage

Whole blood samples were received at the ICVC either by Royal Mail, by Courier or by special delivery with a pre-arranged drop-off time (e.g. one of the major clinical centres used a daily taxi service to deliver their samples to the ICVC). On arrival of the samples, they were transferred to 'Lab 1' where each sample was carefully removed from its packaging: laboratory staff had to adhere to strict universal precautions when unpacking and handling the samples. Each sample was allocated a unique identifier number (RV number). Samples were processed and separated by centrifugation with the resultant plasma transferred to labelled (RV number and hospital number) Sarstedt 2mL sample tubes with the laboratory technician ensuring no blood was transferred to the tubes. These plasma samples could either be sent straight for VL/resistance testing or could be stored between -60°C and -80°C until testing took place. The ICVC strove to separate and store the plasma samples within six hours of reaching the laboratory to ensure the integrity of the sample for VL/resistance testing.

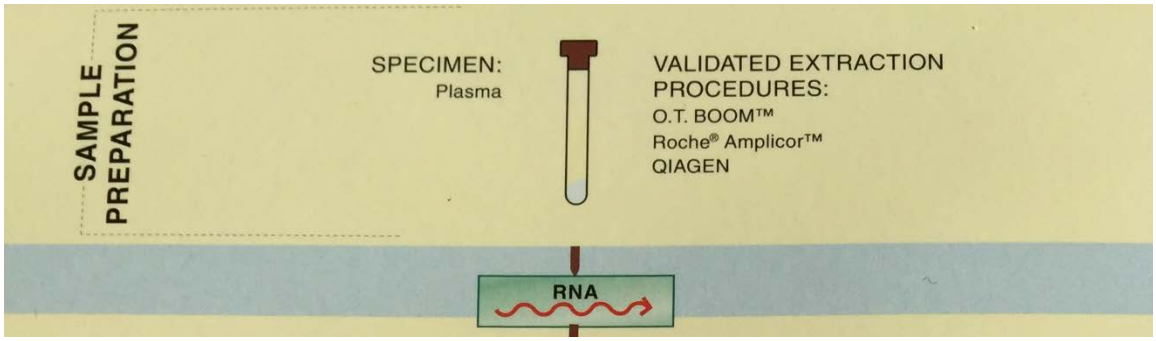
Genotype resistance testing at the ICVC

The ICVC laboratory undertook research into the application of new technologies that would allow the development and use of high-throughput genotypic resistance testing. The TRUGENE® HIV-1 assay was utilised (initially Visible Genetics Inc. (1996 to 2003), Bayer HealthCare LLC (2003 to 2007), and Siemens (2007 to current)). The TRUGENE® HIV-1 assay is a

molecular approach using HIV-1 from infected human plasma for HIV-1 viral RNA reverse transcription; polymerase chain reaction (PCR) amplification to provide complementary DNA (cDNA) for sequencing; and analysis of selected areas of the viral genome in the PR gene (codons 10-99) and in the RT gene (codons 38-247), in order to determine the presence or absence of mutations associated with resistance to the antiretroviral drugs.

The method involved a five step process:

Figure 2.2a: Step 1_SAMPLE PREPARATION: The extraction of HIV-1 viral RNA from infected human plasma (Visible Genetics Inc. 2001).



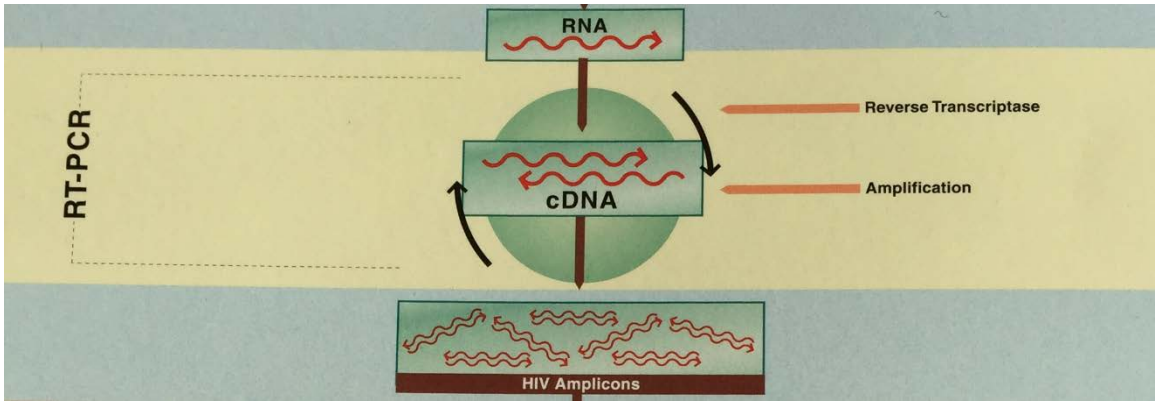
At the ICVC, this extraction process was carried out based on the QIAGEN QIAamp Viral RNA Mini Kit procedure (as shown above) and used the TruPrep™ kit with the ICVC's extraction protocol following the procedure described in the QIAGEN QIAamp Viral RNA Mini Handbook (see Appendix 1 for copy of protocol F10: ROCHE COBAS AMPLICOR HIV-1 MONITOR v1.5 ASSAY (STANDARD)).

Using the TruPrep™ kit, 140 microlitres (µL) of patient plasma (with a VL >1000c/mL) was required for the extraction of HIV-1 RNA. The process involved:

- viral lysis in the presence of RNA inhibitors to preserve viral HIV-1 RNA during the isolation process (due to the presence of ribonucleases (RNases)**,** these needed to be inactivated using Buffer AVL to ensure the isolation of intact viral RNA containing carrier RNA (cRNA))
- binding of viral RNA to the membrane in the TruPrep™ column

- washing of the TruPrep™ column with buffers (AW1 and AW2 to wash away any contaminants)
- the resulting RNA eluted in a special RNase-free buffer (AVE)
- product stored in the freezer between -60°C and -80°C.

Figure 2.2b: Step 2_RT-PCR: Reverse transcription and polymerase chain reaction (RT-PCR) of patients' extracted ribonucleic acid (RNA), to transcribe viral RNA into complementary deoxyribonucleic acid (cDNA) (Visible Genetics Inc. 2001).

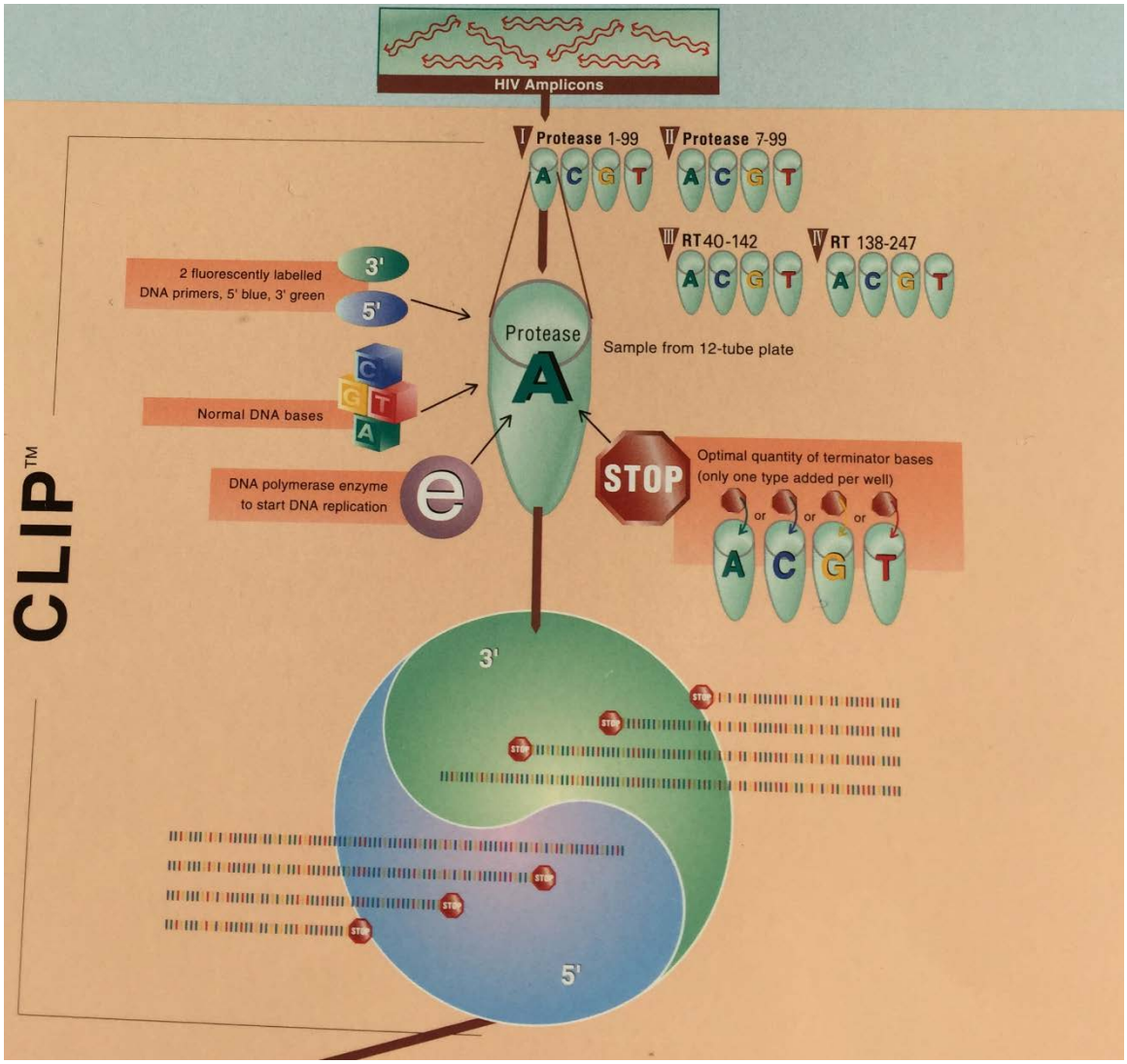


The process involved:

- introducing 17µL of the patient's HIV-1 RNA extract into PCR tubes containing RT-PCR master mix I (comprised of RT-PCR primers, deoxyribonucleotides (DNTPs), dithiothreitol (DTT) and RNase inhibitors)
- heating these tubes in a thermocycler to a temperature of 90°C for two minutes, followed by a cycle of 50°C for five minutes
- adding 14µL of RT-PCR master mix II (RT-PCR buffer, RNase inhibitor, RT-enzyme and DNA polymerase) to the bottom of each tube
- continuing with the 50°C cycle for 55 minutes, then 94°C for two minutes and reheating and cooling the sample through a further 38 cycles of the RT-PCR program:

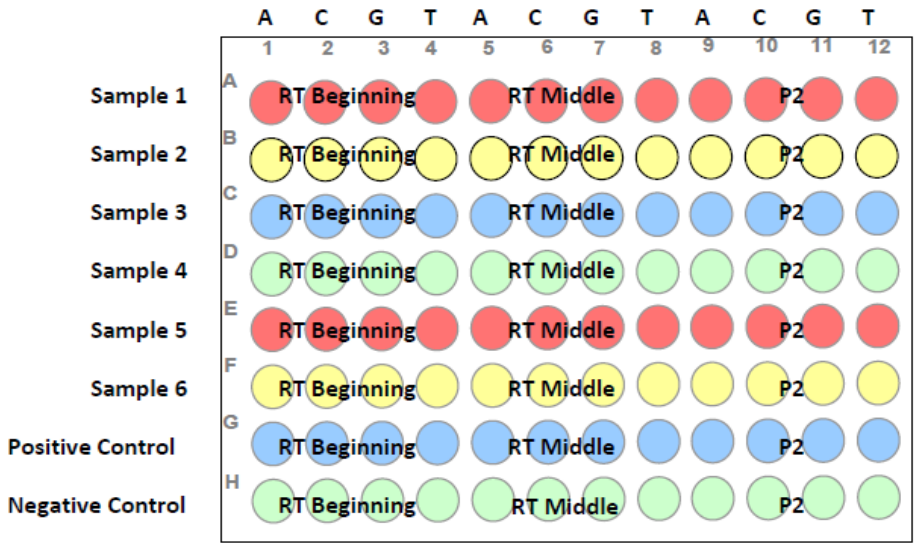
One cycle of:	20 cycles of:	17 cycles of:	One cycle of:
90°C for 2 min 50°C for 60 min 94°C for 2 min	94°C for 30 sec 57°C for 30 sec 68°C for 2 min	94°C for 30 sec 60°C for 30 sec 68°C for 2.5 min	68°C for 7 min 4°C Hold

Figure 2.2c: Step 3_CLIP™: The complementary amplified deoxyribonucleic acid (DNA) undergoes the CLIP™ reaction to sequence both sense (positive) and antisense (negative) strands of this double-stranded DNA; the method compares the sequence of sense and antisense to determine the true sequence of the clinical sample (Visible Genetics Inc. 2001).



The process involved:

- setting-up a 96-well plate containing 0.2mL thin walled strip tubes (see below)

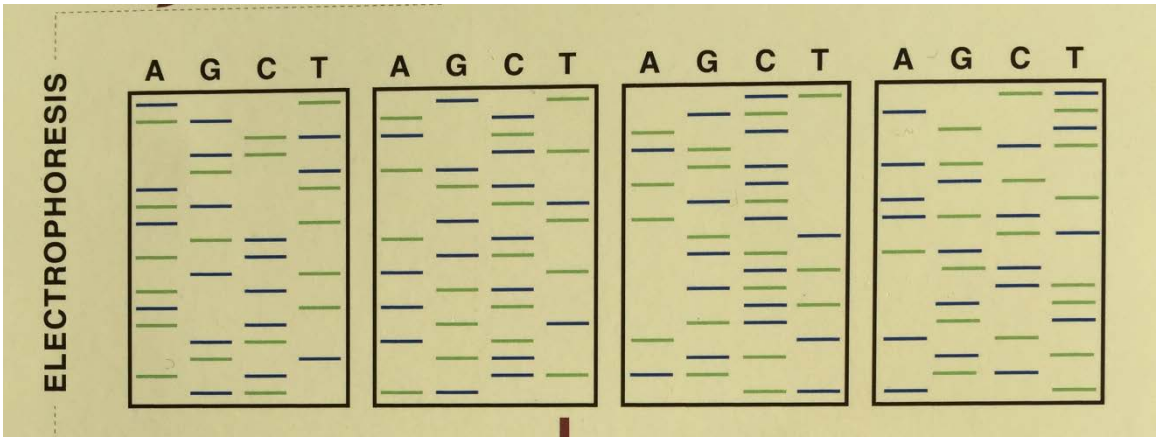


- adding 7µL of CLIP™ terminator mix to the bottom of each strip tube in the 96-well plate
- adding 5µL of RT-PCR product into a CLIP™ tube already containing 72µL of CLIP™ master mix (water, CLIP Buffer 1 and AmpliTaq FS)
- adding 5µL from each of these CLIP™ tubes to each appropriate well
- placing the plate in the thermocycler to undergo the CLIP™ cycle program:

94°C for 5 min	⇨	**30 cycles of:** 94°C for 20 sec 56°C for 20 sec 70°C for 1.5 min	⇨	**One cycle of:** 70°C for 5 min 4°C Hold

- adding 14µL of well mixed ‘Stop Loading Dye’ at the end of the cycle to each well
- denaturing the CLIP™ samples in the thermocycler for three minutes at 85°C to 95°C, to prevent any further reaction.

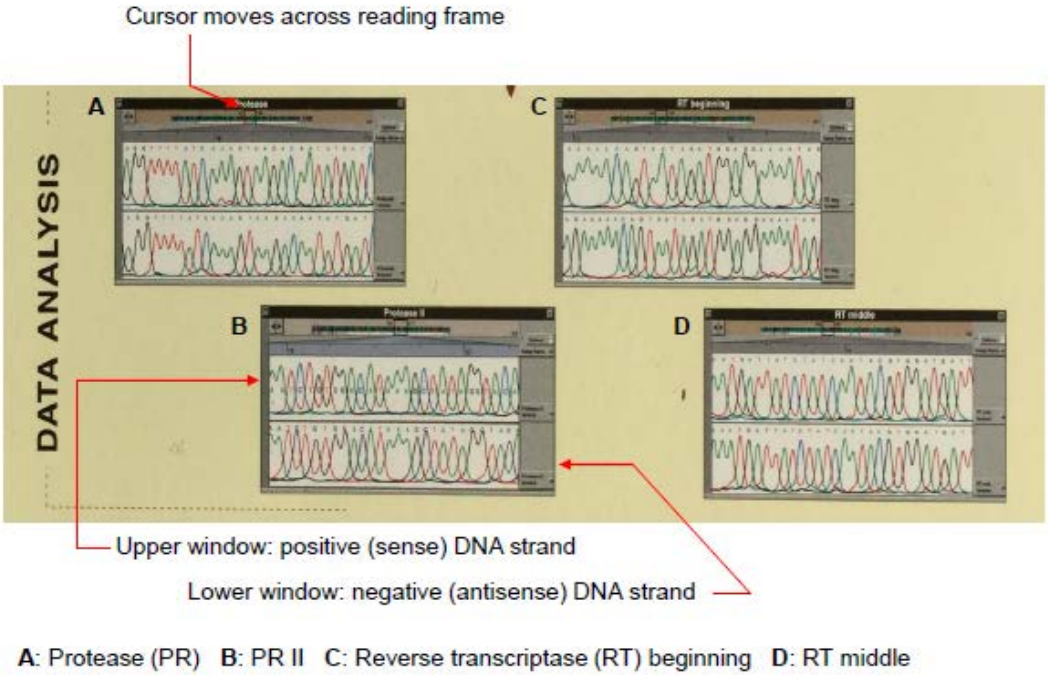
Figure 2.2d: Step 4_ELECTROPHORESIS: The heating of the sequencing reactions to denature the double-stranded deoxyribonucleic acid (DNA) fragments into two single-strands was undertaken on a thermocycler at 95°C (Visible Genetics Inc. 2001).



The process involved:

- filling a double-glass, ultra-thin sequencing plate (MicroCel 500 cassette) with polyacrylamide gel of 6.0% (SureFill®)
- placing the cassette into a sequencer for electrophoresis (Long-Read Tower® sequencer)
- adding fresh TBE buffer to the upper and lower chambers in the sequencer to complete the circuit
- pre-running the sequencer at 60°C for a maximum of 5-10 minutes to equilibriate the buffer and electrophoretic gel
- flushing the cassette with the buffer
- adding 1.5µL from each well, corresponding to one patient's sample, to the correct channel in the sequencing plate
- allowing the electrophoresis to run for 30 minutes.

Figure 2.2e: Step 5_DATA ANALYSIS: Bi-directional CLIP™ sequences were produced (Protease (PR), PR II, Reverse transcriptase (RT) beginning and RT middle) and interpreted by the laboratory technician to ensure consistency to produce a consensus sequence and ultimately a resistance report (Visible Genetics Inc. 2001).



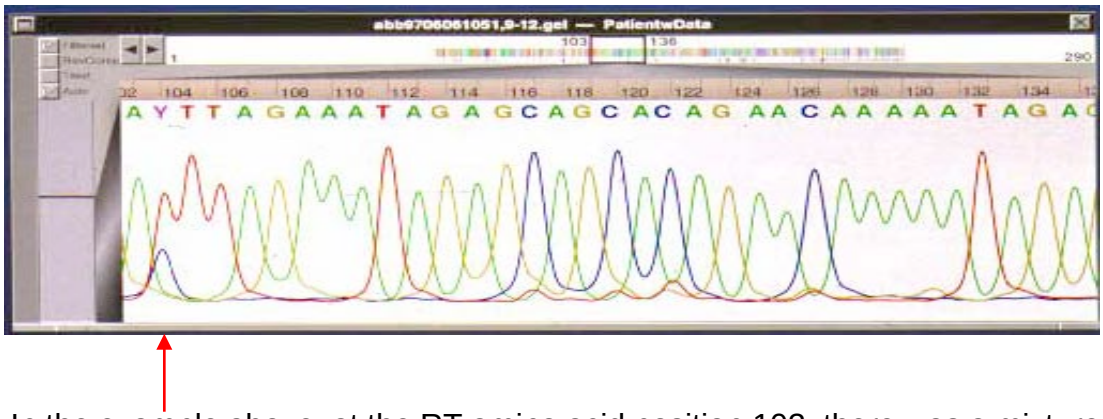
A: Protease (PR) **B**: PR II **C**: Reverse transcriptase (RT) beginning **D**: RT middle

The process involved:

- downloading the bi-directional sequences which were transferred automatically from the Towers and stored on the computer database (GeneObject™ Database) for the PR, PR II, RT beginning and RT middle sequences
- using the cursor to look across the reading frame to check the consistency of the sense versus antisense peaks (upper window: positive (sense) DNA strand, lower window: negative (antisense) DNA strand) for good alignment, good resolution and no significant baseline deviations
- using the dedicated software package (GeneObject™) to analyse for consistency against the HIV-1_{LAV-1} reference sequence (subtype B) and check for any insertions and/or deletions.

Once all checks were completed, the sense and antisense sequences were combined and a consensus sequence was produced and stored on the database (GeneObject™ Database).

Figure 2.3: An example of a consensus sequence of a patient's protease (PR) and reverse transcriptase (RT) genotype (Figure used with kind permission from Prof Clive Loveday and adapted by myself).



In the example above, at the RT amino acid position 103, there was a mixture evident in the peaks (C and T), indicating a mixture of mutant and WT at position 103.

If any base changes were identified, there were three possible outcomes:

- no change in the coded amino acid (silent polymorphism)
- a change in the coded amino acid at a non-relevant resistance site (polymorphism)
- a change in the coded amino acid at a resistance site (mutation).

The consensus sequence was further checked by the laboratory technician who could manually change the sequence: if this occurred, it was signaled by the computer by a change in the case of the identifying letter (small letter rather than capital). Although interpretation and analysis was dependent upon a number of defined rules and guidelines, the technique had a subjective element and the experience and skill of the laboratory technician was essential for the generation of high quality results. The checked consensus sequence was then submitted, interpreted and formatted into a resistance report by the

software package (GeneLibrarian™) producing a list of mutations present from codons 10 to 99 in the PR and 38 to 247 in the RT (Figure 2.4).

Figure 2.4: A patient's mutational profile. To exemplify, the Resistance Mutation highlighted in yellow indicates: the protease base position (PR 28), the wild-type (WT) codon (CTC), the amino acid position (L10I, with 'L' indicating the amino acid WT and 'I' the amino acid substitution conferring resistance), the change in the codon from the WT with the small letters indicating a manual change in the sequence by the laboratory technician (aTc) (Bayer HealthCare LLC 2003).

Resistance Mutations	**Silent Mutations (at all positions)**	**Polymorphisms: Coding changes not at resistance sites**	**Unexpected Mutations at resistance sites**
PR 28 (CTC) L10I (aTc) 136 (ATG) M46L (tTg) 187 (CTC) L63P (ccC) 268 (TTG) L90M(aTG) RT 541 (TAT) Y181Y/C (TrT) 550 (ATG) M184I/V (rTa) 628 (TTG) L210W (TGG) 643 (ACC) T215Y (TAC)	PR 52 (CAA) Q18Q (CAG) 97 (TTA) L33L (YTA) ** 151 (GGA) G51G (GGG) 280 (GGT) G94G (GGC) RT 133 (GGG) G45G (GGA) 232 (AGA) R78R (AGR) 250 (ACT) T84T (ACC) 289 (CCC) P97P (CCT) 310 (AAA) K104K (AAG) 319 (ACA) T107T (ACG) 346 (TTT) F116F (TTY) ** 394 (ATA) I132I (ATT) 412 (GAG) E138E (GAR) 418 (CCA) P140P (CCR) 502 (TTA) L168L (CTA) 580 (GAA) E194E (GAG) 589 (CAG) Q197Q (CAA) 610 (GAG) E204E (GAA) 637 (GGA) G213G (GGG) 658 (AAA) K220K (AAG) 679 (TTC) F227F (TTT) ** 709 (GAT) D237D (GAC)	PR 43 (ATA) I15V (GTA) 103 (GAA) E35E/D (GAM) 109 (AGT) S37N (AAT) 121 (AGA) R41K/R (ARA) 172 (CAG) Q58E (GAG) 214 (ATA) I72V (GTA) RT 127 (AAG) K43Q (CAG) 178 (GTA) V60I (ATA) 361 (GAT) D121Y (TAT) 403 (ATA) I135T (ACA) 406 (AAC) N136K/N/T (AMM) 454 (GGA) G152G/X (KGA) 484 (AGT) S162A (GCT) 586 (GGG) G196E (GAG) 607 (GAG) E203K (AAG) 619 (CAA) Q207E (GAA) 631 (AGG) R211K (AAG) 640 (CTT) L214F (TTT) 724 (CAG) Q242Q/L (CWG) 733 (GTG) V245A/V (GYG)	NONE

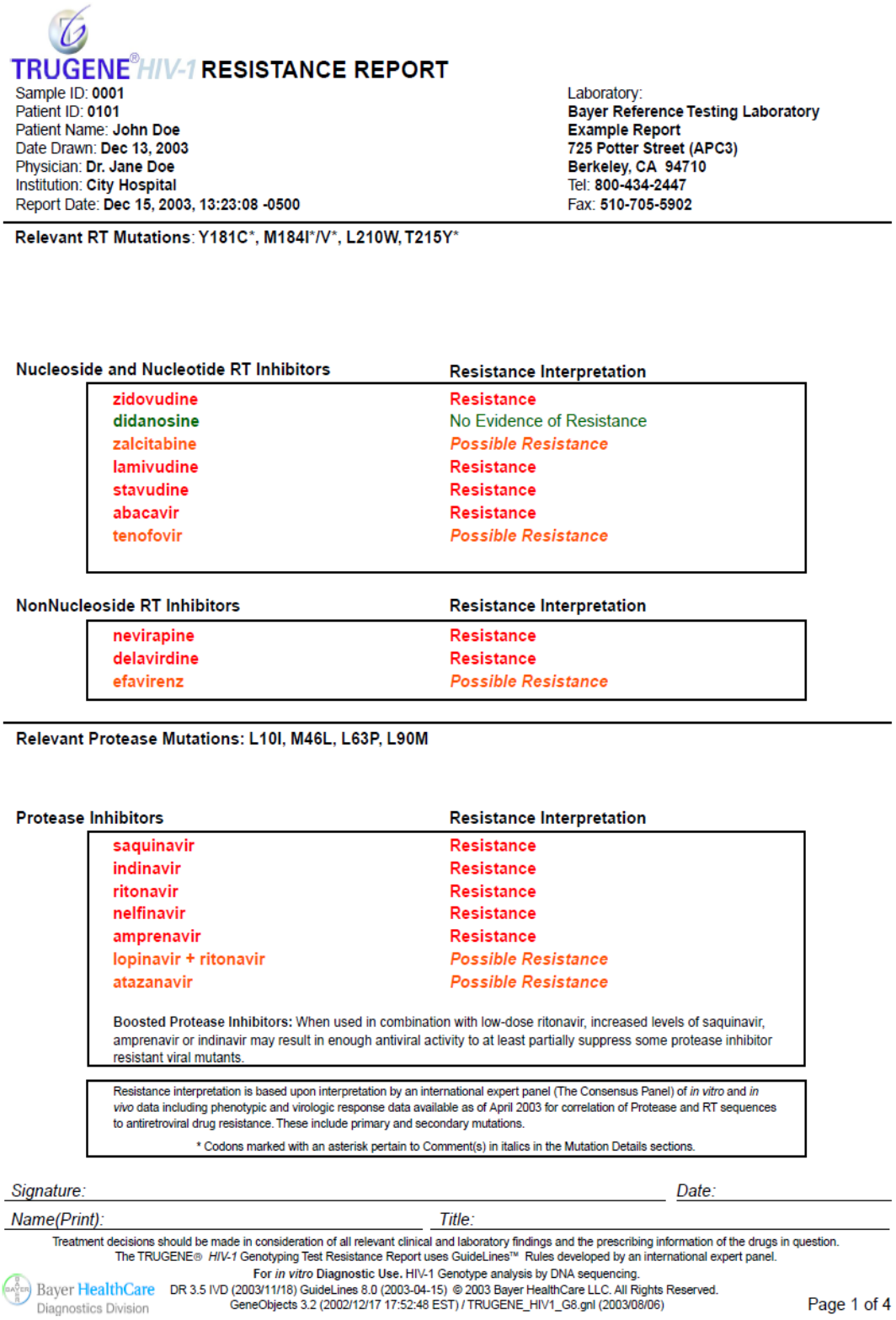
**Indicates a silent mutation at a resistance site.

The effects of these mutations on the individual antiretroviral therapies were interpreted by the software (GuideLines Rules) to determine whether the mutations conferred 'Resistance' (the patient's virus was no longer susceptible to the specific drug); 'Possible Resistance' (the patient's virus may no longer be susceptible to the specific drug) or 'No Evidence of Resistance' (the mutations present do not confer resistance). The software (GeneLibrarian™) produced a resistance report providing: a colour-coded, easy-to-read front

page consisting of the resistance interpretation (Figure 2.5a); the evidence source on which the software's algorithm based its interpretation; and an indication of the strength of the evidence used to determine the interpretation rules (Figure 2.5b-d).

These GuideLines Rules were updated annually, according to the Food and Drug Administration (FDA) regulations, by a selected panel of 'world-renowned HIV experts'. The experts met once a year and considered the latest clinical and research resistance data, including *in vivo* virological response and *in vitro* phenotypic data (published or abstracted); they then altered the rules for the TRUGENE® GeneLibrarian™ system accordingly and ran dummy cases to ensure there was no ambiguity in the interpretation. The new database for interpretation was then circulated worldwide to everyone running the TRUGENE® system (http://www.medical.siemens.com).

Figure 2.5a: Front cover of the TRUGENE® HIV-1 resistance report providing a colour co-ordinated, at-a-glance resistance summary of mutations present and the interpreted resistance level (Resistance, *Possible Resistance*, No Evidence of Resistance) per drug class (Bayer HealthCare LLC 2003).



TRUGENE® *HIV-1* RESISTANCE REPORT

Sample ID: **0001**
Patient ID: **0101**
Patient Name: **John Doe**
Date Drawn: **Dec 13, 2003**
Physician: **Dr. Jane Doe**
Institution: **City Hospital**
Report Date: **Dec 15, 2003, 13:23:08 -0500**

Laboratory:
Bayer Reference Testing Laboratory
Example Report
725 Potter Street (APC3)
Berkeley, CA 94710
Tel: **800-434-2447**
Fax: **510-705-5902**

Relevant RT Mutations: Y181C*, M184I*/V*, L210W, T215Y*

Nucleoside and Nucleotide RT Inhibitors	Resistance Interpretation
zidovudine	Resistance
didanosine	No Evidence of Resistance
zalcitabine	*Possible Resistance*
lamivudine	Resistance
stavudine	Resistance
abacavir	Resistance
tenofovir	*Possible Resistance*

NonNucleoside RT Inhibitors	Resistance Interpretation
nevirapine	Resistance
delavirdine	Resistance
efavirenz	*Possible Resistance*

Relevant Protease Mutations: L10I, M46L, L63P, L90M

Protease Inhibitors	Resistance Interpretation
saquinavir	Resistance
indinavir	Resistance
ritonavir	Resistance
nelfinavir	Resistance
amprenavir	Resistance
lopinavir + ritonavir	*Possible Resistance*
atazanavir	*Possible Resistance*

Boosted Protease Inhibitors: When used in combination with low-dose ritonavir, increased levels of saquinavir, amprenavir or indinavir may result in enough antiviral activity to at least partially suppress some protease inhibitor resistant viral mutants.

Resistance interpretation is based upon interpretation by an international expert panel (The Consensus Panel) of *in vitro* and *in vivo* data including phenotypic and virologic response data available as of April 2003 for correlation of Protease and RT sequences to antiretroviral drug resistance. These include primary and secondary mutations.

* Codons marked with an asterisk pertain to Comment(s) in italics in the Mutation Details sections.

Signature: *Date:*

Name(Print): *Title:*

Treatment decisions should be made in consideration of all relevant clinical and laboratory findings and the prescribing information of the drugs in question.
The TRUGENE® *HIV-1* Genotyping Test Resistance Report uses GuideLines™ Rules developed by an international expert panel.
For *in vitro* Diagnostic Use. HIV-1 Genotype analysis by DNA sequencing.

Bayer HealthCare Diagnostics Division

DR 3.5 IVD (2003/11/18) GuideLines 8.0 (2003-04-15) © 2003 Bayer HealthCare LLC. All Rights Reserved.
GeneObjects 3.2 (2002/12/17 17:52:48 EST) / TRUGENE_HIV1_G8.gnl (2003/08/06)

Page 1 of 4

Figure 2.5b: List of specific TRUGENE® HIV-1 resistance report rules and Evidence Basis for the interpretation of the mutations seen and the resistance level assigned, per drug (nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs)) (Bayer HealthCare LLC 2003).

TRUGENE® *HIV-1* RESISTANCE REPORT

Sample ID: **0001**
Patient ID: **0101**
Patient Name: **John Doe**
Date Drawn: **Dec 13, 2003**
Physician: **Dr. Jane Doe**
Institution: **City Hospital**
Report Date: **Dec 15, 2003, 13:23:08 -0500**

Laboratory:
Bayer Reference Testing Laboratory
Example Report
725 Potter Street (APC3)
Berkeley, CA 94710
Tel: **800-434-2447**
Fax: **510-705-5902**

Mutation Details for Nucleoside and Nucleotide RT Inhibitors:	Evidence Basis
zidovudine	
RT M184V may reduce the level of zidovudine, stavudine or tenofovir resistance measured in vitro. Some trials suggest it may delay emergence of zidovudine or stavudine resistance and improve virologic responses to zidovudine or stavudine for an uncertain duration. (NRTI-33)	IIa
RT Y181C may reduce the level of zidovudine resistance measured in vitro. (NRTI-35)	IIIa
T215Y indicates Resistance to zidovudine (NRTI-16)	Ia
didanosine	
RT M184I/V may cause diminished didanosine susceptibility of uncertain impact on clinical response. (NRTI-05)	IIb
zalcitabine	
There are limited comprehensive clinical data to determine a resistance effect determination for zalcitabine.	
M184I/V indicate Possible Resistance to zalcitabine (NRTI-06)	IIIb
lamivudine	
M184I/V indicate Resistance to lamivudine (NRTI-10)	Ia
stavudine	
RT M184V may reduce the level of zidovudine, stavudine or tenofovir resistance measured in vitro. Some trials suggest it may delay emergence of zidovudine or stavudine resistance and improve virologic responses to zidovudine or stavudine for an uncertain duration. (NRTI-33)	IIIa
T215Y indicates Resistance to stavudine (NRTI-16)	IIa
abacavir	
M184I/V, L210W, and T215Y indicate Resistance to abacavir (NRTI-02)	IIa
M184I/V, L210W, and T215Y indicate Resistance to abacavir (NRTI-03)	IIa
L210W and T215Y indicate Possible Resistance to abacavir (NRTI-01)	IIa
M184I/V, L210W, and T215Y indicate Possible Resistance to abacavir (NRTI-07)	IIa
tenofovir	
RT M184V may reduce the level of zidovudine, stavudine or tenofovir resistance measured in vitro. Some trials suggest it may delay emergence of zidovudine or stavudine resistance and improve virologic responses to zidovudine or stavudine for an uncertain duration. (NRTI-33)	IIIa
L210W and T215Y indicate Possible Resistance to tenofovir (NRTI-20)	IIa

Mutation Details for NonNucleoside RT Inhibitors:	Evidence Basis
There is extensive clinical cross-resistance within the NNRTI drug class.	
nevirapine	
Y181C indicates Resistance to nevirapine (NNRTI-04)	IIa
Y181C indicates Possible Resistance to nevirapine (NNRTI-01)	IIIa
delavirdine	
There are limited comprehensive clinical data to determine a resistance effect determination for delavirdine.	
Y181C indicates Resistance to delavirdine (NNRTI-05)	IIa

NOTE: The strongest resistance effect will be reported on the profile page if different resistance levels are detailed.

Treatment decisions should be made in consideration of all relevant clinical and laboratory findings and the prescribing information of the drugs in question.
The TRUGENE® *HIV-1* Genotyping Test Resistance Report uses GuideLines™ Rules developed by an international expert panel.
For *in vitro* Diagnostic Use. HIV-1 Genotype analysis by DNA sequencing.
Bayer HealthCare Diagnostics Division
DR 3.5 IVD (2003/11/18) GuideLines 8.0 (2003-04-15) © 2003 Bayer HealthCare LLC. All Rights Reserved.
GeneObjects 3.2 (2002/12/17 17:52:48 EST) / TRUGENE_HIV1_G8.gnl (2003/08/06)
Page 2 of 4

Figure 2.5c: List of specific TRUGENE® HIV-1 resistance report rules and Evidence Basis for the interpretation of the mutations seen and the resistance level assigned, per drug (non-nucleoside reverse transcriptase inhibitors (NNRTIs) contd. and protease inhibitors (PIs)) (Bayer HealthCare LLC 2003).

TRUGENE® *HIV-1* **RESISTANCE REPORT**

Sample ID: **0001**
Patient ID: **0101**
Patient Name: **John Doe**
Date Drawn: **Dec 13, 2003**
Physician: **Dr. Jane Doe**
Institution: **City Hospital**
Report Date: **Dec 15, 2003, 13:23:08 -0500**

Laboratory:
Bayer Reference Testing Laboratory
Example Report
725 Potter Street (APC3)
Berkeley, CA 94710
Tel: **800-434-2447**
Fax: **510-705-5902**

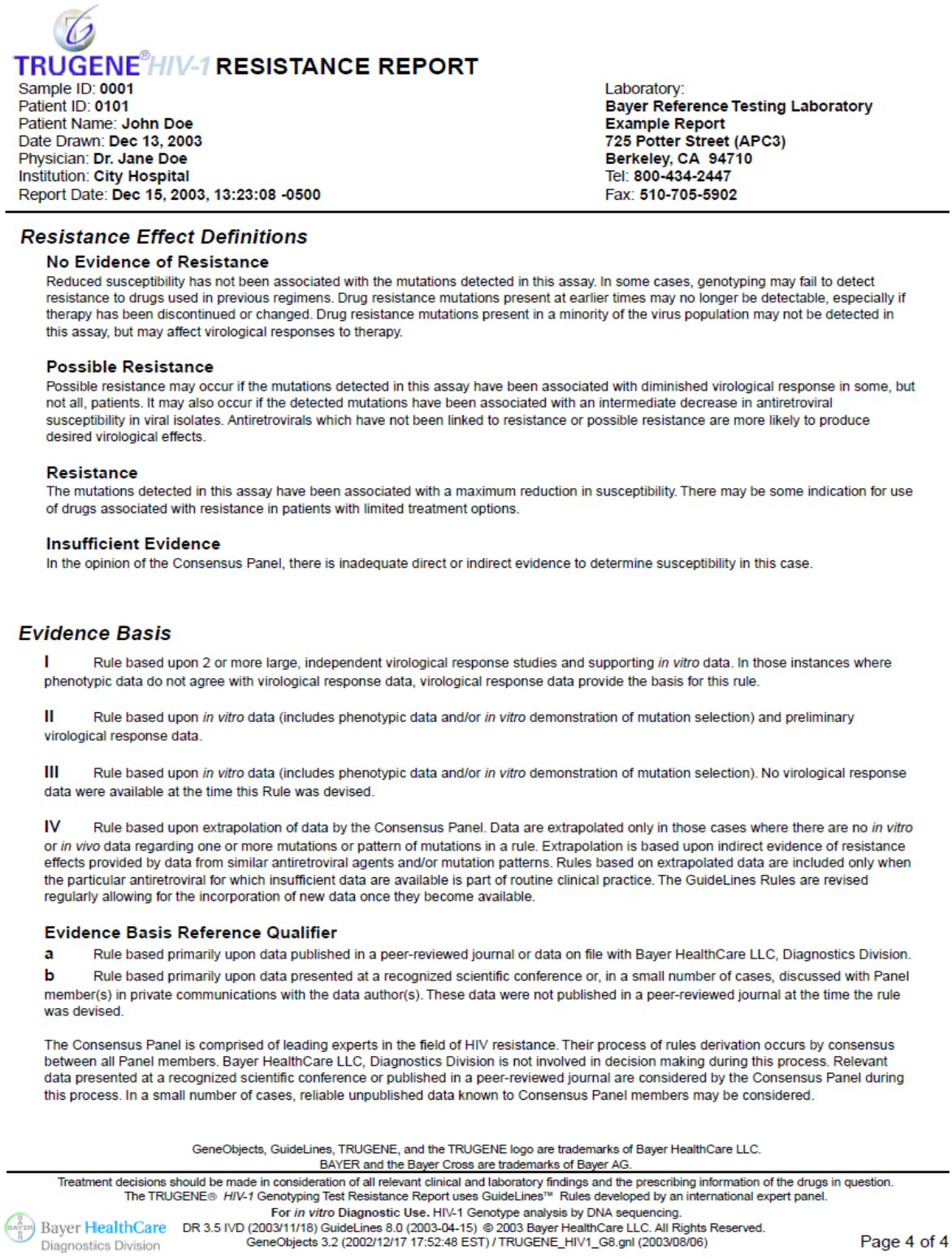
Mutation Details for NonNucleoside RT Inhibitors:	**Evidence Basis**
Y181C indicates Possible Resistance to delavirdine (NNRTI-01)	IIIa
efavirenz	
There is no evidence of long-term virologic benefit in sequential use of NNRTIs in the setting of NNRTI mutations.	
Y181C indicates Possible Resistance to efavirenz (NNRTI-01)	IIIa

Mutation Details for Protease Inhibitors:	**Evidence Basis**
Boosted Protease Inhibitors: When used in combination with low-dose ritonavir, increased levels of saquinavir, amprenavir or indinavir may result in enough antiviral activity to at least partially suppress some protease inhibitor resistant viral mutants. Data do not yet allow reliable prediction of which sets of multiple protease gene mutations can mediate resistance to in vivo suppressive effects of ritonavir-boosted saquinavir, amprenavir or indinavir.	
saquinavir	
L90M indicates Resistance to saquinavir (PI-03)	IIa
M46L and L90M indicate Resistance to saquinavir (PI-14)	Ia
M46L and L90M indicate Resistance to saquinavir (PI-32)	Ia
indinavir	
M46L and L90M indicate Resistance to indinavir (PI-12)	Ia
M46L and L90M indicate Resistance to indinavir (PI-32)	IIa
M46L indicates Possible Resistance to indinavir (PI-09)	IIa
L90M indicates Possible Resistance to indinavir (PI-31)	Ia
ritonavir	
M46L and L90M indicate Resistance to ritonavir (PI-12)	Ia
M46L and L90M indicate Resistance to ritonavir (PI-32)	IIa
M46L indicates Possible Resistance to ritonavir (PI-09)	IIa
L90M indicates Possible Resistance to ritonavir (PI-31)	IIa
nelfinavir	
L90M indicates Resistance to nelfinavir (PI-02)	IIa
M46L and L90M indicate Resistance to nelfinavir (PI-12)	IIa
M46L and L90M indicate Resistance to nelfinavir (PI-32)	IIa
amprenavir	
M46L and L90M indicate Resistance to amprenavir (PI-32)	IIIa
M46L indicates Possible Resistance to amprenavir (PI-09)	IIIa
lopinavir + ritonavir	
M46L and L90M indicate Possible Resistance to lopinavir + ritonavir (PI-13)	IIIa
M46L and L90M indicate Possible Resistance to lopinavir + ritonavir (PI-33)	IIIa
atazanavir	
Mutations associated with resistance to atazanavir have not yet been well characterized.	
L90M indicates Possible Resistance to atazanavir (PI-41)	IIIb

NOTE: The strongest resistance effect will be reported on the profile page if different resistance levels are detailed.

Treatment decisions should be made in consideration of all relevant clinical and laboratory findings and the prescribing information of the drugs in question.
The TRUGENE® *HIV-1* Genotyping Test Resistance Report uses GuideLines™ Rules developed by an international expert panel.
For *in vitro* Diagnostic Use. HIV-1 Genotype analysis by DNA sequencing.
Bayer HealthCare Diagnostics Division
DR 3.5 IVD (2003/11/18) GuideLines 8.0 (2003-04-15) © 2003 Bayer HealthCare LLC. All Rights Reserved.
GeneObjects 3.2 (2002/12/17 17:52:48 EST) / TRUGENE_HIV1_G8.gnl (2003/08/06)
Page 3 of 4

Figure 2.5d: Definitions of the TRUGENE® HIV-1 resistance report Resistance Effect levels and Evidence Basis levels (Bayer HealthCare LLC 2003).



TRUGENE® *HIV-1* RESISTANCE REPORT

Sample ID: **0001**
Patient ID: **0101**
Patient Name: **John Doe**
Date Drawn: **Dec 13, 2003**
Physician: **Dr. Jane Doe**
Institution: **City Hospital**
Report Date: **Dec 15, 2003, 13:23:08 -0500**

Laboratory:
Bayer Reference Testing Laboratory
Example Report
725 Potter Street (APC3)
Berkeley, CA 94710
Tel: **800-434-2447**
Fax: **510-705-5902**

Resistance Effect Definitions

No Evidence of Resistance
Reduced susceptibility has not been associated with the mutations detected in this assay. In some cases, genotyping may fail to detect resistance to drugs used in previous regimens. Drug resistance mutations present at earlier times may no longer be detectable, especially if therapy has been discontinued or changed. Drug resistance mutations present in a minority of the virus population may not be detected in this assay, but may affect virological responses to therapy.

Possible Resistance
Possible resistance may occur if the mutations detected in this assay have been associated with diminished virological response in some, but not all, patients. It may also occur if the detected mutations have been associated with an intermediate decrease in antiretroviral susceptibility in viral isolates. Antiretrovirals which have not been linked to resistance or possible resistance are more likely to produce desired virological effects.

Resistance
The mutations detected in this assay have been associated with a maximum reduction in susceptibility. There may be some indication for use of drugs associated with resistance in patients with limited treatment options.

Insufficient Evidence
In the opinion of the Consensus Panel, there is inadequate direct or indirect evidence to determine susceptibility in this case.

Evidence Basis

I Rule based upon 2 or more large, independent virological response studies and supporting *in vitro* data. In those instances where phenotypic data do not agree with virological response data, virological response data provide the basis for this rule.

II Rule based upon *in vitro* data (includes phenotypic data and/or *in vitro* demonstration of mutation selection) and preliminary virological response data.

III Rule based upon *in vitro* data (includes phenotypic data and/or *in vitro* demonstration of mutation selection). No virological response data were available at the time this Rule was devised.

IV Rule based upon extrapolation of data by the Consensus Panel. Data are extrapolated only in those cases where there are no *in vitro* or *in vivo* data regarding one or more mutations or pattern of mutations in a rule. Extrapolation is based upon indirect evidence of resistance effects provided by data from similar antiretroviral agents and/or mutation patterns. Rules based on extrapolated data are included only when the particular antiretroviral for which insufficient data are available is part of routine clinical practice. The GuideLines Rules are revised regularly allowing for the incorporation of new data once they become available.

Evidence Basis Reference Qualifier

a Rule based primarily upon data published in a peer-reviewed journal or data on file with Bayer HealthCare LLC, Diagnostics Division.

b Rule based primarily upon data presented at a recognized scientific conference or, in a small number of cases, discussed with Panel member(s) in private communications with the data author(s). These data were not published in a peer-reviewed journal at the time the rule was devised.

The Consensus Panel is comprised of leading experts in the field of HIV resistance. Their process of rules derivation occurs by consensus between all Panel members. Bayer HealthCare LLC, Diagnostics Division is not involved in decision making during this process. Relevant data presented at a recognized scientific conference or published in a peer-reviewed journal are considered by the Consensus Panel during this process. In a small number of cases, reliable unpublished data known to Consensus Panel members may be considered.

GeneObjects, GuideLines, TRUGENE, and the TRUGENE logo are trademarks of Bayer HealthCare LLC.
BAYER and the Bayer Cross are trademarks of Bayer AG.

Treatment decisions should be made in consideration of all relevant clinical and laboratory findings and the prescribing information of the drugs in question.
The TRUGENE® *HIV-1* Genotyping Test Resistance Report uses GuideLines™ Rules developed by an international expert panel.
For *in vitro* Diagnostic Use. HIV-1 Genotype analysis by DNA sequencing.

Bayer HealthCare Diagnostics Division

DR 3.5 IVD (2003/11/18) GuideLines 8.0 (2003-04-15) © 2003 Bayer HealthCare LLC. All Rights Reserved.
GeneObjects 3.2 (2002/12/17 17:52:48 EST) / TRUGENE_HIV1_G8.gnl (2003/08/06)

Page 4 of 4

Interpretation of the resistance report by the ICVC's Clinical Director

At the ICVC, before any resistance reports were disseminated to the collaborating centres, each report generated was checked and signed-out by the Clinical Director who would interpret the resistance report with reference to the clinical data provided by the clinician requesting the resistance test. The collaborating centres were encouraged to contact the Clinical Director to discuss any of their patients' resistance reports and future options in order to ensure the best clinical care for their patient.

The ICVC Clinical Cohort Resistance Database

Using the Statistical Package for the Social Sciences (SPSS, initially version 12.1 for Windows through to version 22), I designed and developed the ICVC Clinical Cohort Resistance Database to integrate the clinical and resistance data for each patient.

Before data entry, each patients' resistance 'package' (request form and resistance report) was assigned a unique SPSS identifier. One 'package' was entered at a time. Clinical data provided on the request form were entered first: no patient names were included on the ICVC Clinical Cohort Resistance Database. The patients were uniquely identified based on the SPSS identifier assigned to them, along with the unique sample identifier (RV number) assigned by the ICVC's laboratory, and their hospital number. If the clinician did not complete a field on the resistance request form, the clinical variable was left blank on the database. The corresponding list of PR and RT resistance mutations and polymorphisms were then entered (see Figure 2.4).

Using the clinical data provided, the resistance database provided a powerful tool for conducting cohort analyses. Simple analyses could be conducted e.g. male/female groupings; looking at a hospital's resistance profile over time; looking at a geographical region's resistance profile over time; looking at the frequency of specific mutations/polymorphisms in the database.

The database could be interrogated, allowing more complex analyses e.g. using the 'major reason for test' and 'drug' variables or patients could be identified and grouped as treatment-naïve or treatment-experienced. Patients who were classed as treatment-experienced, based on the clinical data provided, could be further grouped into those on their first-line treatment regimen, but who were failing, or those on their second or third-line treatment regimens. The resistance profiles of these groups could then be compared.

Using FASTA sequences to determine patients' subtype

The TRUGENE® system generated a FASTA '*pol*' (PR and RT) sequence for each patients' genotype. This is a shorthand sequential description of the sequence carried out in the machine which can be easily transmitted between laboratory sites and online analytical tools.

Figure 2.6: An example of a patient's FASTA sequence as generated by the TRUGENE® genotype system (Figure used with kind permission from Prof Clive Loveday).
The first line of the sequence indicated the hospital number of the patient (M94-0099), the unique sample identifier (RV number) assigned by the ICVC's laboratory (65165), the date and time the report was generated (200401261109) and the nucleotides which were analysed: PR (protease) (10-297), RT (reverse transcriptase) (112-741). After this, the first part of the sequence represented the PR nucleotides with the long list of 'N's' confirming the transition between the PR and RT sequence.

>M94-0099|65165|200401261109||VGI nucleotide|PR(10-297),RT(112-741)I+D0
NNNNNNNNNACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGcCAgATAAA
GGAGGCTCTCTTAGACACAGGAGCAGATGATACAGTATTAGARGArATAAATTTRCCAG
GAAAATGGAAACCAAAAATGATAGGRGGAATTGGAGGTTTTATCAAAGTAAGACAGTAT
GATCAAATACTCATAGAAATTTGTGGAAAAAAGGCTATAGGTACAGTATTAGTAGGACC
TACACCTGTCAACATAATTGGAAGAAAyATGTTGACTCAGCTTGGATGCACACTAAACTT
YNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
TGTGAAGAAATGGAGAAGGAAGGAAAAATTACAAAAATWGGGCCTgAAAatCCATATAA
CACTCCAGTGTTTGCCATAAAAAAGAAGGACAGTACTAARTGGAGAAAATTAGTAGATT
TCAGGGARCTTAATAARAGAACTCAAGACTTYTGGGAAGTTCAATTAGGAATACCCCAC
CCAGCAGGGTTAAAAAAGAAAAAATCAGTGACAGTACTAGAYGTGGGAGATGCATATTT
TTCAGTTCCTTTAGATAAAGACTTCAGGAARTATACTGCATTcACcATACCTAGTATAAAC
AATGAAACACcAGGGATTAGATATCAATAYAATGTRCTTCCACAGGGATGGAAAGGATC
ACCAGCAATATTCCAGAGTAGCATGACAAAAATCTTAGAGCCCTTTAGGGCACAAAATC
CAGGAATAGTCATCTATCAATATATGGATGACTTGTATGTAGGATCTGACTTAGAAATAG
GGCAACATAGAGCAAAAATAGAAGAGTTAAGAGAACATCTATTGAAGTGGGGATTTACC
ACACCAGATAAGAAACATCAGAAAGAACCCCCATTTCTTTGGATGGGGTATGAACTCCA
TCCTGACAAATGGACAGTACAGCCTATAGAGCTGCCA

Although the gold standard for classification of HIV-1 subtypes should be based on phylogenetic analysis of full-length genome sequences (Robertson et al 1999) in clinical practice, this is not practical. FASTA '*pol*' (PR and RT) sequences can be downloaded from GeneLibrarian™ and entered into subtype tools available freely on the internet to determine subtype. Subtype characterisation (using five online tools to determine subtype) and interpretation are presented in Chapter 7: the evolution of subtype profiles in the clinical cohort.

Quality control of the data

All data analysed in this thesis were entered into the ICVC Clinical Cohort Resistance Database by myself. I ensured spot-checks of the data entry occurred every three months and that audits of the data were conducted to ensure accuracy. As data collection spanned 10 years, some of the patients on the database had a number of resistance reports conducted over this timeframe; this too was a good way of checking the clinical information provided, as well as the reported mutations and polymorphisms, as I could determine if there were any major discrepancies in the data that needed to be reviewed.

In conclusion, using the ICVC Clinical Cohort Resistance Database, I conducted a series of clinical cohort studies to determine whether the clinical care of patients with HIV-1 could be improved and was responsible for:

- forming the research idea/question
- designing how to identify sub-groups of patients in the database
- setting the inclusion/exclusion criteria
- devising the analysis plan including the clinical variables/demographics to analyse; the relevant mutations (polymorphisms) to analyse
- interpreting and presenting the findings.

The ICVC Clinical Cohort Resistance Database collection commenced in 1996 and, for the purposes of this thesis, continued up to the end of 2006, providing ten years of continuous data collection. The ICVC Clinical Cohort Resistance Database holds 3,573 entries for 2,785 patients with HIV-1 infection, whose plasma samples were submitted for investigation of genotypic resistance as part of their clinical care.

Distribution of the ICVC Collaborative Research Group

The ICVC conducted resistance testing for a wide, geographically distributed group of clinics (n=39), of varying sizes, throughout the UK. Figure 3.1 highlights the areas across the UK which formed the ICVC Collaborative Research Group with the specific city/town/borough listed by geographical region in Table 3.1.

Figure 3.1: Map showing the distribution of the clinical centres across the United Kingdom (UK) that formed the International Clinical Virology Centre (ICVC) Collaborative Research Group.

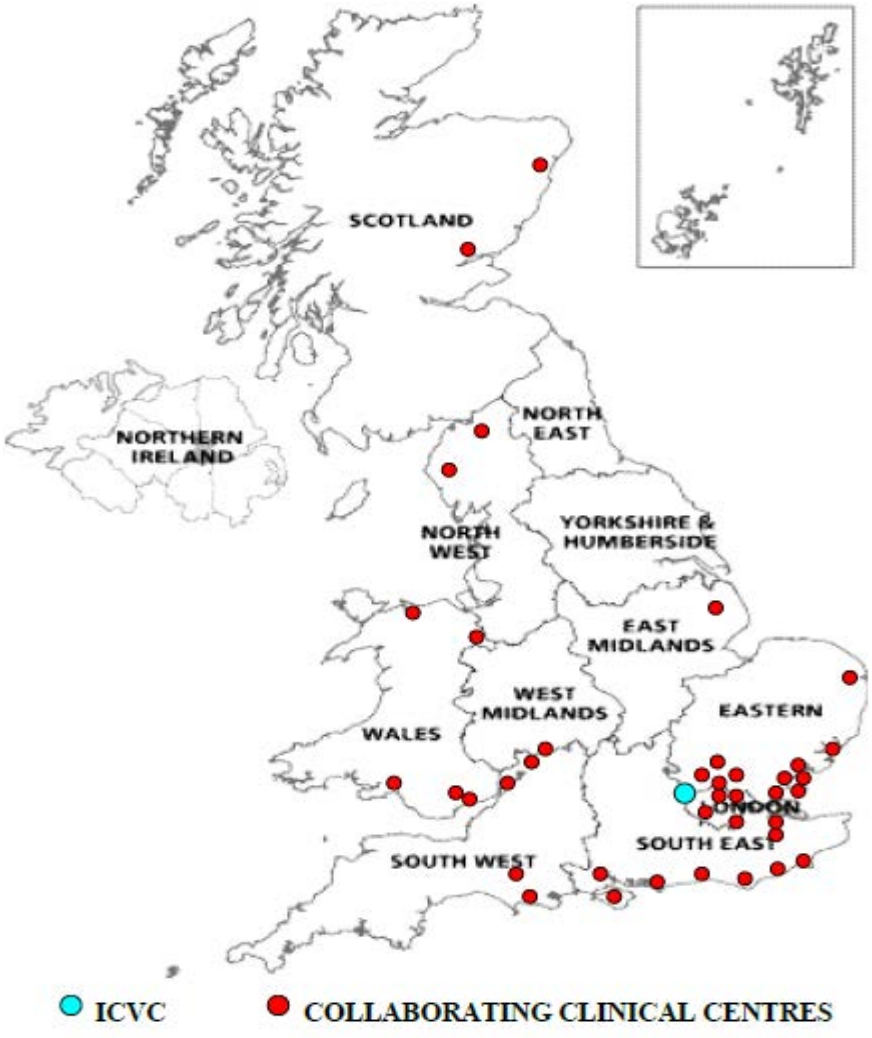


Table 3.1: Breakdown of the International Clinical Virology Centre (ICVC) Collaborative Research Group by geographical region and city/town/borough where the clinical centre was based. The majority of the clinical centres were from the Greater London area (64.3%).

Geographical region: city/town/borough where clinical centre based	n	%
Scotland	**35**	**1.0**
Aberdeen	34	
Dundee	1	
North West	**30**	**0.8**
Carlisle	20	
Workington	10	
Wales	**153**	**4.3**
Wrexham	5	
Rhyl	16	
Swansea	55	
Cardiff	75	
Newport, Gwent	2	
East Midlands	**51**	**1.4**
Lincoln	51	
Eastern	**325**	**9.1**
Great Yarmouth	38	
Colchester	84	
Chelmsford	5	
Hertford/Lister	117	
Watford	81	
Greater London	**2,299**	**64.3**
Barnet	4	
Beckenham	63	
Ealing	374	
Lewisham	115	
North Middlesex	29	
Romford	30	
Hampstead	1,022	
West Smithfield, City of London	457	
Whipps Cross	205	
South East	**590**	**16.5**
Southampton	2	
Isle of Wight	46	
Portsmouth	258	
Worthing	29	
Brighton	128	
Eastbourne	10	
St. Leonards-on-Sea	4	
Slough	88	
High Wycombe	20	
Aylesbury	5	
South West	**90**	**2.5**
Bristol	1	
Gloucester	32	
Cheltenham	28	
Dorset	4	
Weymouth	25	
Total	**3,573**	100.0

Of the 2,785 patients, the majority had one resistance entry only n=2,237 (80.3%), with the complete breakdown presented in Table 3.2.

Table 3.2: Number of resistance entries per patient in the International Clinical Virology Centre (ICVC) Clinical Cohort Resistance Database showed that the majority only had one resistance data entry in the database.

Number of resistance entries per patient	n	%
1 entry	2,237	80.3
2 entries	382	13.7
3 entries	114	4.1
4 entries	34	1.2
5 entries	14	0.5
6 entries	4	0.1
Total	2,785	100.0

Resistance request form

Table 3.3 illustrates the completion rates of the different clinical variables on the resistance request form. Apart from the clinicians indicating the 'major reason for requesting a genotypic resistance test', there was less than 100.0% completion of the fields on the resistance request forms by clinicians.

Table 3.3: Completion of the resistance request form showed great variability in data provided by the clinicians requesting a genotypic resistance test for their patients.

Clinical variable	n=2,785 patients with HIV-1			
	Available data		Missing data	
	n	%	n	%
Sex	2,276	81.7	509	18.3
Date of Birth	2,258	81.1	527	18.9
Origin of Infection	1,794	64.4	991	35.6
Ethnic Origin	982	35.3	1,803	64.7
Risk Exposure Group	1,009	36.2	1,776	63.8
Date of Diagnosis	892	32.0	1,893	68.0
Clinical variable	**n=3,573 resistance entries**			
	Available data		**Missing data**	
	n	%	n	%
Major reason for test	3,573	100.0	0	0.0
Sample viral load	1,209	33.8	2,364	66.2
Recent viral load	1,844	51.6	1,729	48.4
Most recent CD4	1,951	54.6	1,622	45.4
Current drug therapy	2,707	75.8	866	24.2
Adherence comment	2,260	63.3	1,313	36.7

'Valid percentages' were used throughout the thesis to present the clinical data, that is the denominators for the percentages (unless stated otherwise),

were based on the available data and excluded those patients where the data was missing.

Epidemiological data

The majority of patients with HIV-1 infection were male n=1,596 (70.1%) with 680 (29.9%) females. The age range was from 7 years to 87 years of age, with an overall mean age of 42 years (43 years in males, 37 years in females).

The majority of the infections were of European origin n=926 (51.6%), with 725 (40.5%) of African origin, 48 (2.7%) from Asia, 34 (1.9%) from North America, and the remainder had a prevalence of less than 1.0% including Australasia, South America, the Caribbean, the Middle East and Russia.

From the data provided, 467 (47.6%) patients were classed as White non-Hispanic, 432 (44.0%) as Black African, 21 (2.1%) as White Hispanic, 20 (2.0%) as Black Afro-Caribbean, 13 (1.3%) as Asian Sub-Continent, and all other categories had a prevalence below 1.0% including Asian Middle Eastern, Asian Oriental, Black British, Burmese, Filipino, Indian, Mauritian, Thai, Turkish and White South African.

The main risk exposure group was due to heterosexual contact n=560 (55.5%), while 370 (36.7%) were classified as at risk due to MSM, 23 (2.3%) due to IDU, 20 (2.0%) due to mother-to-child transmission, 16 (1.6%) due to blood/tissue transfer, and all other exposure groups had a prevalence below 1.0% including as a result of a sharps injury, MSM/IDU, heterosexual/IDU, bisexual/commercial sex/MSM/heterosexual.

Table 3.4: Crosstabulation showing Ethnic origin by Origin of infection by Risk exposure group. The majority of men who have sex with men (MSM) cases originated in Europe (90.0%) and were of White non-Hispanic ethnicity (92.3%). The majority of heterosexual cases were of African origin (81.2%) and were classed as Black African ethnicity (77.5%). All injecting drug users (IDU) cases (n=19) originated in Europe and were of White non-Hispanic/White Hispanic ethnic origin.

Ethnic origin	**Origin of infection**	**Risk exposure group**												**Total**
		MSM		Heterosexual		IDU		Blood/tissue transfer		Mother-to-child		Other		
		n	%	n	%	n	%	n	%	n	%	n	%	n
White non-Hispanic	Europe	297	91.7	40	52.6	17	100.0	8	100.0	2	100.0	9	81.8	373
	Africa	6	1.9	22	28.9	0	0.0	0	0.0	0	0.0	0	0.0	28
	Asia	1	0.3	9	11.8	0	0.0	0	0.0	0	0.0	0	0.0	10
	North America	12	3.7	1	1.3	0	0.0	0	0.0	0	0.0	0	0.0	13
	Other	8	2.5	4	5.3	0	0.0	0	0.0	0	0.0	2	18.2	14
	Total	324	100.0	76	100.0	17	100.0	8	100.0	2	100.0	11	100.0	438
White Hispanic	Europe	9	64.3	1	25.0	2	100.0	0	0.0	0	0.0	1	100.0	13
	North America	1	7.1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1
	Other	4	28.6	3	75.0	0	0.0	0	0.0	0	0.0	0	0.0	7
	Total	14	100.0	4	100.0	2	100.0	0	0.0	0	0.0	1	100.0	21
Asian Sub-Continent	Europe	1	100.0	1	12.5	0	0.0	0	0.0	0	0.0	0	0.0	2
	Africa	0	0.0	2	25.0	0	0.0	0	0.0	0	0.0	0	0.0	2
	Asia	0	0.0	5	62.5	0	0.0	1	100.0	0	0.0	0	0.0	6
	Total	1	100.0	8	100.0	0	0.0	1	100.0	0	0.0	0	0.0	10
Black African	Europe	1	50.0	6	1.5	0	0.0	0	0.0	0	0.0	0	0.0	7
	Africa	1	50.0	384	97.2	0	0.0	2	100.0	9	100.0	3	75.0	399
	Other	0	0.0	5	1.3	0	0.0	0	0.0	0	0.0	1	25.0	6
	Total	2	100.0	395	100.0	0	0.0	2	100.0	9	100.0	4	100.0	412
Black Afro-Caribbean	Europe	1	100.0	4	30.8	0	0.0	0	0.0	0	0.0	0	0.0	5
	Africa	0	0.0	2	15.4	0	0.0	0	0.0	0	0.0	0	0.0	2
	North America	0	0.0	2	15.4	0	0.0	0	0.0	0	0.0	0	0.0	2
	Other	0	0.0	5	38.5	0	0.0	0	0.0	1	100.0	0	0.0	6
	Total	1	100.0	13	100.0	0	0.0	0	0.0	1	100.0	0	0.0	15

Table 3.4 contd.: Crosstabulation showing Ethnic origin by Origin of infection by Risk exposure group. The majority of men who have sex with men (MSM) cases originated in Europe (90.0%) and were of White non-Hispanic ethnicity (92.3%). The majority of heterosexual cases were of African origin (81.2%) and were classed as Black African ethnicity (77.5%). All injecting drug users (IDU) cases (n=19) originated in Europe and were of White non-Hispanic/White Hispanic ethnic origin.

Ethnic origin	**Origin of infection**	**Risk exposure group**												**Total**
		MSM		Heterosexual		IDU		Blood/tissue transfer		Mother-to-child		Other		
		n	%	n	%	n	%	n	%	n	%	n	%	n
Other	Europe	7	77.8	2	14.3	0	0.0	0	0.0	0	0.0	1	100.0	10
	Africa	1	11.1	4	28.6	0	0.0	0	0.0	0	0.0	0	0.0	5
	Asia	1	11.1	6	42.9	0	0.0	0	0.0	0	0.0	0	0.0	7
	North America	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
	Other	0	0.0	2	14.3	0	0.0	0	0.0	0	0.0	0	0.0	2
	Total	9	100.0	14	100.0	0	0.0	0	0.0	0	0.0	1	100.0	24
Total	Total Europe	316	90.0	54	10.6	19	100.0	8	72.7	2	16.7	11	64.7	410
	Total Africa	8	2.3	414	81.2	0	0.0	2	18.2	9	75.0	3	17.6	436
	Total Asia	2	0.6	20	3.9	0	0.0	1	9.1	0	0.0	0	0.0	23
	Total North America	13	3.7	3	0.6	0	0.0	0	0.0	0	0.0	0	0.0	16
	Total Other	12	3.4	19	3.7	0	0.0	0	0.0	1	8.3	3	17.6	35
	Total Origin of Infection	351	100.0	510	100.0	19	100.0	11	100.0	12	100.0	17	100.0	920

Sixty-three (7.1%) patients were diagnosed with HIV-1 infection in the 1980s, 282 (31.6%) in the 1990s and 547 (61.3%) in the 2000s.

Table 3.5: Year of diagnosis, by sex, for patients in the International Clinical Virology Centre (ICVC) Clinical Cohort Resistance Database showed female infection was delayed, compared to males, but showed expansion since 1998.

Year of diagnosis	Male		Female	
	n	%	n	%
1981	1	100.0	0	0.0
1983	2	100.0	0	0.0
1984	5	100.0	0	0.0
1985	13	92.9	1	7.1
1986	5	62.5	3	37.5
1987	11	100.0	0	0.0
1988	6	85.7	1	14.3
1989	10	71.4	4	28.6
1990	13	72.2	5	27.8
1991	9	69.2	4	30.8
1992	22	75.9	7	24.1
1993	24	92.3	2	7.7
1994	21	75.0	7	25.0
1995	24	77.4	7	22.6
1996	26	76.5	8	23.5
1997	21	63.6	12	36.4
1998	15	57.7	11	42.3
1999	33	76.7	10	23.3
2000	20	55.6	16	44.4
2001	37	51.4	35	48.6
2002	77	67.5	37	32.5
2003	56	58.3	40	41.7
2004	29	51.8	27	48.2
2005	37	51.4	35	48.6
2006	47	50.0	47	50.0
Total	564		319	

Table 3.6: Major reasons for requesting a resistance test.

Major reason	**n**	**%**
Any resistance/extent of resistance?	1,397	39.1
Treatment-naïve patient, newly diagnosed	591	16.5
Viral load raised	450	12.6
Poor response to treatment	446	12.5
Viral load rebound	251	7.0
Restarting therapy	123	3.4
Primary HIV-1 infection (PHI)	112	3.1
Pregnant	85	2.4
Other**	118	3.3
Total	3,573	100.0

Key:
** Other reasons included: treatment interruption, simplification of treatment, PCR negative, low CD4 count, issues with adherence, post-exposure prophylaxis (PEP) (all frequencies <1.0% per reason).

Table 3.7: Sample and/or most recent viral load (VL) data.

VL levels (c/mL)	**Sample VL** (c/mL)		**Recent VL** (c/mL)	
	n	**Valid %**	**n**	**Valid %**
≤50	27	2.2	43	2.3
≥51 to ≤500	59	4.9	61	3.3
≥501 to ≤1,000	36	3.0	49	2.7
≥1,001 to ≤5,000	211	17.5	418	22.7
≥5,001 to ≤10,000	90	7.4	212	11.5
≥10,001 to ≤50,000	266	22.0	457	24.8
≥50,001 to ≤100,000	153	12.7	228	12.4
≥100,001 to ≤250,000	185	15.3	193	10.5
≥250,001 to ≤750,000	114	9.4	131	7.1
≥750,001	68	5.6	52	2.8
Total	1,209	100.0	1,844	100.0

Table 3.8: Most recent CD4 cell count data showed that 38.3% of patients had CD4 counts below 200 cells/µL with a total of 67.8% of patients with a CD4 count ≤350 cells/µL; as per the British HIV Association (BHIVA) treatment guidelines (Gazzard on behalf of the BHIVA Treatment Guidelines Writing Group 2008), these patients should be taking ART.

CD4 cell count (cells/µL)	**n**	**%**
≤50	202	10.4
≥51 to ≤200	544	27.9
≥201 to ≤350	576	29.5
≥351 to ≤500	326	16.7
≥500	303	15.5
Total	1,951	100.0

Antiretroviral therapy (ART) treatment data

Of ART treatment data provided, 1,953 (72.2%) were treatment-experienced (1,575 (58.2%) were currently taking some form of ART and 378 (14.0%) were not currently on treatment but had previous ART experience) and 754 (27.9%) were treatment-naïve.

Of those currently taking ART (n=1,575), the majority were on a combination of three drugs (n=1,073, 68.1%). A minority were only taking one (n=11, 0.7%) or two (n=108, 6.9%) drugs whilst it was indicated that 20 patients (1.3%) were currently taking six drugs.

Table 3.9: Adherence comment: of those patients currently taking antiretroviral therapy (ART), it was indicated the majority of patients (78.0%) had excellent/reasonable adherence to their ART regimens.

Adherence comment	n	%
Excellent	694	60.7
Reasonable	198	17.3
Poor	190	16.6
Suspect/erratic/intermittent	61	5.3
Total	1,143	100.0

‘First failures’

Of those who were on treatment, 128 patients (12.9%) were currently on their first ART regimen and had no other treatment experience. Of these, 27 patients (21.1%) were currently only taking NRTIs; 97 (75.8%) were taking two drugs classes (71 NRTIs+NNRTIs and 26 NRTIs+PIs) whilst four patients (3.1%) were on a regimen including all three classes.

‘Drug-experienced’

Of 859 patients currently taking ART who had specific data provided with regards to their previous drug experience, 254 (29.6%) had experience of one drug class (178 NRTIs, 40 NNRTIs, 36 PIs); 374 (43.5%) had experience of two drugs classes (154 NRTIs+NNRTIs, 213 NRTIs+PIs, 7 NNRTIs+PIs) whilst 231 (26.9%) had previous treatment experience with all three drugs classes.

Of the 378 not currently on any ART but with previous treatment experience, 59 (16.3%) had experience of one drug class (55 NRTIs, 1 NNRTIs, 3 PIs); 216 (59.8%) had experience of two drugs classes (120 NRTIs+NNRTIs, 94 NRTIs+PIs, 2 NNRTIs+PIs) whilst 86 (23.8%) had previous treatment experience with all three drugs classes.

There was a wide range of drug usage for those currently taking or who had previously taken ART.

Table 3.10: Frequencies of each of the antiretroviral drugs by current antiretroviral therapy (ART) usage and previous drug experience; each of the antiretroviral drugs showed wide use of nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) and less use of the protease inhibitors (PIs).

		Currently on ART				Not currently on ART	
Drug class	Antiretroviral drug	Currently on ART (n=1,575)		Previous drug experience (n=859)		Previous drug experience (n=378)	
		n	%	n	%	n	%
NRTIs	ZDV	586	37.2	567	66.0	265	70.1
	ddI	488	31.0	335	39.0	137	36.2
	ddC	29	1.8	164	19.1	44	11.7
	3TC	964	61.2	478	55.6	274	72.5
	d4T	575	36.5	373	43.3	178	47.1
	ABC	347	22.0	117	13.6	76	20.1
	TDF	279	17.7	22	2.6	32	8.5
	FTC	13	0.8	4	0.5	3	0.8
NNRTIs	NVP	398	25.3	295	34.3	141	37.3
	DLV	9	0.6	18	2.1	7	1.9
	EFV	343	21.8	194	22.5	90	23.8
PIs	SQV	123	7.8	180	20.9	65	17.2
	IDV	93	5.9	235	27.3	60	15.9
	RTV	164	10.4	215	25.0	76	20.1
	NFV	203	12.9	173	20.1	77	20.4
	APV	26	1.7	22	2.5	8	2.1
	LPV	26	1.7	7	0.8	6	1.6
	LPV/r	213	13.5	54	6.3	28	7.4
	TPV	2	0.1	-	-	-	-
	ATV	15	1.0	2	0.2	1	0.3
	FPV	2	0.1	-	-	-	-
Fusion inhibitor	T-20	8	0.5	1	0.1	-	-

Table 3.11: Number of nucleoside reverse transcriptase inhibitors (NRTIs) in a patient's regimen: including those currently taking and with previous experience of taking NRTIs.

Number of NRTIs in regimen						
	Currently on ART				Not currently on ART	
	Current drug usage (n=1,575)		Previous drug experience (n=859)		Previous drug experience (n=378)	
	n	%	n	%	n	%
0	58	3.7	83	9.7	31	8.2
1	109	6.9	127	14.8	33	8.7
2	1,093	69.4	302	35.2	170	45.0
3	273	17.3	142	16.5	67	17.7
4	42	2.7	139	16.2	51	13.5
5	-	-	50	5.8	21	5.6
6	-	-	15	1.7	5	1.3
7	-	-	1	0.1	-	-

Table 3.12: Number of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in a patient's regimen: including those currently taking and with previous experience of taking NNRTIs.

Number of NNRTIs in regimen						
	Currently on ART				Not currently on ART	
	Current drug usage (n=1,575)		Previous drug experience (n=859)		Previous drug experience (n=378)	
	n	%	n	%	n	%
0	832	52.8	427	49.7	174	46.0
1	736	46.7	361	42.0	179	47.4
2	7	0.4	67	7.8	23	6.1
3	-	-	4	0.5	2	0.5

Table 3.13: Number of protease inhibitors (PIs) in a patient's regimen: including those currently taking and with previous experience of taking PIs.

Number of PIs in regimen						
	Currently on ART				Not currently on ART	
	Current drug usage (n=1,575)		Previous drug experience (n=859)		Previous drug experience (n=378)	
	n	%	n	%	n	%
0	902	57.3	372	43.3	195	51.6
1	487	30.9	243	28.3	102	27.0
2	179	11.4	140	16.3	50	13.2
3	6	0.4	67	7.8	23	6.1
4	1	0.1	26	3.0	7	1.9
5	-	-	6	0.7	1	0.3
6	-	-	5	0.6	-	-

Of the 3,573 entries, 2,995 (83.8%) had a full resistance report, 38 (1.1%) only had a PR sequence, 59 (1.7%) only had a RT sequence and 481 (13.5%) had complete PCR failure and the samples were unable to be amplified.

Review of the PCR failures

For the successful extraction of HIV-1 RNA from a patient's plasma sample and for genotypic testing to occur, it was recommended that the VL of the sample submitted be >1,000c/mL. Of the PCR failures with available VL data, 81/374 (21.7%) had a sample/recent VL ≤1,000c/mL. Of the samples submitted with origin of infection data, 181 (49.1%) were of European origin, with 156 (42.3%) of African origin, 12 (3.3%) were from Asia, 11 (3.0%) from North America, and the remainder had a prevalence of <1.0% including Australasia, South America and Russia. For those from an African, Asian, South American and Russian origin, they may have had a non-B subtype and the primers used in the genotypic kit (based on subtype B) were not able to amplify these samples.

Protease (PR) and reverse transcriptase (RT) mutations data

Of the 3,092 successful resistance entries, 2,911 (94.1%) were identified as having PR and/or RT mutations. One hundred and eighty-one (5.9%) had no evidence of resistance mutations. Of the 2,911 entries, 2,744 (94.3%) had PR mutations with a minimum of one, a maximum of 12 and a mean of 2.4 PR mutations. With the RT mutations, 1,707 (58.6%) had such mutations with a minimum of one, a maximum of 13 and a mean of 3.7. Overall, the minimum number of PR and/or RT mutations was 1 and the maximum was 22, with a mean of 4.4.

A detailed tabulation of the frequencies of specific PR and RT mutations found in the cohort, using the TRUGENE® resistance guidelines (Genelibrarian version current at time of testing) are presented in Appendix 2. This detailed tabulation of all the research information may be of value to other researchers in the future, and as such, has been noted here.

Discussion

As detailed in the overview above, the ICVC Clinical Cohort Resistance Database holds a wealth of data for patients with HIV-1 who have had resistance tests conducted as part of their clinical care. In addition, these resistance results have been performed over an extended period of time, providing a longitudinal view of the evolution of the patients' virus. This data was used to answer the research questions posed in the following chapters and ultimately, to attempt to improve the clinical care of patients with HIV-1.

Chapter 4: Resistance in a UK HIV-1 treatment-naïve clinical cohort

In 1996, the introduction of HAART for the treatment of HIV-1 infection generated a period of optimism in the HIV-1 treatment field (Chesney et al, 1999). Dramatic improvements in the mortality and morbidity of patients with HIV-1 infection were witnessed, with a decrease in the incidence of opportunistic infections, tumours and deaths (Rubbert and Ostrowski 2003). This early optimism waned however as a result of the emergence of resistance mutations to the antiretroviral therapies. Resistance emerges when the PR and RT enzymes that the ART should be inhibiting are able to continue operating and aiding the replication of HIV-1, even in the presence of the drugs. This high rate of error-prone replication, particularly the RT enzyme, allows rapid selection of mutations associated with drug resistance. These drug-resistant viruses are selected in the presence of sub-optimal therapy and may be horizontally and vertically transmitted to new hosts.

Genotyping resistance testing is an important tool in selecting optimal therapy for patients on ART. At the time this study was designed, there was a poor understanding in the clinical field with regards to the use of genotypic resistance testing with treatment-naïve patients. Initially, international treatment guidelines did not advocate testing treatment-naïve patients prior to their first therapy; then the IAS-USA convened a panel to review antiretroviral drug resistance testing in adults and the implications for clinical management. The panel members (who had expertise in ART drug resistance and in the care of patients with HIV-1) opinionated: ‘routine testing for certain patients e.g. treatment-naïve pregnant women and persons with PHI, should be considered for testing when the prevalence of drug resistance in that population is increased’ (Hirsch et al 1998). In 2000, the same panel convened again and concluded: ‘prior to starting treatment in patients with established infection, the use of resistance testing should be considered, particularly in areas where the local prevalence of primary drug resistance is appreciable’ (Hirsch et al 2000). The panel considered that if a population exhibited a transmitted resistance level of 10.0%, then this was a

trigger for concern and resistance testing for anyone who was treatment-naïve in this population should be considered (personal communication with IAS-USA panel member).

In 1999, an audit was conducted at the ICVC of consecutive treatment-naïve patients following genotypic resistance testing for clinical care (n=89). These 89 patients were compared to an 'absolute treatment-naïve' patient population sequenced in 1986 (n=7), (Loveday et al 1999). The pre-ART cohort in 1986 revealed a homogeneous population of viruses in the patients tested whereas there was an increase in the heterogeneity in the virus genome in the 1999 cohort: no major PR mutations were seen and only one RT mutation (M41L) but there was a low prevalence of minor PR mutations associated with ART resistance in the later cohort. There was a marked increase in genetic heterogeneity relative to the 1986 cohort which suggested ongoing and increasing genetic mobility of the genome due to the prolific replication rate and high error rate.

Research aims

At the time this study was conducted, the majority of HIV-1 clinics were not actively utilising resistance testing for their treatment-naïve patients; they would start their patients on ART and if the VL had not become undetectable in three months, then a resistance test would be requested. Often, the resistance results highlighted the presence of mutations which impacted on the future treatment options for the patient.

In order to determine the best possible clinical care for those identified as treatment-naïve in the ICVC Clinical Cohort Resistance Database, I investigated:

- the evolving and transmitted drug resistance mutations in treatment-naïve patients, prior to them starting their first ART regimen
- the prevalence of resistance over time between 2001 and the end of

June 2004

- the regional variability in resistance in treatment-naïve patients across the ICVC UK cohort
- the implications of pre-existing drug mutations for patients about to start their first-line ART regimen and how this could be managed in the clinic.

Method

Patient selection

All treatment-naïve patients including PHI (infected with HIV-1 <6 months); chronic-naïve (CN, infected with HIV-1 for >6 months and treatment-naïve) and pregnant and treatment-naïve; who had a genotypic resistance test performed as part of their clinical care from 2001 to the end of June 2004, were identified on the ICVC Clinical Cohort Resistance Database. The inclusion of PHI and CN patients in the study was important as it reflected the treatment-naïve population attending HIV-1 clinics for care i.e. the majority of patients were unaware of their HIV-1 infection and by the time they were diagnosed, they had already experienced the PHI stage and were now deemed as CN.

For those whose genotypic test outcome was PCR-negative, they were excluded from the study. For those who had more than one resistance test conducted during the time period, their earliest resistance test was included in the analyses.

Determining resistance using the TRUGENE® HIV-1 Resistance Report

Resistance was determined based on the TRUGENE® HIV-1 resistance reports generated for each patient as part of their clinical care. The TRUGENE® algorithm was used to determine resistance in this treatment-naïve study, and not a different interpretation tool e.g. the IAS-USA Drug Resistance Mutations Figures; as the TRUGENE® resistance report output

reflected the genotypic resistance profiles that were disseminated to the clinicians in the cohort and therefore the resistance patterns that were occurring across the UK clinics which the ICVC served.

Results

Patients' demographics

Three hundred and eighty treatment-naïve patients were sequenced between January 2001 and the end of June 2004: 2001 n=37, 2002 n=89, 2003 n=167, 2004 n=87. Seventy-six (20.0%) were PHI, 279 (73.4%) CN, and 25 (6.6%) pregnant and naive.

Table 4.1: Treatment-naïve patients derived from 26 of 39 denominator hospitals with the majority from clinical centres within the Greater London geographical region (64.2%).

Geographical region	n	%
Greater London	244	64.2
South East	83	21.8
Eastern	27	7.1
South West	8	2.1
North West	8	2.1
Wales	6	1.6
East Midlands	4	1.1

The majority of the patients were male (n=280, 73.7%), with a mean age of 38.6 years, ranging from 20 to 77 years. One hundred and twenty-five (50.8%) were infected through MSM, 117 (47.6%) through heterosexual contact, two (0.8%) through blood/tissue transfer, one (0.4%) bisexual and one (0.4%) IDU.

Europe was the predominant origin of infection for 192 (58.9%) of the patients, Africa (n=111, 34.0%); Asia (n=6, 1.8%); North America (n=5, 1.5%); South Africa (n=3, 0.9%); Australasia, Europe/South America (all n=2, 0.6%); the Caribbean, the Middle East, Asia/Europe and Africa/Europe (all n=1, 0.3%).

Two hundred and thirty (60.5%) treatment-naïve patients presented with a subtype B virus whilst 150 (39.5%) had a non-B subtype virus, with subtype C being the most predominant (n=97, 25.6%).

Evidence of resistance to ART

Of the 380 patients, 266 (70.0%) presented with listed mutations associated with ART (excluding 69 (18.2%) with L63P only). Forty-five patients (11.8%) had no listed mutations.

From the 266 patients, 44 (16.5%) were found to have at least one major mutation:

- 39 were male
- mean age was 38.9 years, ranging from 28 to 68
- 11 were PHI, 31 were CN, two pregnant and naïve
- 24 were MSM, seven heterosexual
- 10 presented with non-B subtypes
- 34 derived from the Greater London region
- major mutations conferred resistance to:
    - NNRTIs for 31 patients (11.7%)
    - NRTIs for six patients (2.3%)
    - PIs for five patients (1.9%)
    - two drug classes (NNRTI and NRTI) for one patient (0.4%)
    - all three drug classes for one patient (0.4%).

Resistance by year, associated demographics, mutations and drug class affected

In 2001, four patients had evidence of resistance (4/37, 10.8%), 15 patients had resistance in 2002 (15/89, 16.9%), 12 patients in 2003 (12/167, 7.2%) and 13 patients to the end of June 2004 (13/87, 14.9%).

Table 4.2: Prevalence of resistance in primary HIV-1 infection (PHI), chronic-naïve (CN), pregnant and treatment-naive patients with resistance, by year.

	2001		2002		2003		2004		Total	
	n	%	n	%	n	%	n	%	n	%
PHI	1/10	10.0	7/26	26.9	1/29	3.5	2/11	18.2	11/76	14.5
CN	3/24	12.5	8/58	13.8	9/127	7.1	11/70	15.7	31/279	11.1
Pregnant & naïve	0/3	0.0	0/5	0.0	2/11	18.2	0/6	0.0	2/25	8.0

A detailed tabulation for each individual patient with resistance mutations detected: their associated demographic data, the specific mutations identified and the drug class affected, by year; are presented in Appendix 3. This detailed tabulation of all the research information may be of value to other researchers in the future, and as such, has been noted here.

For those with VL data available (VL of the sample tested or most recent VL level i.e. in the past three months), **Table 4.3a** broadly illustrates the VL levels associated with the cohort. **Table 4.3b** presents in detail the VL of the patients presenting as PHI, CN, pregnant and naive, and evidence of resistance.

Table 4.3a: Viral load and evidence of resistance.

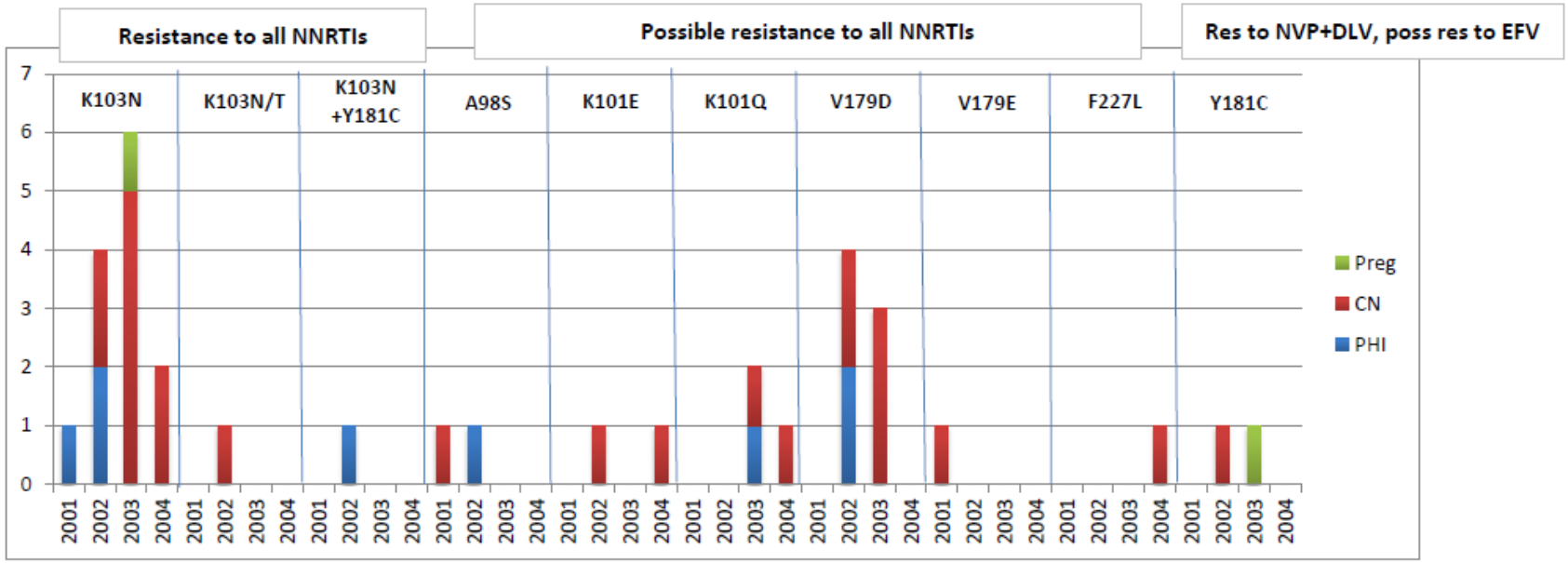
Resistance	**n**	**Mean VL** (c/mL)	**Range VL** (c/mL)
No resistance	297	293,799	400 – 15,000,000
Evidence of resistance	41	236,713	5910 – 892,000
To NNRTIs	29	248,566	5910 – 892,000
To NRTIs	5	144,314	7570 – 329,000
To PIs	5	296,708	7940 - >750,000
To NNRTIs + NRTIs	1	80,700	-
To all 3 drug classes	1	211,000	-

Table 4.3b: Viral load (VL), evidence of resistance (R) to the non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs); and presentation as primary HIV-1 infection (PHI), chronic-naive (CN) or pregnant and treatment-naïve.

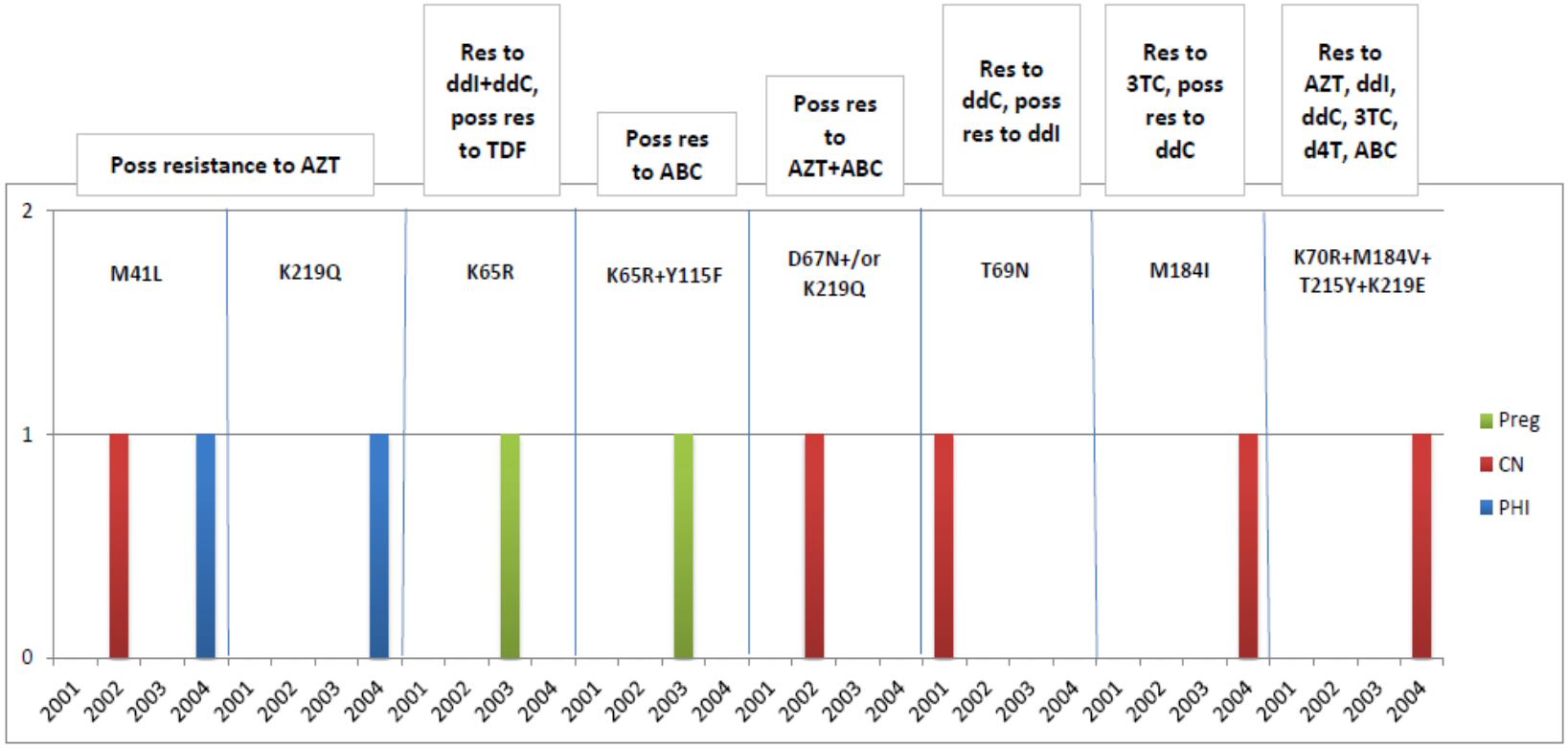
Treatment-naive grouping	**No resistance**			**R to the NNRTIs**			**R to the NRTIs**			**R to the PIs**		
	n	**Mean VL** (c/mL)	**Range VL** (c/mL)	**n**	**Mean VL** (c/mL)	**Range VL** (c/mL)	**n**	**Mean VL** (c/mL)	**Range VL** (c/mL)	**n**	**Mean VL** (c/mL)	**Range VL** (c/mL)
PHI	59	386,016	400 – 8,000,000	8	238,200	29,800–830,000	2	218,000	107,000–329,000	1	368,000	-
CN	218	293,936	400 – 15,000,000	20	220,541	5910 – 750,000	3	95,190	7570 – 203,000	4	278,885	7940 – 750,000
Pregnant and naive	20	20,266	400 – 158,000	1	892,000	-	-	-	-	-	-	-

Graphs 4.1a-c highlight the resistance mutations identified, by year, by treatment-naïve grouping (PHI, CN, pregnant and naïve) and the impact of the resistance mutations for the drug class affected. The two patients' with multi-class resistance were included in the separate drug class graphs: **Graph 4.1a** shows the NNRTI data, **Graph 4.1b** shows the NRTI data and **Graph 4.1c** shows the PI data.

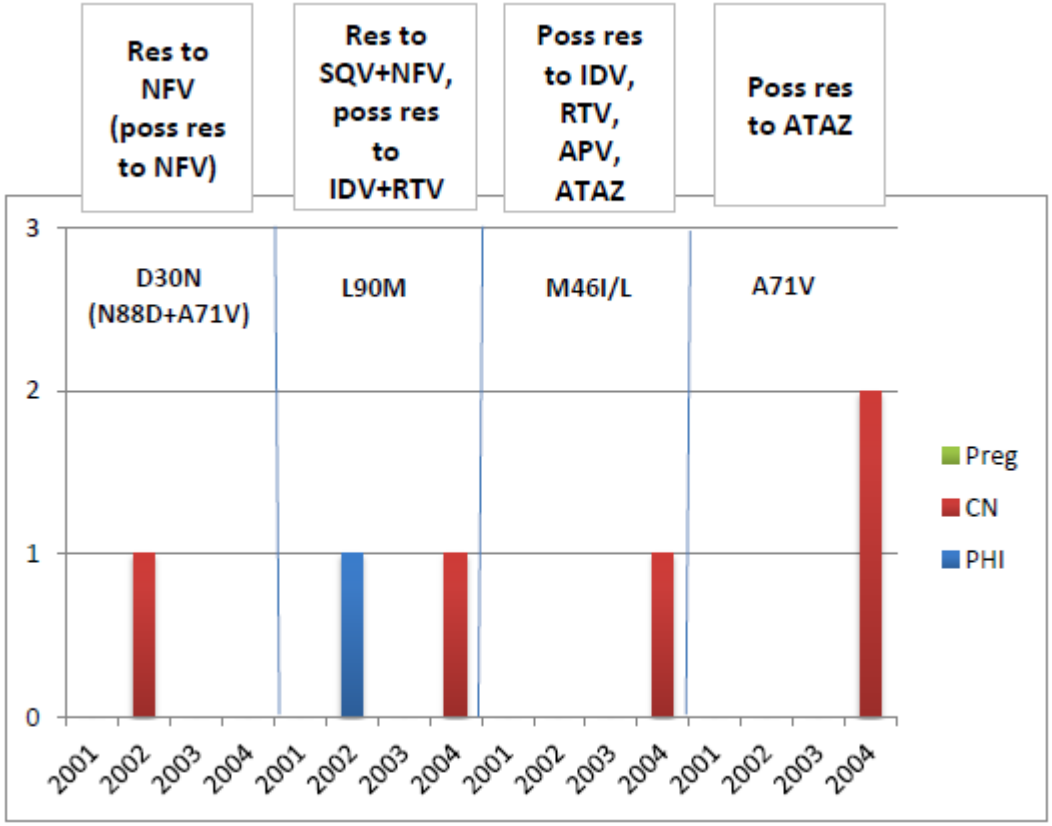
Graph 4.1a: Non-nucleoside reverse transcriptase inhibitors (NNRTIs), associated mutations and prevalence of resistance over the study (and presentation as primary HIV-1 infection (PHI), chronic-naive (CN) or pregnant and treatment-naïve). K103N was the most prevalent of the NNRTI mutations impacting use of the whole of the NNRTI drug class. V179D mutation was the next most frequent mutation which translated as possible resistance to all of the NNRTIs.



Graph 4.1b: Nucleoside reverse transcriptase inhibitors (NRTIs), associated mutations and prevalence of resistance over the years (and presentation as primary HIV-1 infection (PHI), chronic-naive (CN) or pregnant and treatment-naïve). The graph depicts low frequencies of NRTI mutations with treatment options within this drug class available to the majority of the patients with these mutations. However, for the patient with four NRTI resistance mutations (K70R+M184V+T215Y+K219E) the accumulation of these mutations impacted a number of the specific drugs available within this class.



Graph 4.1c: Protease inhibitors (PIs), associated mutations and prevalence of resistance over the years (and presentation as primary HIV-1 infection (PHI), chronic-naive (CN) or pregnant and treatment-naïve). The graph depicts low frequencies of PI mutations with other PI treatment options within this drug class available to the majority of the patients with these mutations.



To determine the persistence of the transmitted resistance mutations, the date of diagnosis was subtracted from the sample date to provide an approximation of the length of time the resistance associated mutations had persisted for in the patient's circulating virus population.

Table 4.4: The persistence of resistance associated mutations circulating in a patient's virus population (sample date minus date of diagnosis) by drug class (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs)). The table illustrates that some of these related mutations can persist for >12 months.

Resistance mutation by drug class	Persistence of mutation (in months)	n
NNRTIs		
A98S	<3	1
K101E	>12 <18	1
K101Q	>6 <12	1
K103N	<3	3
	>6 <12	1
	>18 <24	1
	>36	1
V179D	>6 <12	2
	>30 <36	1
V179E	<3	1
Y181C	>6 <12	1
NRTIs		
D67N	>12 <18	1
M184I	>6 <12	1
K219Q	>12 <18	1
PIs		
D30N	>12 <18	1
M46I/L	>12 <18	1
A71V	>12 <18	1
N88D	>12 <18	1

Discussion

The overall resistance prevalence rate in a cohort of 380 treatment-naïve patients with HIV-1 was 16.5%. In patients presenting with PHI, the overall resistance prevalence rate was 14.5%, for CN patients it was 11.1%, and for women who were pregnant and treatment-naïve it was 8.0%. Adopting the IAS-USA rule that if a population exhibited a transmitted resistance level of 10.0%, resistance testing should be considered (IAS-USA panel 1998 personal communication), all these patient classes provided evidence that all treatment-naïve patients should be tested for resistance at baseline. At the ICVC, we presented our findings to all 39 clinical centres and advocated that their treatment-naïve patients, irrespective of their treatment-naïve grouping (PHI/CN/pregnant and naïve), should have a genotypic resistance test conducted before initiating treatment.

Overall, for those patients with resistance to one drug class only, the NNRTI drug class was most affected with 31 patients (11.7%) presenting with mutations associated with resistance to this drug class (three in 2001, 12 in 2002, 11 in 2003, five to the end of June 2004). Resistance to NNRTIs only requires the development of one major mutation and the patient is said to be resistant to all three drugs within this class. For example, 14 patients presented with a K103N(T) mutation; eight with V179D/E; two with A98S; one with Y181C; one with K103N+Y181C; four with K101E/Q and one with F227L. For these 31 patients, the clinician would not be able to utilise the NNRTI drug class, even for the patients with possible resistance only, therefore the first treatment regimen for these patients would have included drugs available in the NRTI and PI classes.

The BHIVA Treatment Guidelines (Gazzard on behalf of the BHIVA Writing Committee 2005) recommended initial first-line HAART regimens contained two NRTIs and one NNRTI (see Table 4.6). This would not be suitable for these 31 patients with the mutations above and the BHIVA Guidelines next

recommendation was that a two NRTI and boosted PI regimen was considered; a combination that had more side effects. For the two NRTIs, Combivir (ZDV+3TC) was the most popular combination used in the UK, although clinicians also had TDF+FTC or ABC+3TC combinations available, which both offered similar efficacy to Combivir. The BHIVA Guidelines recommended that LPV, boosted by RTV (LPV/r) was used as the PI, although other alternative options were available if LPV/r was not a suitable option for a specific patient.

Table 4.5: Preferred first-line antiretroviral therapy (ART) regimens from the British HIV Association (BHIVA) Guidelines for the treatment of HIV-infected adults with antiretroviral therapy: a combination of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) was preferred (Gazzard on behalf of the BHIVA Writing Committee 2005).

Regimen	A	B	C
Preferred	EFV LPV/r	ZDV ABC TDF ddI	3TC FTC
Alternative	FOS/r SQV/r		
Specific groups	NVP ATAZ ATAZ/r		

Prevalence of resistance associated mutations related to the NRTI drug class (2.3%) and PI drug class (1.9%) remained at low levels over the years in this clinical cohort. For these drug classes it was important to consider the resistance associated mutations on a drug by drug basis as, unlike the NNRTIs, the presence of one major NRTI or PI mutation did not necessarily translate as resistance to all drugs within the classes.

In this study, six patients (2.3%) had mutations associated with the NRTI class, with four patients presenting in 2004. Of the six, only one patient had resistance to the majority of the drugs within the class including ZDV, ddI,

ddC, 3TC, d4T and ABC. Using the BHIVA treatment guidelines, this patient only had TDF+FTC available to use as a two NRTI backbone. Three patients had resistance to ZDV only, one patient had resistance to ddC and possible resistance to ddI, and one had resistance to 3TC and possible resistance to ddC. The clinicians for these five patients therefore had options available to employ as a two NRTI backbone.

Five patients presented with PI related mutations (1.9%), with four seen in 2004. Two patients had a mutation affecting resistance to SQV+NFV and possible resistance to IDV+RTV. One patient had possible resistance to IDV, RTV, APV and ATV. Two patients had the PR mutation A71V which in 2004 and interpreted using TRUGENE® GuideLines 8.0, translated as possible resistance to ATV. However, on sign-out of the resistance report, the Clinical Director of the ICVC commented that the prevalence/appearance of this mutation should not influence the use of this drug in these patient's treatment regimens.

The presentation of PI resistance in these patients did not affect first-line treatment options recommended by the BHIVA Guidelines and a two NRTI and NNRTI initial regimen could be utilised. However, subsequent treatment options may be affected by the presence of the PI mutations.

Specific cases exemplifying clinical issues around transmitted resistance evolving from this study

i) Potential for high levels of resistance being transmitted

Two patients' resistance profiles were quite striking in this study. The first patient presented as CN and had evidence of resistance to all three drugs classes, including resistance to the PI NFV (D30N; A71V+N88D), possible resistance to the NRTIs ZDV and ABC (D67N+K219Q) and possible resistance to all NNRTIs (K101E). The patient was diagnosed as HIV-1 positive in 2001 and this baseline resistance test was conducted in

September 2002. His resistance profile suggested that a person with a high level of treatment-experience infected him and that the resistance mutations had persisted since he was infected. As per the BHIVA Guidelines, the clinician did have options available to treat this patient including using a two NRTI backbone of TDF+FTC, or using ddI+3TC or ddI+FTC, which are both well tolerated options, but ddI does have food restrictions and there is a potential for long term mitochondrial toxicity. A boosted PI, probably LPV/r, would complete the regimen as the NNRTI class was not available to use.

The second striking resistance profile was for a patient who presented as pregnant and treatment-naïve. She was found to have resistance to the NRTIs ddI, ddC (K65R), possible resistance to TDF (K65R) and ABC (K65R+Y115F), and resistance to the NNRTIs NVP, DLV (Y181C) and possible resistance to EFV (Y181C). Once again, the patient's resistance profile indicated that a person with quite a high level of treatment-experience infected her. Another suggestion may be that she had previously taken some antiretroviral drugs but was not willing to divulge. Using the BHIVA Guidelines, the clinician could use a two NRTI backbone of ZDV+3TC, and a boosted PI, probably LPV/r. However, this patient was pregnant and the clinician would need to consider the suitability of these drugs for both the patient and her baby.

ii) Demonstration of patient to patient transmission

Whilst liaising with one of our clinical collaborators, it became evident that a couple of patients' resistance profiles in the treatment-naive cohort were epidemiologically linked (Kinloch et al 2003). It transpired that patient SPSS 92 (the 'donor') and patient SPSS 99 (the 'recipient') were partners practicing unprotected sexual intercourse. SPSS 92 was diagnosed as PHI in June 2002 whilst his partner SPSS 99 was diagnosed in September 2002. Their resistance profiles were similar; both presented with the major RT mutation K103N. The only difference in their resistance profiles was that

SPSS 99 had one additional polymorphism (RT: T165T/I) to SPSS 92's profile. Sequencing of both patients' strains showed that there was <1.0% difference between the two strains, suggesting a strong epidemiological link between the two infections. This 'case study' reflected that horizontal transmission of the K103N resistant mutation was possible amongst a person who was treatment-naïve to another person who was treatment-naive.

iii) Importance of resistance testing at baseline

Another important case on the ICVC Clinical Cohort Resistance Database highlighted the importance of clinicians requesting resistance testing for their treatment-naïve patients before initiating therapy. Patient SPSS 3315 was diagnosed with HIV-1 on 13/09/2005. The clinician prescribed a first-line treatment regimen of 3TC, ABC and EFV which the patient started on 18/10/2005. A resistance test was requested in December 2005 as the patient's virus was not responding to the treatment, even though it was deemed his adherence was excellent. The patient's VL was 105,552c/mL and his CD4 count was 31cells/µL. The resistance report showed the patient had the minor PR mutation L10I and the RT mutations V106M, V179D and M184V. These RT mutations translated as conferring resistance to the NRTIs 3TC/FTC, possible resistance to ddC, and resistance to all the NNRTIs (NVP, DLV and EFV). If the clinician had requested the resistance test before initiation of ART, perhaps this first-line treatment failure could have been averted.

This is not a straightforward issue however and the clinical situation will influence whether a baseline, pre-initiation of ART resistance test can be requested. For example, patient SPSS 3083 was diagnosed in June 2004 and had a VL of 172,000c/mL and a CD4 count of only two cells/µL. The clinician started ART immediately as the CD4 count was so low, and could not wait for the results of a resistance test. The patient was prescribed ABC,

3TC and NVP on 18/06/2004. The clinician requested a resistance test on a sample from the patient in September 2004 as the patient had a poor response to the treatment regimen, even though they stated their adherence was excellent. The patient's VL in September was 66,500c/mL with a CD4 count of nine cells/µL. The resistance test showed the patient had mutations which conferred resistance to 3TC (NRTI), NVP and DLV (NNRTIs) as well as possible resistance to ddC (NRTI) and EFV (NNRTI). Following these results, the clinician requested a retrospective resistance test was conducted using a sample stored at the ICVC from before the patient had started on ART. This retrospective analysis only highlighted the minor PR mutation M36I. In this case, a resistance test before initiating ART would not have highlighted the mutations which occurred once treatment was started. The resistant variants transmitted were not identified at baseline testing: explanations for this include reversion of the transmitted resistant virus to WT and that the resistant variants were archived and only came to the forefront once treatment was initiated. Another possible explanation includes the fact that resistance tests are unable to detect minority variants that comprise <20.0% of the viral population, and although techniques are now available to detect these minorities, they are complex and not appropriate for use in clinical practice (Haubrich 2005).

In conclusion, this clinical study identified a significant level of transmitted resistance mutations circulating in the treatment-naïve population and this warranted the recommendation that all treatment-naïve patients (irrespective of whether they were PHI, CN or pregnant and naïve) should have baseline genotypic resistance testing conducted before the initiation of their first-line treatment regimens, to ensure the best possible clinical care.

Chapter 5: Resistance in a UK HIV-1 treatment-experienced clinical cohort

When treatment first became available for patients with HIV-1 it was initially with NRTI monotherapy (Fischl et al 1987; Larder and Kemp 1989); then a combination of two NRTIs were used (Schlomo et al 1996; Katlama et al 1996; Delta Coordinating Committee 1996; Hammer et al 1996); and there was every opportunity for HIV-1 replication to continue as the virus was able to mutate and evolve resistance to these drugs that were being used to attempt to suppress HIV-1 replication. Even though great advances were seen when HAART (NRTIs + PIs +/or NNRTIs) were introduced to treat patients with HIV-1 (Hammer et al 1997; Gulick et al 1997; Cameron et al 1998), the high pill burden (patients were required to take up to 30 tablets a day) and side-effects, often resulted in suboptimal adherence rates; HIV-1 was able to replicate, even in the presence of low concentrations of the drugs, leading to the development of resistance mutations and drug failure (Paterson et al 2000; Dunbar et al 2000).

The ICVC Clinical Cohort Resistance Database included a large proportion of patients with HIV-1 who were highly treatment-experienced and who had lived through the early years of the HIV-1 treatment developments leading to HAART. Genotypic resistance tests were conducted for these treatment-experienced patients and they provided an opportunity to understand the resistance mutations that were currently circulating within this treatment-experienced clinical cohort and the UK community. These mutation data could be used to interpret the next treatment options available for the patients currently failing therapy or wishing to re-start treatment.

Research aims

Of those identified as treatment-experienced in the ICVC Clinical Cohort Resistance Database, the aims were to investigate:

- the frequency of important and specific major NRTI, NNRTI and PI resistance mutations

- the prevalence of these resistance mutations over time: were there any significant differences observed in the early cohort (EC, 1996-2000) and the late cohort (LC, 2001-2006)
- the geographical distribution of these resistance mutations across the cohort
- the cumulative resistance profiles over time of those treatment-experienced patients who had more than one genotypic report.

Method

Patient selection

All treatment-experienced patients who were currently on treatment or had previous treatment-experience, were eligible for inclusion. Complete PR and RT sequences were required for analysis, so treatment-experienced patients whose genotypic analyses provided only a partial sequence (PR only (1.2%) or RT only (0.9%)) or were PCR-negative (16.5%) were excluded from the study.

Determining resistance

The IAS-USA Update of the Drug Resistance Mutations in HIV-1: December 2009 list (Johnson et al 2009) was used to determine major resistance mutations prevalent in this study.

NRTI mutations included: M41L, A62V, K65R, D67N, 69Insert, K70R, L74V, V75I, F77L, Y115F, F116Y, Q151M, M184I/V, L210W, T215F/Y, K219E/Q.

NNRTI mutations included: V90I, A98G, L**100**I, K**101**E/H/P, K**103**N, V**106**A/M, V**108**I, E138A, V179D/F/T, Y**181**C/I/V, Y**188**C/L/H, G**190**A/S, P**225**H, M230L.

PI mutations included: L10C/F/I/R/V, V11I, I13V, G16E, K20I/M/R/T/V, L24I, D**30**N, V**32**I, L33F/I/V, E34Q, E35G, M36I/L/V, K43T, M**46**I/L, I**47**A/V, G**48**V, I**50**L/V, F53L/Y, I**54**A/L/M/S/T/V, Q**58**E, D60E, I62V, L63P, I64L/M/V, H69K,

PI mutations included (contd.): A71I/L/T/V, G73A/C/S/T, T**74**P, L**76**V, V77I, V**82**A/F/L/S/T, N83D, I**84**V, I85V, N**88**D/S, L89V, L**90**M, I93L/M.

Key:
For those NNRTI and PR amino acid positions highlighted in bold, they were considered major (primary) mutations which occur first and the non-bold were minor (secondary mutations) that are supportive to the molecular changes which are associated with the primary mutations.

Data analyses

Two approaches to data analysis were adopted once it was determined, at patient selection, that there were a group of treatment-experienced patients who had more than one genotypic resistance entry available on the database. Patients with one genotype resistance report were pooled and their data analysed together to determine the prevalence of major resistance mutations (and their impact on the drugs classes) by calendar year. Patients with more than one genotype resistance report where reports were conducted in different years (1996-2006), were initially analysed as per the first group (i.e. reports in different years in the same patient were treated as independent); but a second analytical approach (Pillay et al 2005) was also utilised to determine the accumulation of major resistance mutations in this group over the time period analysed. For example, a patient had a resistance report from 2000 which showed major mutations which conferred resistance to the NNRTI drug class. This patient had another resistance report available from 2002 which showed major mutations to the NRTI and PI drugs classes. Using the first approach (one resistance report per year) this patient would be classed as having NNRTI resistance in 2000 and NRTI+PI resistance in 2002. Using the cumulative approach, this patient would be classed as having NNRTI resistance in 2000 and NNRTI+NRTI+PI resistance in 2002; once mutations have developed, even if they are not detected by the resistance test they have not ‘disappeared’ and no longer exist, but have been archived and would become the dominant virus population once treatment related to the archived resistance mutations was initiated again (Lambotte et al 2004, Pillay et al 2005).

Statistical analyses were conducted using SPSS (version 22). Chi^2 (χ^2) analyses were used to test for any significant differences in the distribution of mutations between the EC and the LC. Significance was assigned at the 0.05 level ($p<0.05$).

Results

Overall, 1,786 treatment-experienced patients were eligible to take part in this study including 1,488 with one genotypic resistance report available and 298 patients with more than one report available (725 reports in 298 patients making a total of 2,213 genotypic resistance reports altogether).

Patients' demographics

Of the 1,786 treatment-experienced patients, the majority were male (968 males, 72.6%, and the minority 366 females, 27.4%). The mean age of the male patients was 44.7, ranging from 18 to 78 years while the mean female age was 38.9, ranging from 18 to 87 years. Of risk exposure group data provided (n=469): 250 (52.1%) of patients were infected through heterosexual contact, 170 (35.4%) through MSM, 21 IDU (4.4%), 14 through blood/tissue transfer (2.9%), 11 mother-to-child (2.3%), two MSM/IDU (0.4%) and one sharps injury (0.2%). Of origin of infection data provided (n=1,040): Europe was the predominant origin (559 patients, 53.5%) while the remainder were from: Africa (n=411, 39.4%); Asia (n=24, 2.3%); North America (n=23, 2.2%); South America (n=9, 0.9%); Australasia (n=6, 0.6%); Caribbean (n=4, 0.4%); South Africa (n=3, 0.3%); and the Middle East (n=1, 0.1%).

Evidence of resistance in the 1,488 treatment-experienced patients with one genotypic report available

Of the 1,488 patients in the treatment-experienced cohort with one genotypic report available: 680 (45.7%) were currently on ART treatment, 213 (14.3%) were not currently on ART but had previous experience and 595 (40.0%)

were treatment-experienced but it was not indicated whether they were on a current ART regimen and/or had previous ART experience.

Of the 680 currently on ART treatment, 584 (85.8%) were taking three or four drugs in their regimen, three (0.2%) were on one drug only, 50 (3.4%) were on two, 15 (1.0%) were on five drugs and seven (0.5%) were on six drugs. Thirty-two different combinations were prescribed with 257 (39.0%) on a combination of 2 NRTIs + 1 NNRTI, 142 (21.5%) on 2 NRTIs + 1 PI, 48 (7.3%) on 3 NRTIs, 44 (6.7%) on 2 NRTIs + 2 PIs, 39 (5.9%) on 2 NRTIs, 25 (3.8%) on 3 NRTIs + 1 NNRTI, 18 (2.7%) on 3 NRTIs + 1 PI, 15 (2.3%) on 2 NRTIs + 1 NNRTI + 1 PI, and 13 (2.0%) on 1 NRTI + 1 NNRTI + 1 PI. There were a further 23 combinations of specific drugs within the drugs classes prescribed to 58 patients, all with <1.0% of patients on the combination.

Half of the 680 currently on ART treatment also had previous treatment experience (n=344, 50.6%). The mean number of previous ART drugs was 3.9 with a minimum of one and a maximum of 15 other drugs.

Of the 213 who were not on ART but had previous experience, the mean number of previous ART drugs was 4.2 with a minimum of one and a maximum of 12 other drugs. All 213 patients had previous NRTI experience with 116 (54.5%) having previous NNRTI drugs and 109 (51.2%) previous PI drugs.

Table 5.1: Frequencies of antiretroviral drugs by current usage and previous experience; showed wide use of nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) and less use of protease inhibitors (PIs).

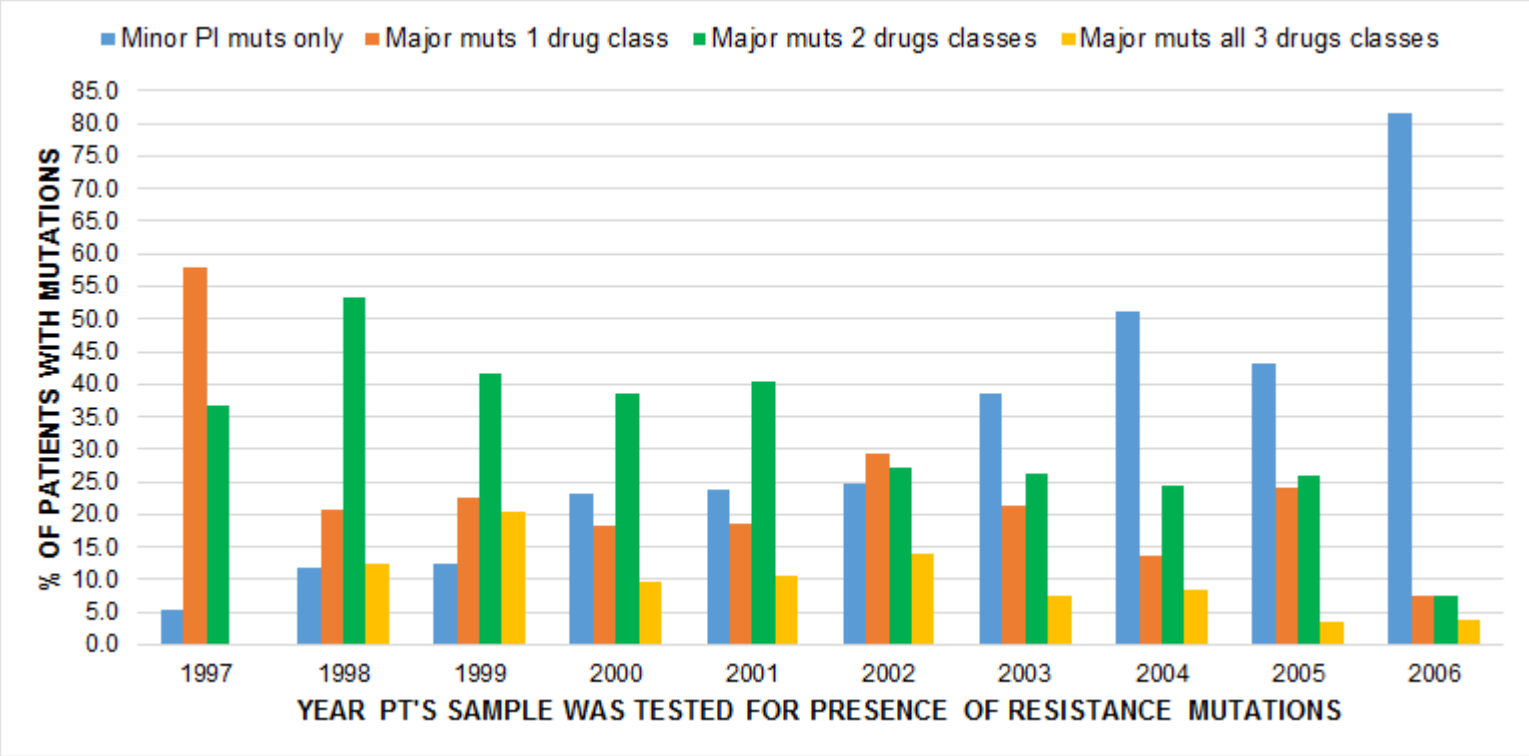
		Currently on ART				Not currently on ART	
Drug class	Antiretroviral drug	Current usage (n=680)		Previous drug experience (n=344)		Previous drug experience (n=213)	
		n	%	n	%	n	%
NRTIs	ZDV	272	41.3	269	78.2	182	83.9
	ddI	190	28.8	157	45.6	93	42.9
	ddC	19	2.9	74	21.5	20	9.2
	3TC	443	67.2	204	59.3	178	82.0
	d4T	252	38.2	172	50.0	113	52.1
	ABC	132	20.0	42	12.2	38	17.5
	TDF	85	12.9	9	2.6	14	6.5
	FTC	3	0.5	1	0.3	2	0.9
NNRTIs	NVP	198	30.0	108	31.4	82	37.8
	DLV	6	0.9	9	2.6	2	0.9
	EFV	131	19.9	69	20.1	49	22.6
PIs	SQV	63	9.6	70	20.3	44	20.3
	IDV	41	6.2	96	27.9	38	17.5
	RTV	65	9.9	81	23.5	41	18.9
	NFV	91	13.8	77	22.4	44	20.3
	APV	7	1.1	9	2.6	2	0.9
	LPV/r	72	10.9	13	3.8	14	6.5
	LPV	11	1.7	3	0.9	3	1.4
	TPV	0	0.0	0	0.0	0	0.0
	ATV	3	0.5	1	0.3	1	0.5
	FPV	1	0.2	0	0.0	0	0.0

Overall, 977 (65.7%) of the 1,488 patients in the treatment-experienced cohort with one genotypic report available presented with major resistance mutations which impacted one or more drug class:

- 319 (21.5%) had major mutations which impacted one drug class (12.9% NRTIs; 7.1% NNRTIs; 1.5% PIs)
- 491 (33.0%) had major mutations which impacted two drugs classes (21.6% NRTI+NNRTI; 10.9% NRTI+PI; 0.5% NNRTI+PI)
- 167 (11.2%) had major mutations to all three drugs classes.

Four hundred and thirty-eight patients (29.4%) presented with minor PI mutations only and 73 (4.9%) had no mutations.

Graph 5.1: Percentage of patients, per year, identified with major mutations (muts) which conferred resistance to the antiretroviral therapies within one drug class, two drugs classes or all three drugs classes. The graph also shows the frequency of minor protease inhibitor (PI) mutations over the years with a clear increase from 1997 to 2006.



** 1996 not presented in the graph above as the denominator was small (n=2).*

Of the 1,488 treatment-experienced cohort with one genotypic report available: 525 patients (35.3%) had genotypic resistance tests conducted during 1996-2000 and formed the early cohort (EC) whilst 963 (64.7%) were tested during 2001-2006 and formed the late cohort (LC).

Table 5.2: Comparison of the prevalence of major mutations in the early cohort (EC, 1996-2000) and the late cohort (LC, 2001-2006) by drug classes affected. There was a decrease in the frequency of major mutations impacting all three drugs classes between the EC (14.9%) and LC (9.2%); and an overall decrease in major mutations affecting two drugs classes (EC: 43.0%, LC 27.5%), particularly the nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs) drugs classes between the EC (22.5%) and LC (4.6%).

Major mutations and drug class affected	Early cohort (EC) (n=525)		Late cohort (LC) (n=963)		Total (n=1,488)	
	n	%	n	%	n	%
All three drugs classes	78	14.9	89	9.2	167	11.2
Two drugs classes	226	43.0	265	27.5	491	33.0
NRTI+PI	118	22.5	44	4.6	162	10.9
NNRTI+PI	3	0.5	4	0.4	7	0.5
NRTI+NNRTI	105	20.0	217	22.5	322	21.6
One drug class	117	22.3	202	21.0	319	21.4
NRTI	80	15.3	112	11.6	192	12.9
NNRTI	29	5.5	76	7.9	105	7.1
PI	8	1.5	14	1.5	22	1.5
Minor PI mutations only	79	15.0	359	37.3	438	29.4
No mutations	25	4.8	48	5.0	73	4.9
Total	525	100.0	963	100.0	1,488	100.0

Geographical distribution of resistance mutations

The majority of the clinical cases were from the Greater London region (n=1,011, 67.9%) with 203 (13.6%) from the South East, 89 (6.0%) seen in both the Eastern region and Wales, 35 (2.4%) from the South West, 23 (1.5%) from Scotland, 22 (1.5%) from the East Midlands and 16 (1.1%) from the North West region.

Table 5.3: Major mutations and drug class affected as per the Geographical regions where the 39 clinical centres were based. Major mutations were identified across all regions, impacting all three drugs classes.

Major mutations and drug class affected	Geographical regions where the clinical centres were based															
	Scotland		North West		Wales		East Midlands		Eastern		Greater London		South East		South West	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
All three drugs classes	2	8.7	5	31.3	18	20.2	1	4.5	3	3.4	117	11.6	19	9.4	2	5.7
Two drugs classes	10	43.5	3	18.8	42	47.2	5	22.7	12	13.5	327	32.3	80	39.4	12	34.3
NRTI+PI	5	21.7	0	0.0	15	16.9	1	4.5	2	2.2	109	10.8	27	13.3	3	8.6
NNRTI+PI	0	0.0	0	0.0	2	2.2	0	0.0	0	0.0	5	0.5	0	0.0	0	0.0
NRTI+NNRTI	5	21.7	3	18.8	25	28.1	4	18.2	10	11.3	213	21.0	53	26.1	9	25.7
One drug class	4	17.4	3	18.8	16	18.0	6	27.3	15	16.9	221	21.9	52	25.6	2	5.7
NRTI	3	13.0	3	18.8	5	5.6	3	13.6	7	7.9	133	13.2	37	18.2	1	2.8
NNRTI	1	4.4	0	0.0	10	11.2	2	9.1	6	6.8	72	7.1	14	6.9	0	0.0
PI	0	0.0	0	0.0	1	2.2	1	4.6	2	2.2	16	1.6	1	0.5	1	2.8
Minor PI mutations only	6	26.1	5	31.3	11	12.4	9	40.9	50	56.2	299	29.6	41	20.2	17	48.6
No mutations	1	4.3	0	0.0	2	2.2	1	4.5	9	10.1	47	4.6	11	5.4	2	5.7
Total	23	100.0	16	100.0	89	100.0	22	100.0	89	100.0	1,011	100.0	203	100.0	35	100.0

Resistance to the NRTI drug class

Overall, 843 (56.7%) of the cohort presented with one or more NRTI drug resistance mutations. The majority had one NRTI resistance mutation (n=274, 18.4%), 136 (9.1%) had two, 132 (8.9%) three, 157 (10.6%) four, 80 (5.4%) five, 44 (3.0%) six, 15 (1.0%) seven, four (0.3%) with eight and one patient (0.1%) presented with 10 NRTI mutations.

Table 5.4: Prevalence of specific nucleoside reverse transcriptase inhibitors (NRTI) "lifetime mutations" in the treatment-experienced clinical cohort. M184V was the most common mutation, followed by T215Y and M41L.

Prevalence of NRTI mutations	Currently on ART (n=680)		Not on ART but previous experience (n=213)		Treatment-experienced but not indicated whether current or previous ART experience (n=595)		Total (n=1,488)	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
M41L	189	27.8	46	21.6	64	10.8	299	20.1
A62V	12	1.8	5	2.3	8	1.3	25	1.7
K65R	12	1.8	6	2.8	25	4.2	43	2.9
D67N	185	27.2	41	19.2	58	9.7	284	19.1
69Insert								
T69D	38	5.6	3	1.4	7	1.2	38	2.6
T69N	14	2.1	0	0	3	0.5	17	1.1
K70R	125	18.4	33	15.5	58	9.7	216	14.5
L74V	43	6.3	7	3.3	26	4.4	76	5.1
V75I	10	1.5	4	1.9	7	1.2	21	1.4
F77L	10	1.5	3	1.4	4	0.7	17	1.1
Y115F	6	0.9	4	1.9	15	2.5	25	1.7
F116Y	8	1.2	2	0.9	2	0.3	12	0.8
Q151M	9	1.3	2	0.9	4	0.7	15	1.0
M184I	15	2.2	2	0.9	19	3.2	36	2.4
M184V	305	44.9	78	36.6	167	28.1	550	37.0
L210W	128	18.8	28	13.1	33	5.5	189	12.7
T215F	51	7.5	11	5.2	16	2.7	78	5.2
T215Y	205	30.1	48	22.5	57	9.6	310	20.8
K219E	37	5.4	14	6.6	23	3.9	74	5.0
K219Q	59	8.7	12	5.6	22	3.7	93	6.3

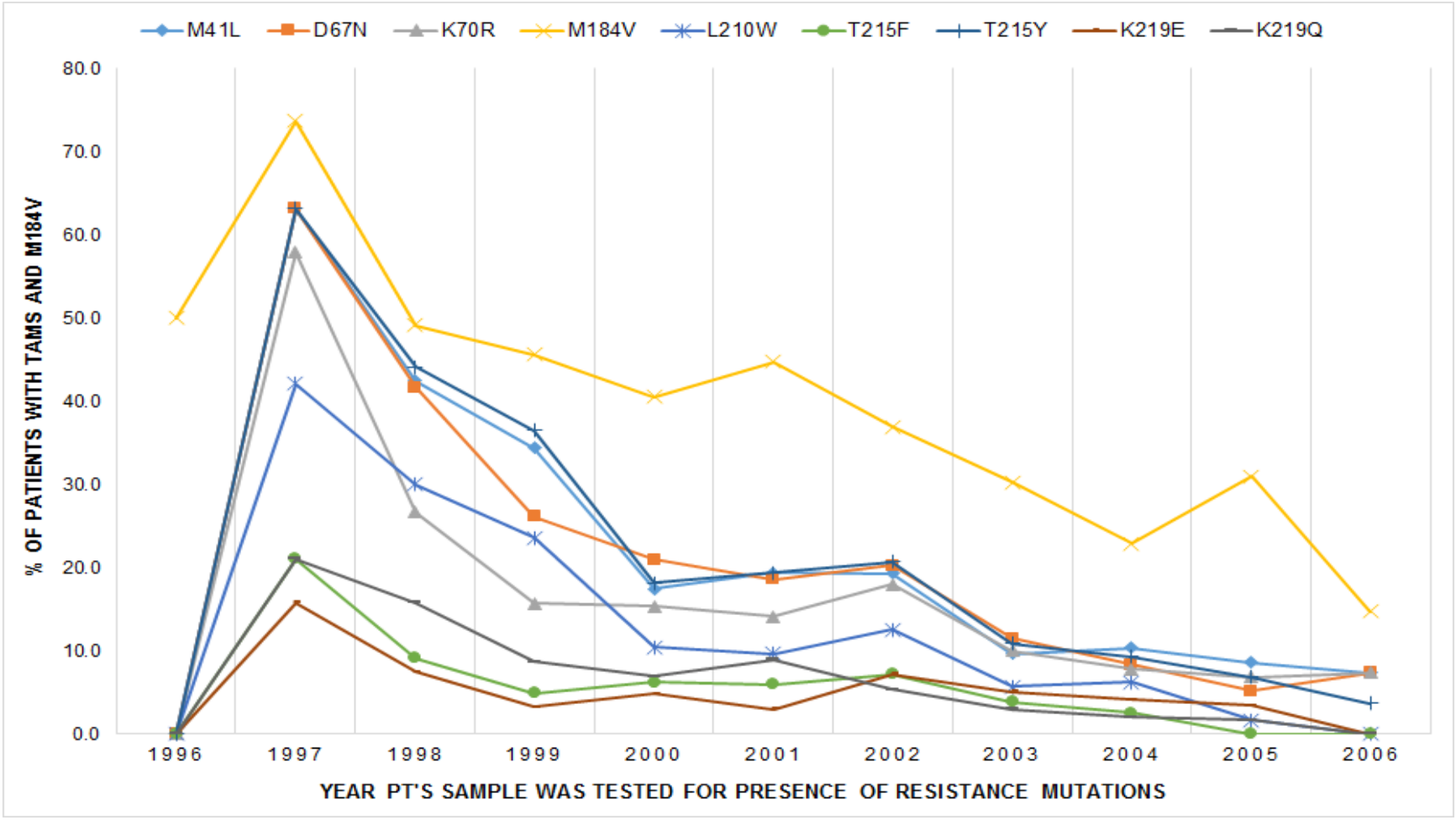
Combinations of major NRTI mutations including TAMs

Of the 843 patients with major NRTI mutations, 330 (39.1%) had three or more TAMs: 165 (50.0%) had three TAMs, 116 (35.2%) had four, 36 (10.9%) had five and 13 (3.9%) had six. Of the 330, 180 (54.5%) had three or more TAMs and the M184V mutation; 170 (51.5%) had a combination of M41L+L210W+T215F/Y and 14 (4.2%) had a combination of K65R+M184V (seven of these patients had one other NRTI mutation including two with A62V, two with L74V and three with Y115F; one patient had two other NRTI mutations including L74V+Y115F).

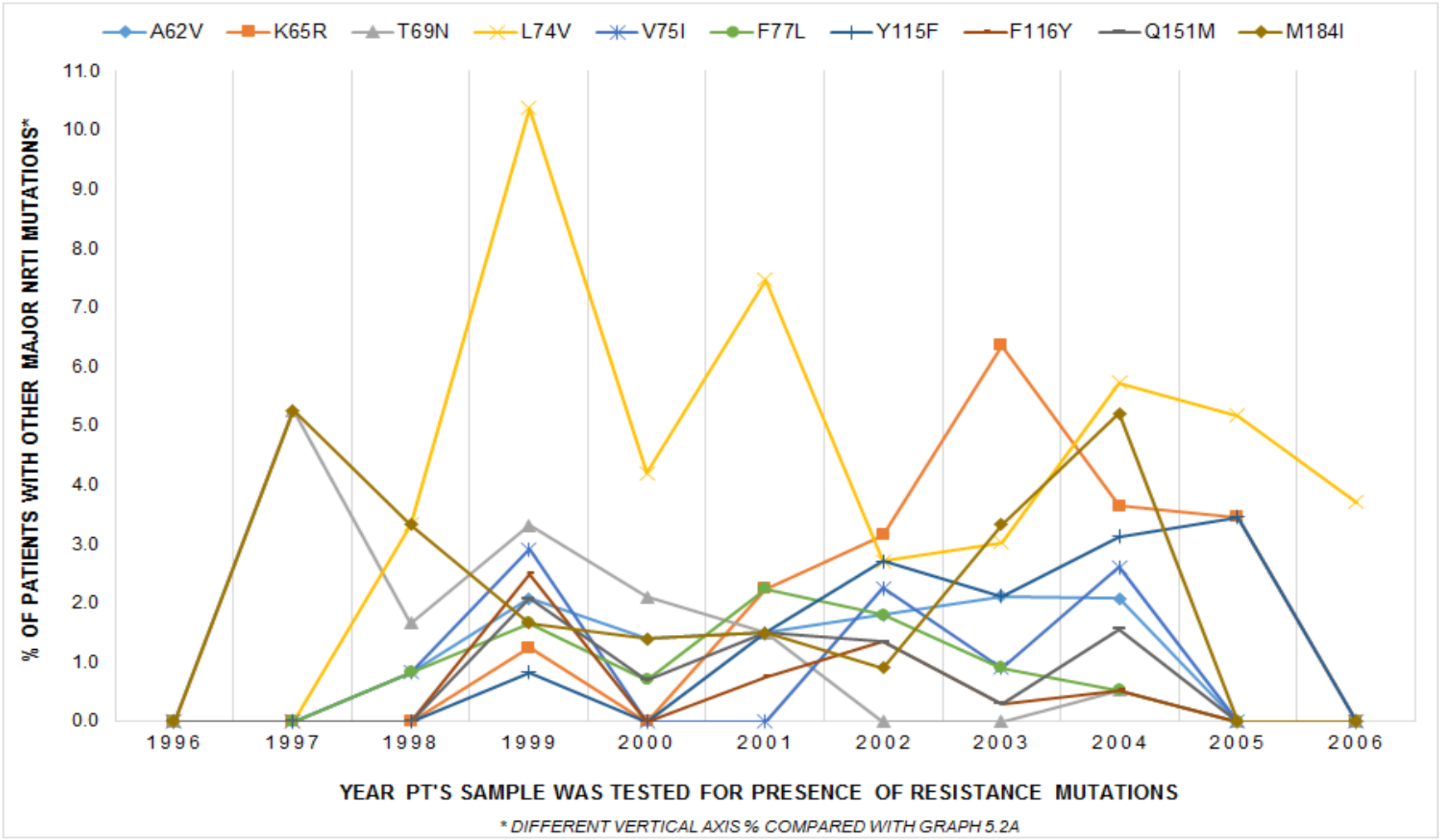
There was a significant difference ($p<0.05$) between the EC and the LC with 72.6% of the EC and 48.0% of the LC presenting with one or more major NRTI mutations. There was a significant decline in the prevalence of the following NRTI mutations in the LC compared with the EC: M41L, D67N, T69D/N, K70R, L74V, M184V, L210W, T215F/Y, K219Q.

The NRTI mutations K65R and Y115F were the only mutations to significantly increase in prevalence between the EC and LC.

Graph 5.2a: Prevalence of thymidine analogue mutations (TAMs) and M184V (major nucleoside reverse transcriptase inhibitors (NRTI) mutations) from 1996 to 2006. Pattern shows suboptimal treatment in 1996-1997 (stavudine (d4T)/zidovudine (ZDV)). From 1998 onwards, the prevalence of the NRTI mutations decreased as highly active antiretroviral therapy (HAART) was successful in suppressing replicating virus.



Graph 5.2b: Prevalence of other major nucleoside reverse transcriptase inhibitors (NRTI) mutations (contd.) from 1996 to 2006. The frequency of these NRTI mutations was generally lower (<10.0%) compared with the prevalence of the thymidine analogue mutations (TAMs) and M184V illustrated above.



Resistance to the NNRTI drug class

Of the cohort, 566 (38.0%) presented with NNRTI mutations. The majority of the 566 had one NNRTI mutation (n=320, 21.5%), 200 (13.4%) had two, 43 (2.9%) had three and three patients (0.2%) had four NNRTI mutations.

Table 5.5: Prevalence of specific non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations in the treatment-experienced clinical cohort. K103N was the most common mutation, followed by Y181C.

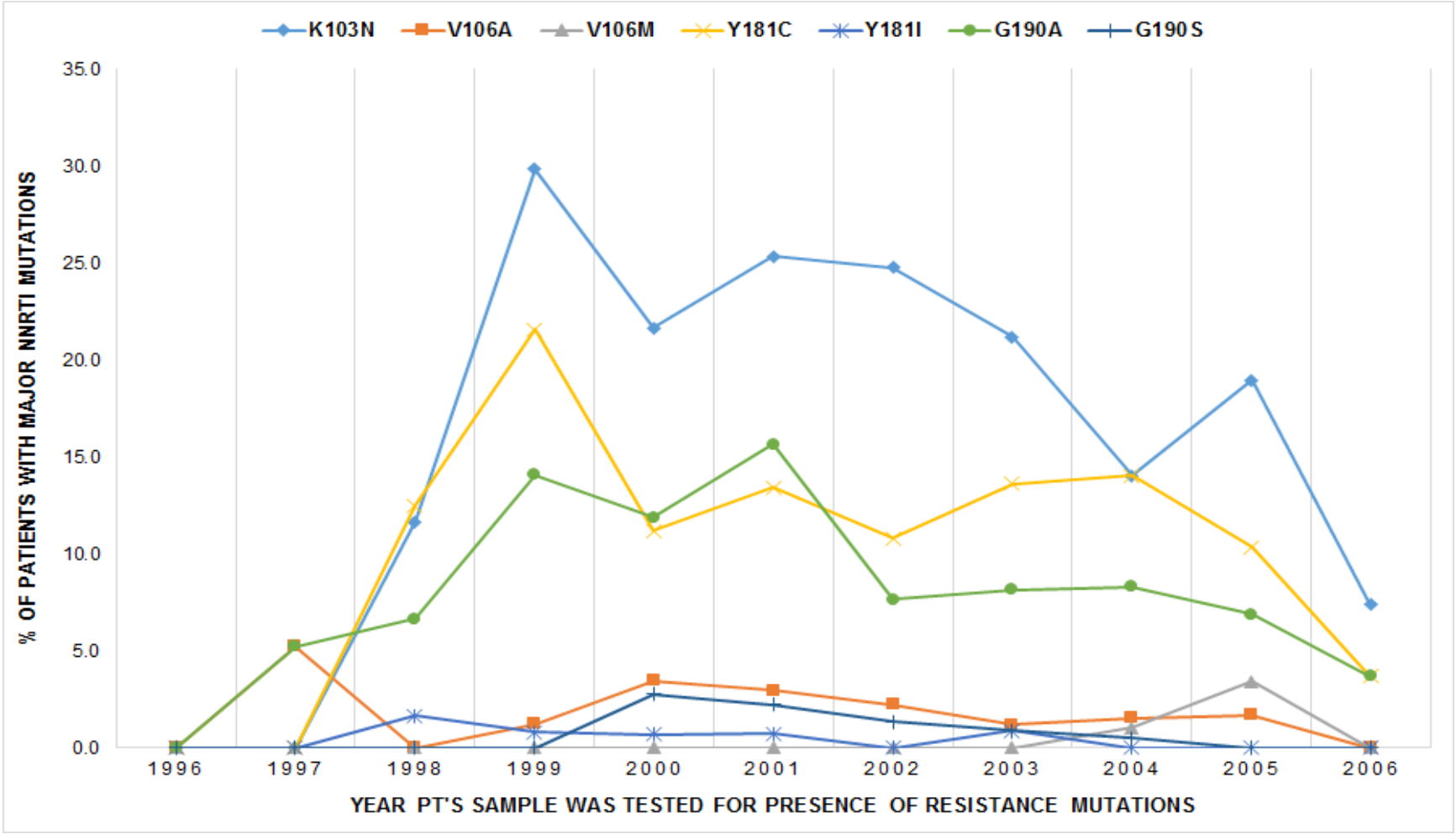
Prevalence of NNRTI mutations	Currently on ART (n=680)		Not on ART but previous experience (n=213)		Treatment-experienced but not indicated whether current or previous ART experience (n=595)		Total (n=1,488)	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
V90I	0	0.0	0	0.0	0	0.0	0	0.0
A98G	31	4.6	4	1.9	16	2.7	51	3.4
L100I	8	1.2	4	1.9	9	1.5	21	1.4
K101E	36	5.3	6	2.8	23	3.9	65	4.4
K101H	0	0.0	0	0.0	0	0.0	0	0.0
K101P	0	0.0	0	0.0	0	0.0	0	0.0
K103N	163	24.0	44	20.7	109	18.3	316	21.2
V106A	17	2.5	4	1.9	5	0.8	26	1.7
V106I	1	0.1	0	0.0	0	0.0	1	0.07
V106M	0	0.0	0	0.0	4	0.7	4	0.3
V108I	27	4.0	8	3.8	31	5.2	66	4.4
E138A	0	0.0	0	0.0	0	0.0	0	0.0
V179D	10	1.5	5	2.3	10	1.7	25	1.7
V179F	0	0.0	0	0.0	0	0.0	0	0.0
V179T	0	0.0	0	0.0	0	0.0	0	0.0
Y181C	108	15.9	22	10.3	74	12.4	204	13.7
Y181I	5	0.7	2	0.9	2	0.3	9	0.6
Y181V	0	0.0	0	0.0	0	0.0	0	0.0
Y188C	3	0.4	0	0.0	1	0.2	4	0.3
Y188H	1	0.1	0	0.0	2	0.3	3	0.2
Y188L	8	1.2	3	1.4	14	2.4	25	1.7
G190A	86	12.6	17	8.0	43	7.2	146	9.8
G190S	6	0.9	3	1.4	5	0.8	14	0.9
P225H	4	0.6	5	2.3	14	2.4	23	1.5
M230L	0	0.0	0	0.0	6	1.0	6	0.4

Key:
NNRTI mutations in bold and highlighted in pink were considered major NNRTI mutations as per the IAS-USA mutation list (Johnson et al 2009).

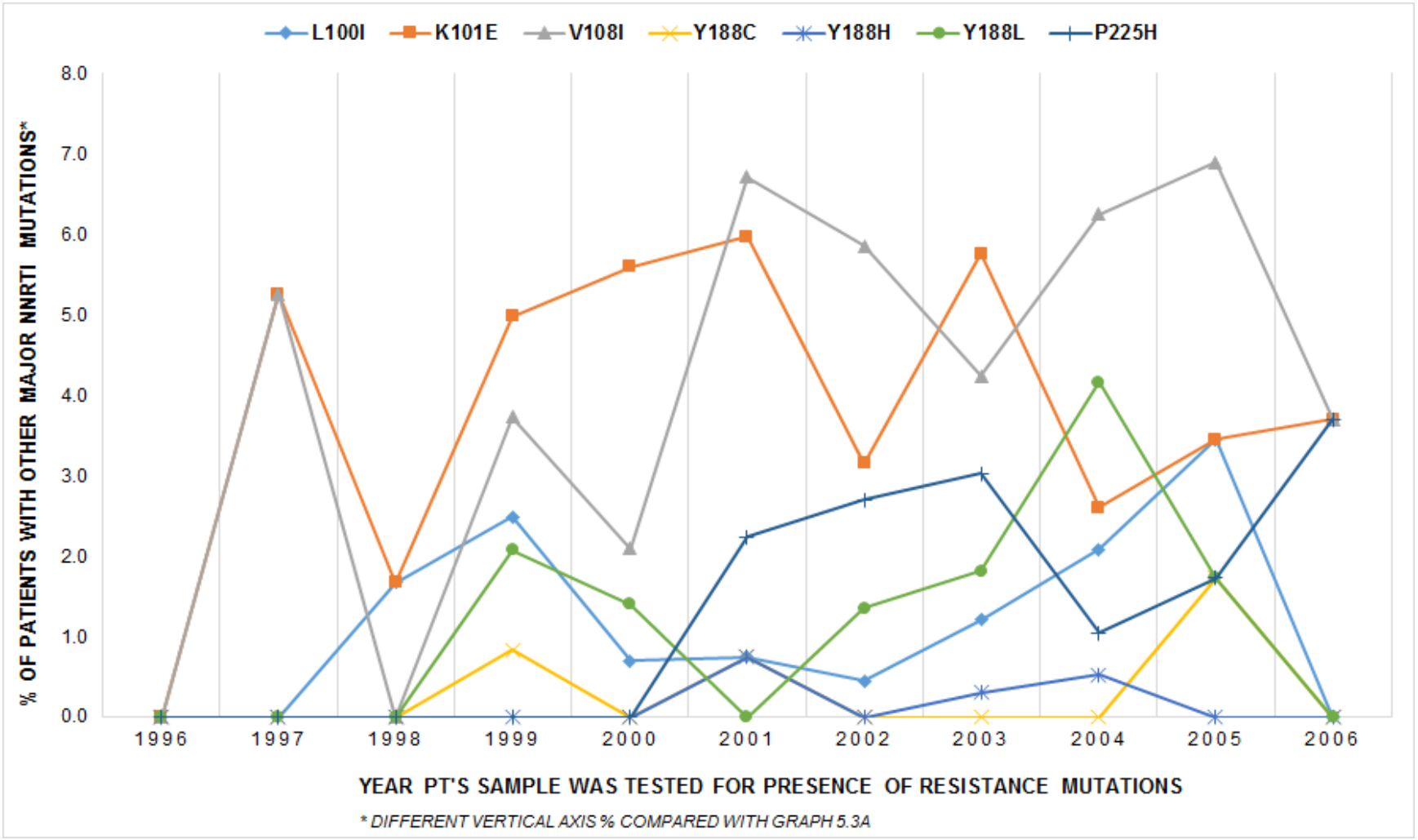
The prevalence of one or more NNRTI mutations was comparable amongst the EC (38.3%) and the LC (37.9%).

Two NNRTI mutations showed a significant difference between the EC and LC: the prevalence of V108I and P225H significantly increased from the EC to the LC.

Graph5.3a: Prevalence of major non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations from 1996 to 2006. K103N was the most prevalent major NNRTI mutation overall, followed by Y181C and G190A.



Graph 5.3b: Prevalence of other major non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations (contd.) from 1996 to 2006. Compared with Graph 5.3a these major NNRTI mutations were prevalent at much lower rates (<7.0%).



Resistance to the PI drug class

The PR mutations were most prevalent: 1,343 (90.3%) of the cohort had such mutations. The most frequent was one PR mutation (n=501, 33.7%), 410 (27.6%) had two, 157 (10.6%) three, 86 (5.8%) four, 63 (4.2%) five, 46 (3.1%) six, 31 (2.1%) seven, 29 (1.9%) eight, 16 (1.1%) nine, and four patients (0.3%) had ≥10 PR mutations (maximum 12).

Of the 1,343 patients with PR mutations, only 358 (26.7%) were classed as having major PR mutations. The majority had one major PR mutation (n=181, 13.5%), 108 (8.0%) had two, 56 (4.2%) three, six (0.4%) four, five (0.4%) five, and two (0.1%) had six major PR mutations.

Table 5.6: Prevalence of specific protease inhibitor (PI) mutations in the treatment-experienced clinical cohort. L90M was the most common major mutation, followed by M46I and V82A. L63P was the most prevalent minor mutation, followed by M36I and L10I.

Prevalence of PI mutations	Currently on ART (n=680)		Not on ART but previous experience (n=213)		Treatment-experienced but not indicated whether current or previous ART experience (n=595)		Total (n=1,488)	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
L10C	0	0.0	0	0.0	0	0.0	0	0.0
L10F	4	0.6	1	0.5	12	2.0	17	1.1
L10I	153	22.5	36	16.9	73	12.3	262	17.6
L10R	2	0.3	1	0.5	0	0	3	0.2
L10V	34	5.0	15	7.0	37	6.2	86	5.8
V11I	0	0.0	0	0.0	0	0.0	0	0.0
I13V	0	0.0	0	0.0	2	0.3	2	0.1
G16E	0	0.0	0	0.0	0	0.0	0	0.0
K20I	0	0.0	0	0.0	0	0.0	0	0.0
K20M	12	1.8	4	1.9	13	2.2	29	1.9
K20R	65	9.6	27	12.7	78	13.1	170	11.4
K20T	0	0.0	0	0.0	0	0.0	0	0.0
K20V	0	0.0	0	0.0	0	0.0	0	0.0
L24I	16	2.4	1	0.5	4	0.7	24	1.4
D30N	38	5.6	7	3.3	10	1.7	55	3.7
V32I	6	0.9	2	0.9	5	0.8	13	0.9
L33F	16	2.4	0	0.0	11	1.8	27	1.8

Table 5.6 (contd.): Prevalence of specific protease inhibitor (PI) mutations in the treatment-experienced clinical cohort. L90M was the most common major mutation, followed by M46I and V82A. L63P was the most prevalent minor mutation, followed by M36I and L10I.

Prevalence of PI mutations	Currently on ART (n=680)		Not on ART but previous experience (n=213)		Treatment-experienced but not indicated whether current or previous ART experience (n=595)		Total (n=1,488)	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
L33I	0	0.0	0	0.0	1	0.2	1	0.1
L33V	0	0.0	0	0.0	0	0.0	0	0.0
E34Q	0	0.0	0	0.0	0	0.0	0	0.0
E35G	0	0.0	0	0.0	0	0.0	0	0.0
M36I	261	38.4	115	54.0	374	62.9	750	50.4
M36L	1	0.1	0	0.0	4	0.7	5	0.3
M36V	1	0.1	1	0.5	2	0.3	4	0.3
K43T	0	0.0	0	0.0	0	0.0	0	0.0
M46I	70	10.3	13	6.1	25	4.2	108	7.3
M46L	28	4.1	3	1.4	10	1.7	41	2.8
I47A	0	0.0	0	0.0	0	0.0	0	0.0
I47V	2	0.3	1	0.5	4	0.7	7	0.5
G48V	20	2.9	2	0.9	4	0.7	26	1.7
I50L	0	0.0	0	0.0	0	0.0	0	0.0
I50V	8	1.2	3	1.4	1	0.2	12	0.8
F53L	4	0.6	3	1.4	7	1.2	14	0.9
F53Y	0	0.0	0	0.0	0	0.0	0	0.0
I54A	1	0.1	0	0.0	0	0.0	1	0.1
I54L	5	0.7	1	0.5	5	0.8	11	0.7
I54M	0	0.0	0	0.0	0	0.0	0	0.0
I54S	0	0.0	0	0.0	0	0.0	0	0.0
I54T	0	0.0	0	0.0	2	0.3	2	0.1
I54V	68	10.0	12	5.6	20	3.4	100	6.8
Q58E	0	0.0	0	0.0	0	0.0	0	0.0
D60E	0	0.0	0	0.0	0	0.0	0	0.0
I62V	0	0.0	0	0.0	0	0.0	0	0.0
L63P	383	56.3	105	49.3	263	44.2	764	51.3
I64L	0	0.0	0	0.0	0	0.0	0	0.0
I64M	0	0.0	0	0.0	0	0.0	0	0.0
I64V	0	0.0	0	0.0	0	0.0	0	0.0
H69K	1	0.1	0	0.0	5	0.8	6	0.4
A71I	3	0.4	0	0.0	1	0.2	4	0.3
A71L	0	0.0	0	0.0	0	0.0	0	0.0
A71T	72	10.6	22	10.3	31	5.2	125	8.4

Table 5.6 (contd.): Prevalence of specific protease inhibitor (PI) mutations in the treatment-experienced clinical cohort. L90M was the most common major mutation, followed by M46I and V82A. L63P was the most prevalent minor mutation, followed by M36I and L10I.

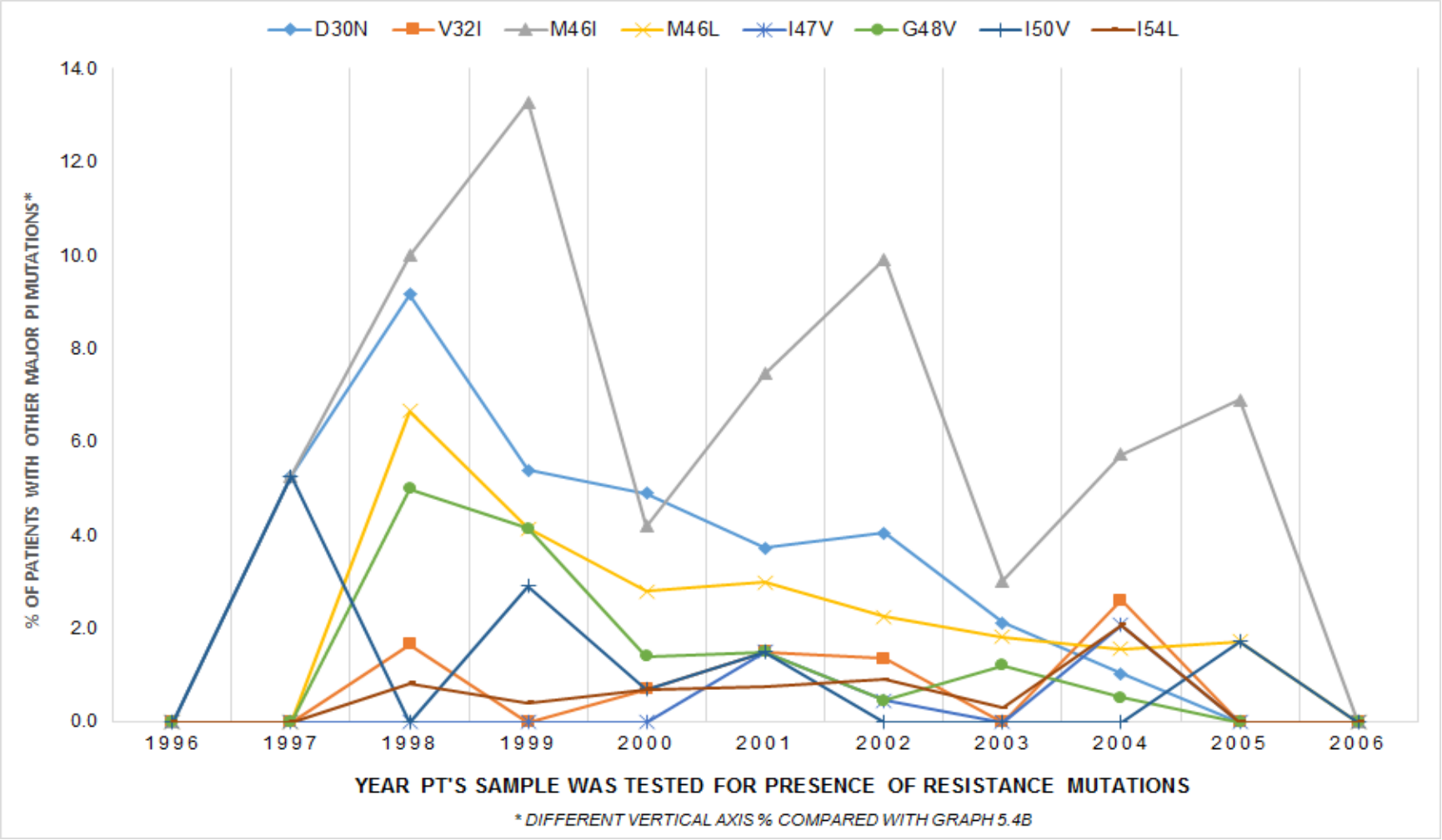
Prevalence of PI mutations	Currently on ART (n=680)		Not on ART but previous experience (n=213)		Treatment-experienced but not indicated whether current or previous ART experience (n=595)		Total (n=1,488)	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
A71V	94	13.8	15	7.0	36	6.1	145	9.7
G73A	0	0.0	0	0.0	0	0.0	0	0.0
G73C	1	0.1	0	0.0	0	0.0	1	0.1
G73S	32	4.7	2	0.9	8	1.3	42	2.8
G73T	0	0.0	0	0.0	1	0.2	1	0.1
T74P	0	0.0	0	0.0	0	0.0	0	0.0
L76V	0	0.0	0	0.0	0	0.0	0	0.0
V77I	142	20.9	11	5.2	4	0.7	157	10.6
V82A	78	11.5	12	5.6	18	3.0	108	7.3
V82F	4	0.6	0	0.0	1	0.2	5	0.3
V82L	0	0.0	0	0.0	0	0.0	0	0.0
V82S	1	0.1	0	0.0	2	0.3	3	0.2
V82T	7	1.0	0	0.0	2	0.3	9	0.6
N83D	0	0.0	0	0.0	0	0.0	0	0.0
I84V	32	4.7	7	3.3	17	2.9	56	3.8
I85V	0	0.0	0	0.0	0	0.0	0	0.0
N88D	23	3.4	6	2.8	9	1.5	38	2.6
N88S	1	0.1	0	0.0	3	0.5	4	0.3
L89V	0	0.0	0	0.0	0	0.0	0	0.0
L90M	116	17.1	23	10.8	32	5.4	171	11.5
I93L	0	0.0	0	0.0	0	0.0	0	0.0
I93M	0	0.0	0	0.0	0	0.0	0	0.0

Key:
PR mutations in bold and highlighted in pink were considered major PR mutations as per the IAS-USA mutation list (Johnson et al 2009).

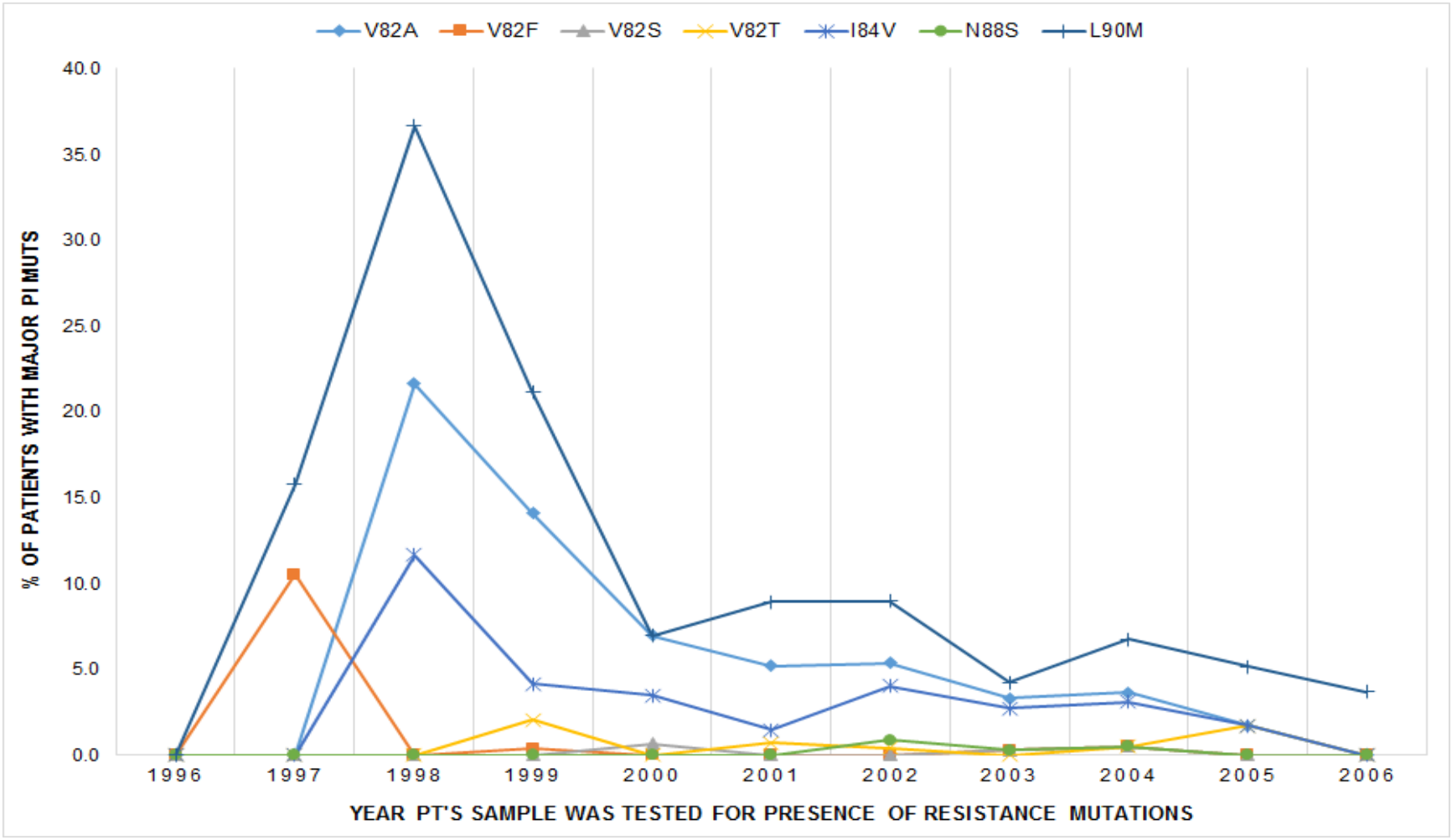
The prevalence of one or more PI mutations was comparable amongst the EC (89.7%) and the LC (90.6%). Major PI mutations were significantly more prevalent in the EC however (39.6%) compared with the LC (15.6%) ($p<0.05$).

There was a significant decline in the prevalence of the following major PI mutations in the LC compared with the EC: D30N, M46I/L, G48V, V82A, I84V, L90M. There was also a significant decline in the prevalence of the following minor PI mutations in the LC compared with the EC: L10I, L24I, L33F, L63P, A71T/V, G73S, V77I, N88D. Only two PI mutations, both minor, showed an increase in prevalence from the EC to the LC: M36I and F53L.

Graph 5.4a: Prevalence of major protease inhibitor (PI) mutations from 1996 to 2006. All mutations excluding M46I had a prevalence of <10.0%, with a clear decline to approximately 2001 and stability afterwards reflecting the use of more potent PIs.



Graph 5.4b: Prevalence of major protease inhibitor (PI) mutations (contd.) from 1996 to 2006. Prevalence of these PI mutations peaked in 1997/1998 and declined to <10.0% (as per the PI mutations in the graph above) from 2001 onwards.



Evidence of resistance in the 298 treatment-experienced patients with more than one genotypic report available

Of the 298 treatment-experienced patients with more than one genotypic resistance report available, conducted across different years: 210 had two reports, 58 had three reports, 22 had four reports, five had five reports and three patients had six reports. A total of 725 resistance reports were available for the 298 treatment-experienced patients.

At the time of genotyping, 525/725 (72.4%) were currently on ART treatment with the majority taking a combination with three to four drugs (462, 63.7%). Of those currently on ART, it was indicated that 320 (61.0%) also had previous experience with other drugs. There were 91 patients (12.6%) not on ART but who had previous experience and 109 (15.0%) were treatment-experienced but it was not indicated whether they were on a current ART regimen and/or had previous ART experience.

Table 5.7: Comparison of the prevalence of major mutations by the type of analysis: year by year or accumulative; by drug classes affected. Year by year analyses show the number of patients with major mutations in that year. Cumulative analyses reflect the accumulation of major resistance mutations over the years.

Major mutations and drug class affected	Type of analysis	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	Total
All three drugs classes	Year by year	0	0	2	15	22	20	24	20	6	1	2	112
	Cumulative	0	0	2	17	39	59	83	103	109	110	112	112
Two drugs classes	Year by year	1	4	15	33	37	32	62	43	18	2	0	247
	Cumulative	1	5	20	53	90	122	184	227	245	247	247	247
NRTI+PI	Year by year	0	4	11	12	8	8	17	14	3	2	0	79
	Cumulative	0	4	15	27	35	43	60	74	77	79	79	79
NNRTI+PI	Year by year	0	0	1	0	0	1	2	1	1	0	0	6
	Cumulative	0	0	1	1	1	2	4	5	6	6	6	6
NRTI+NNRTI	Year by year	1	0	3	21	29	23	43	28	14	0	0	162
	Cumulative	1	1	4	25	54	77	120	148	162	162	162	162
One drug class	Year by year	0	1	6	15	21	28	28	46	14	9	2	170
	Cumulative	0	1	7	22	43	71	99	145	159	168	170	170
NRTI	Year by year	0	1	4	10	8	18	20	25	5	1	1	93
	Cumulative	0	1	5	15	23	41	61	86	91	92	93	93
NNRTI	Year by year	0	0	1	2	12	9	7	21	8	8	1	69
	Cumulative	0	0	1	3	15	24	31	52	60	68	69	69
PI	Year by year	0	0	1	3	1	1	1	0	1	0	0	8
	Cumulative	0	0	1	4	5	6	7	7	8	8	8	8
Total year by year		*1*	*5*	*23*	*63*	*80*	*80*	*114*	*109*	*38*	*12*	*4*	*529*
Total cumulative		*1*	*6*	*29*	*92*	*172*	*252*	*366*	*475*	*513*	*525*	*529*	*529*

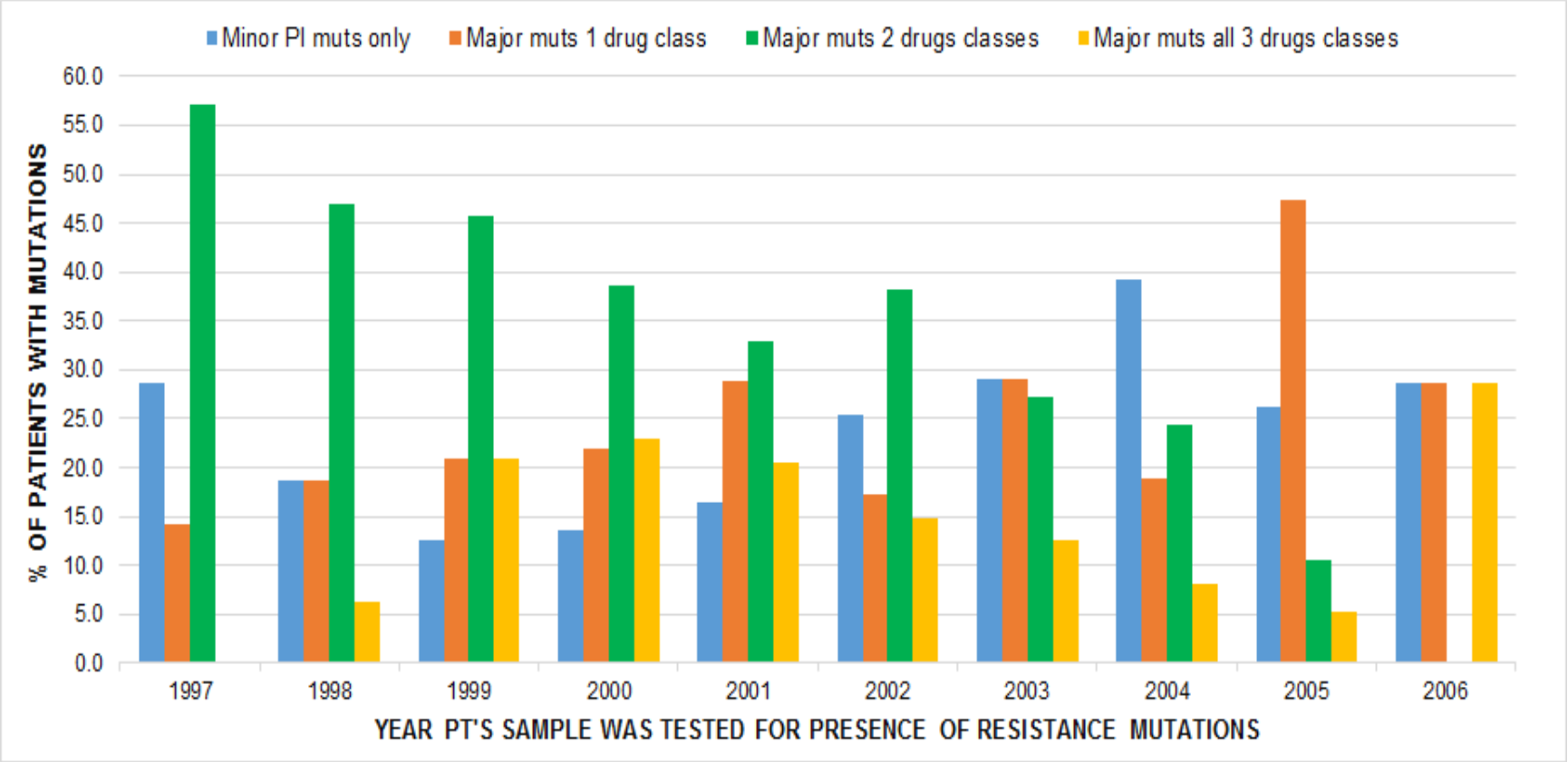
Overall, 529 (73.0%) of the 725 resistance entries for 298 treatment-experienced patients in the cohort with >1 genotypic report available presented with major resistance mutations which impacted one or more drug class:

- 170 (23.4%) had major mutations which impacted one drug class (12.8% NRTIs; 9.5% NNRTIs; 1.1% PIs)
- 247 (34.0%) had major mutations which impacted two drugs classes (22.3% NRTI+NNRTI; 10.9% NRTI+PI; 0.8% NNRTI+PI)
- 112 (15.4%) had major mutations to all three drugs classes.

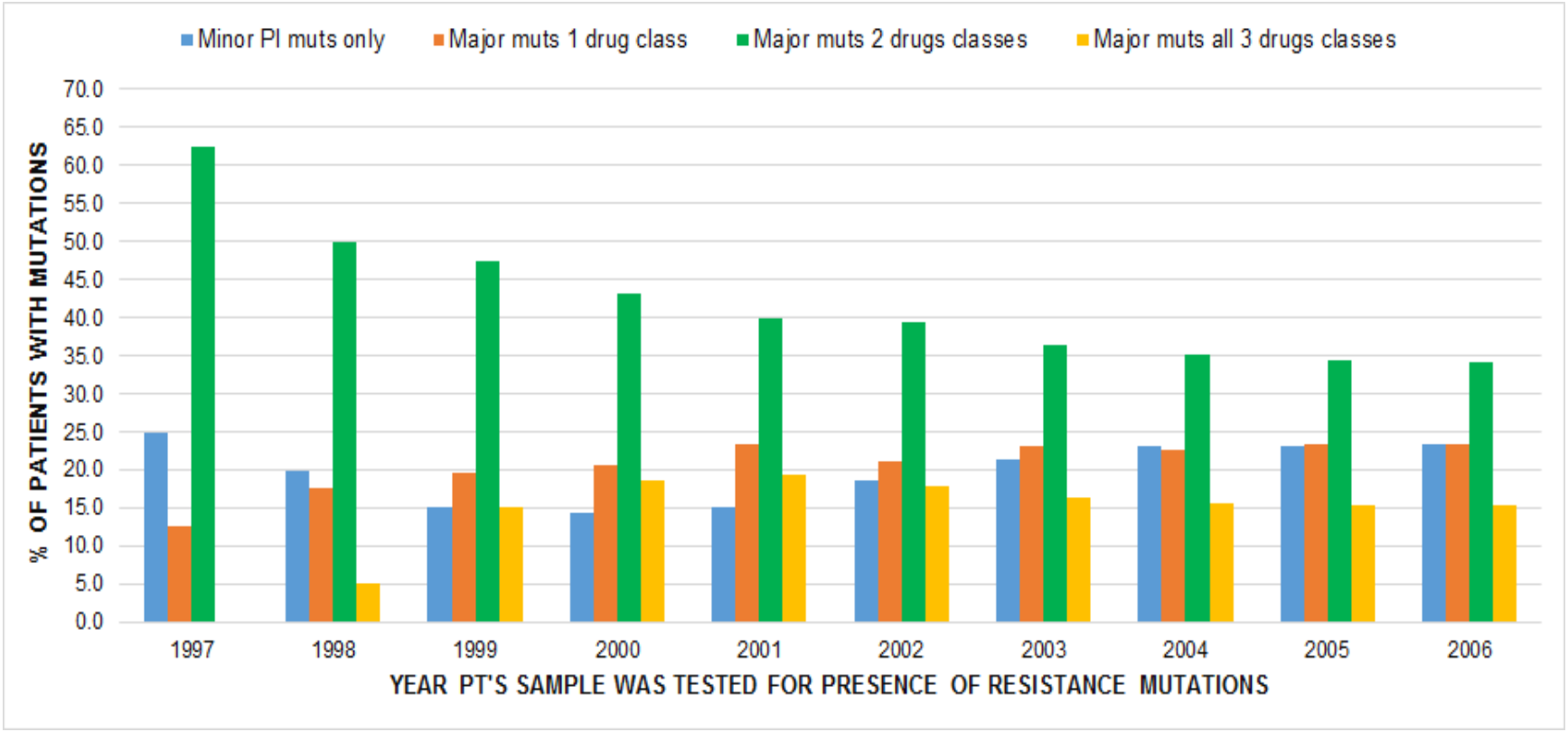
One hundred and sixty-nine (23.3%) presented with minor PI mutations only, while 27 (3.7%) of the cohort had no mutations.

These results were comparable with the findings of the patients with one resistance report only (overall resistance 65.7%, resistance to one drug class 21.5%, to two drugs classes 33.0%, to three drugs classes 11.2%, to minor mutations 29.4%, had no mutations 4.9%).

Graph 5.5a: For 298 treatment-experienced patients (pt's) with more than one genotypic result available (n=725 results), the graph shows the percentage of pt's per year, identified with major mutations (muts) which conferred resistance to the antiretroviral therapies within one drug class, two drugs classes or all three drugs classes. The graph also shows the frequency of minor protease inhibitor (PI) mutations over the years.



Graph 5.5b: For 298 treatment-experienced patients (pt's) with more than genotypic result available (n=725), the graph shows the accumulation of major mutations (muts), year on year, which conferred resistance to the antiretroviral therapies within one drug class, two drugs classes or all three drugs classes; alongside minor protease inhibitor (PI) mutations over the years.



Discussion

The resistance mutation profiles from 2,213 reports for 1,786 treatment-experienced patients were analysed. It was determined 68.1% of the samples submitted for genotypic resistance testing had major mutations; 22.1% had mutations which impacted one drug class, 33.3% had mutations which impacted two drugs classes and 12.6% had mutations which impacted all three drugs classes.

Analyses were conducted independently for those treatment-experienced patients with one resistance report only (n=1,488) and for those patients who had more than one resistance report available over the study time period (n=298, 725 reports).

Resistance in the 1,488 treatment-experienced patients with one genotypic report available

Of the 1,488 treatment-experienced patients, 56.7% harboured major mutations to the NRTI drug class; 38.0% had major mutations to the NNRTI drug class and 26.7% had major mutations to the PI drug class (90.3% had minor PI mutations). At the time of treatment, only three drugs classes were available to treat these patients and for those in the EC, there were fewer treatment options available. For the 38.0% of patients who had resistance mutations to the NNRTIs, this drug class was no longer available to them as one mutation conferred resistance to all of the first-generation NNRTIs within this class. For the 56.7% of patients with NRTI resistance and 26.7% with PI resistance, the clinician would have considered the resistance report output that was provided to them and taken into account any previous drugs that had been used and the potential for the mutations that were identified at this resistance test to impact on future treatment options. At the ICVC, the Clinical Director was available to discuss patient cases and alongside the clinician, review the resistance output and provide expert interpretation of the mutational profile and potential treatment options.

NRTI resistance mutations

There was a significant decline in the prevalence of NRTI resistance mutations from the EC to the LC. Graphs 5.2a-b and statistical analyses of the specific NRTI mutations prevalent in the EC and the LC, determined a significant decline in the prevalence of the TAMs (M41L, D67N, K70R, L210W, T215F/Y, K219Q); mutations associated with the early NRTI drugs ZDV and d4T, that were available for treatment (Shafer and Schapiro 2008). With the introduction of newer and more robust drugs to the NRTI class, there was a more marked suppression of viral replication and thus a decreased opportunity for resistance mutations to emerge, as is evident in the LC.

Two NRTI mutations: K65R and Y115F did significantly increase in prevalence between the EC and LC. K65R is observed in patients treated with ddC (Gu et al 1994), ddI (Zhang et al 1994), ABC (Miller et al 2000) and TDF (Margot et al 2002). Its increase in the LC likely reflects the extent of treatment with NRTI drugs in this cohort: 98.8% currently on treatment and 94.1% with previous ART experience were on NRTI containing regimens. The Y115F mutation confers resistance to ABC (Miller et al 2000) and its appearance in the LC likely reflects the significant increased use of this drug in the LC: 8.6% of the EC had experience with ABC compared with 16.5% of the LC ($p<0.05$).

NNRTI resistance mutations

Of the NNRTI major mutations (Graphs 5.3a-b), K103N was the most prevalent mutation and remained so over time. Graph 5.3a clearly reflects the introduction of NNRTIs to treatment regimens, particularly EFV in 1998, with a peak in 1999 of the major mutations: K103N (30.0% prevalence), Y181C (20.0%) and G190A (14.0%). These three mutations remained dominant, although their prevalence declined over time.

All other major NNRTI mutations (Graph 5.3b) remained at a prevalence of <7.0%: a significant increase in the prevalence of V108I and P225H from the EC to the LC was seen, with P225H first appearing in 2001. V108I is associated with NVP and EFV drug use and reduces susceptibility by two-fivefold (Rhee et al 2003, Shafer and Schapiro 2008). P225H is associated with EFV use and is generally seen in the presence of K103N (Bacheler et al 2001, Shafer and Schapiro 2008): 22 of the 23 patients with P225H in this cohort also had K103N. First-generation NNRTIs used with this group of treatment-experienced patients had a low genetic barrier and once one of these major mutations were established, the drug class was no longer efficacious (Shafer and Schapiro 2008).

PI resistance mutations

Major PI resistance mutations were significantly more prevalent in the EC compared with the LC with Graphs 5.4a-b clearly showing the significant decline in the major PR mutations D30N, M46I/L, G48V, V82A, I84V and L90M over time. All these mutations followed a similar pattern of decline and it likely reflects the introduction of more potent PI drugs, in particular the effect of using RTV to boost the other PI drugs.

The mutation M46I, did not follow this general pattern of decline and as shown on Graph 5.4a, it had a saw-tooth pattern: the mutation was first seen in 1997 (5.0% prevalence) which increased to 13.0% in 1999, declined to 4.0% in 2000, increased to 10.0% in 2002, declined to 3.0% in 2003, increased to 6.0% in 2005 and decreased to 0.0% in 2006. M46I was considered a major PR mutation for resistance to IDV/r and a minor mutation for NFV, LPV/r, ATV/r and FPV/r. These drugs were available for treatment at different time points throughout the span of this study with IDV and RTV introduced in 1996, NFV in 1997, LPV/r in 2000, ATV/r and FPV/r in 2003. The spike in prevalence of M46I coincides with the introduction of these drugs and may reflect this and the development and appearance of this

mutation as a major and minor mutation dependent on the specific PI drug used.

Resistance in the 298 treatment-experienced patients with more than one genotypic report available (n=725)

Of the 725 reports for 298 treatment-experienced patients, 61.5% harboured major mutations to the NRTI drug class; 48.1% had major mutations to the NNRTI drug class and 28.3% had major mutations to the PI drug class (90.9% had minor PI mutations). The prevalence of major NRTI and NNRTI resistance mutations was higher in this sub-group of patients compared with the 1,488 with one report only (56.7% NRTI mutations, 38.0% NNRTI mutations) whilst the prevalence of PI major and minor mutations was comparable (26.7% major PI mutations, 90.3% minor PI mutations).

Table 5.7 highlighted the different interpretation analyses used, which showed the prevalence of resistance mutations over time, either year by year, or cumulative analyses. This cumulative approach was used to illustrate that once mutations are identified in a patient, they may disappear from the plasma and may not be seen in the next resistance test but they are archived and retained in memory cells and can be recalled if the appropriate drug is given: these are “lifetime mutations”. Using Graphs 5.5a-b to consider this concept further: Graph 5.5a (one resistance report per patient, per year) showed major mutations to two drugs classes had declined over time with a 10.0% prevalence in 2005 and no resistance in 2006. However, Graph 5.5b (accumulation of resistance mutations) showed a prevalence of ~35.0% in 2005 of major mutations impacting two drugs classes year on year, which was maintained in 2006. Graph 5.5a showed more fluctuation in the frequencies of patients with mutations impacting the drug classes whereas using the cumulative approach (Graph 5.5b), a more stable interpretation was provided.

Further studies need to be conducted to evaluate this cumulative design and concept. For each patient with >1 resistance report, a cumulative mutational profile could be determined (see Figure 5.1 for an example).

Figure 5.1: The mutational profile of one of our patients who had five resistance entries available on the ICVC Clinical Cohort Resistance Database. The boxes highlighted in red indicate the identification of mutations that year and showed that at some time points, mutations seen previously had been archived. For example, the PI mutation M46I/L was identified in 2000, was undetected in 2001, detected again in 2002 and 2004 and undetected in 2005. The final column shows the accumulated mutations that can be found in the patient's plasma or memory cell archive since the patient started his first treatment.

Resistant mutations		Year resistance test conducted					"Lifetime mutations"
		2000	2001	2002	2004	2005	
PI mutations	M46I/L						
	I47V						
	I54L						
	I84V						
	L90M						
NRTI mutations	M41L						
	T69N						
	L74V						
	M184V						
	L210W						
	T215Y						
NNRTI mutations	K101E						
	K103N						
	Y181C						
	G190A						

Future research will be undertaken to interrogate the ICVC Clinical Cohort Resistance Database to evaluate using mutational profiles e.g. the treatment pathway information for this patient, alongside this mutational profile (Figure 5.1) which illustrated the "lifetime mutations" this patient had; this could provide a powerful tool to aid the clinical management of patients with HIV-1.

In conclusion, this study showed at a population level, the prevalence and trends of resistance mutations in a UK treatment-experienced clinical cohort.

The clinicians would use the resistance reports to determine the best treatment options for their patient and ensure the best possible care for them. These analyses also provided evidence of the key mutations circulating in the treatment-experienced population in a community, and therefore the potential mutations that may be transmitted to newly infected persons.

Chapter 6: The "theoretical susceptibility" of the cohort to new ARVs

As detailed in the previous chapter, high-levels of resistant mutations were circulating within the treatment-experienced community. Failure of the 'traditional' first-generation ART to completely suppress the patients' circulating viruses (due to resistance), led to the development of mutations to the available drugs. Newer and more robust drugs were required for the treatment of patients with numerous mutations. Two second-generation drugs, developed against viruses with resistant mutations, have been approved for use with patients with HIV-1: a new NNRTI TMC 125, Etravirine (ETV) (European Medicines Agency (EMEA) 2007); and a new PI TMC 114, Darunavir (DRV), for use in treatment-experienced (EMEA 2008) and treatment-naive patients (EMEA 2009).

Overall research aims

Using the published resistance mutations profiles for ETV and DRV, I developed a new model for assessing the "theoretical susceptibility" of our treatment-experienced clinical cohort to these new drugs. I investigated:

- the frequency and type of NNRTI resistance mutations present in NNRTI treatment-experienced patients with HIV-1 infection, whether they were currently taking NNRTIs or had previous experience
- the NNRTI mutations present and the "theoretical susceptibility" of the NNRTI treatment-experienced clinical cohort for the potential use of ETV
- the frequency and type of PI resistance mutations present in PI treatment-experienced patients with HIV-1 infection, whether they were currently taking PIs or had previous experience
- the PI mutations present and the "theoretical susceptibility" of the PI treatment-experienced clinical cohort for the potential use of DRV.

The second-generation NNRTI: Etravirine (ETV)

Since 1996, the first-generation NNRTIs NVP, EFV and DLV have formed an essential component of HAART (Jayaweera et al 2008). However, their low genetic barrier to resistance (one mutation produced 100.0% resistance to

all first-generation NNRTIs) resulted in widespread drug failure with resistance. Archiving of these mutations precluded, forever, further use of this drug class, as discussed in the previous chapter, 38.0% of the treatment-experienced cohort had major first-generation NNRTI mutations and could not utilize this drug class again.

The second-generation NNRTI ETV, is a diarylpyrimidine NNRTI and was designed to be active against HIV-1 with resistance mutations from the use of the first-generation NNRTIs (Das et al 2004). Etravirine can bind the RT in multiple conformations, blocking the enzyme’s activity, and has a high genetic barrier compared to the first-generation NNRTIs, i.e. >3 NNRTI mutations are required for resistance to occur to ETV.

The phase III clinical trials DUET 1 and 2, were conducted to determine the efficacy of ETV in treatment-experienced patients. These trials were randomised, double-blind, placebo-controlled multinational trials with 612 patients in the DUET 1 trial (Madruga et al 2007) and 591 patients in the DUET 2 trial (Lazzarin et al 2007). The primary endpoint of both trials was the proportion of patients fully suppressed with a VL <50 c/mL.

Etravirine related mutations were also determined, with 13 mutations identified as associated with a decreased virologic response:

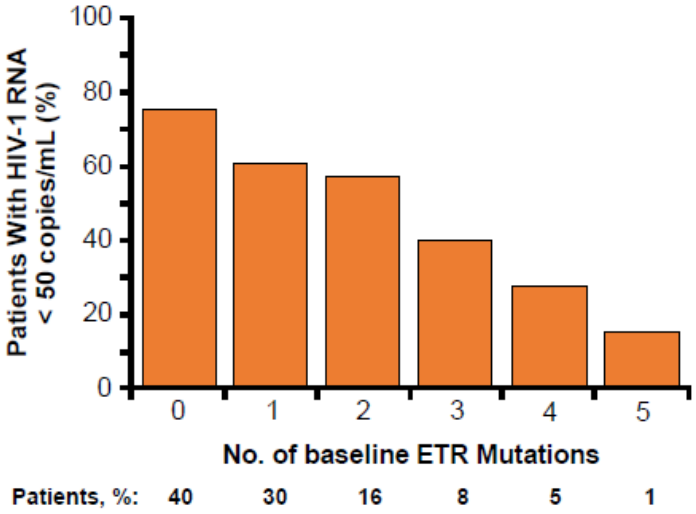
V90I*, A98G*, L100I, K101E*/P, V106I*, V179D*/F*, Y181C/I/V*, G190A/S.

***Key**:* * Novel mutations to ETV, not seen with the first-generation NNRTIs.

High-level resistance was associated with the accumulation of ≥3 of these mutations (Vingerhoets et al 2007, Katlama et al 2007a).

Chapter 6: The “theoretical susceptibility” of the cohort to new ARVs

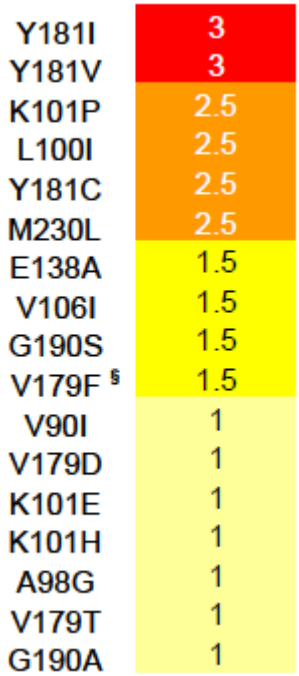
Graph 6.1: Shows the effect of an increased number of etravirine (ETV) mutations on the virological suppression of a patient’s virus: the greater the number of baseline ETV mutations, the fewer the patients with undetectable viral load (VL) (Katlama et al 2007a).



Further analyses of the DUET 1 and 2 data identified four further novel ETV mutations: K101H, E138A, V179T and M230L (Vingerhoets et al 2008).

These 17 mutations were weighted to indicate the relative impact the individual ETV mutations would have on the patient’s total virological response: Y181I and Y181V had the greatest impact (score 3), followed by L100I, K101P, Y181C and M230L (score 2.5), the remaining mutations had a lesser impact (score 1.5 to 1), (Vingerhoets et al 2008; Vingerhoets et al 2010).

Figure 6.1: Genotypic 'weighted score' of individual etravirine (ETV) mutations on virological response. In practice, a patient with an ETV weighted score of '0 to 2', would have the highest response to the drug; if a patient had an ETV score of '2.5 to 3.5' they would have an intermediate response to the drug and if a patient had a score of '≥4', they would have a reduced response (Vingerhoets et al 2008; Vingerhoets et al 2010).



Key: §V179F was never present as single ETR RAM (always with Y181C)

ETV research aim

The aim of this study was to define the frequency and type of NNRTI resistance mutations present in our NNRTI treatment-experienced clinical cohort and determine the "theoretical susceptibility" of this cohort to the future use of ETV.

Method

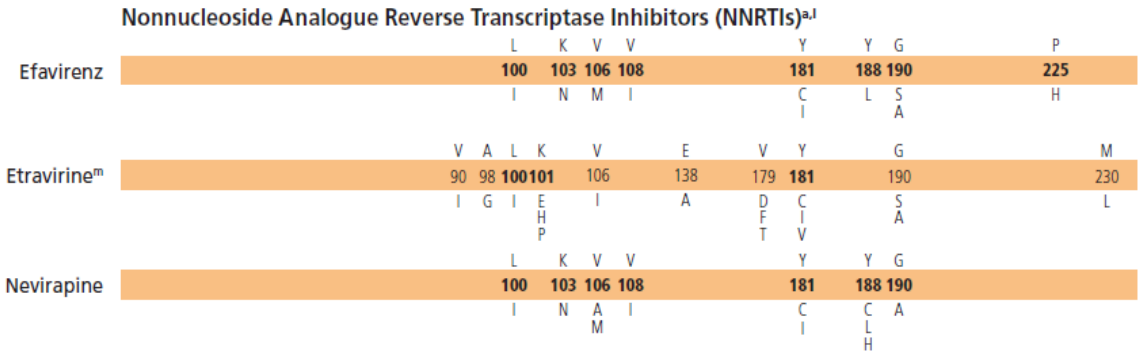
Patient selection

Using the treatment-experienced clinical cohort, all patients whose clinicians indicated on the ICVC resistance request form that their patient had treatment experience with the NNRTIs NVP, DLV and EFV (whether current usage or previous treatment experience), were identified and included in the analyses (1996-2006).

Chapter 6: The "theoretical susceptibility" of the cohort to new ARVs

Determining resistance

The mutational profile for each NNRTI treatment-experienced patient was established, including the prevalence of first-generation NNRTI mutations (EFV, NVP), and the specific ETV related mutations, as identified by the DUET 1 and 2 clinical trials, and confirmed by the IAS-USA drug resistance mutations list (Johnson et al 2009):



The "theoretical susceptibility" of the cohort to the use of ETV was determined as: for patients with ≤2 ETV related mutations, they should be susceptible to ETV. For those identified with ≥3 ETV related mutations, their mutational profiles were further evaluated using the genotypic weighted score (see Figure 6.1, Vingerhoets et al 2008; Vingerhoets et al 2010) to determine the likely clinical response (high response, intermediate response, reduced response) against the specific ETV mutations that were identified.

Results

Nine hundred and sixty-seven NNRTI treatment-experienced patients were identified in the cohort:

- 550 (56.9%) were currently failing (CF) on a NNRTI-containing regimen (NVP 51.6%, EFV 45.7%, DLV 1.5%, NVP+EFV 1.3%)
- 417 (43.1%) had previous treatment experience (PE) with a NNRTI-containing regimen (NVP 48.2%, EFV 29.5%, DLV 1.4%, NVP+EFV 17.3%, NVP+DLV 1.9%, EFV+DLV 0.2%, all three NNRTIs 1.4%), but were not currently on NNRTI treatment.

Chapter 6: The "theoretical susceptibility" of the cohort to new ARVs

Table 6.1: Demographics of patients currently failing (CF) or with previous experience (PE) of non-nucleoside reverse transcriptase inhibitors (NNRTIs) containing regimens.

	Currently failing (CF) pts (n=550)						Previous experience (PE) pts (n=417)					
	All CF		CF with NNRTI muts (n=412)		CF with no NNRTI muts (n=138)		All PE		PE with NNRTI muts (n=188)		PE with no NNRTI muts (n=229)	
	n	%	n	%	n	%	n	%	n	%	n	%
Sex Male	372	72.4	283	73.5	89	69.0	276	68.3	132	71.4	144	65.8
Female	142	27.6	102	26.5	40	31.0	128	31.7	53	28.6	75	34.2
Age Min	18		18		20		20		21		20	
Max	77		75		77		75		69		75	
Mean	44.3		44.6		43.5		43.6		44.2		43.1	
Risk group												
Hetero	136	53.5	99	51.5	37	62.5	99	55.0	46	51.1	53	58.9
MSM	89	35.0	71	37.1	18	30.9	61	33.9	33	36.7	28	31.1
IDU	10	3.9	8	4.1	2	3.3	8	4.4	5	5.6	3	3.3
Blood/tissue transfer	12	4.7	12	6.2	0	0.0	3	1.7	2	2.2	1	1.1
Mother-to-child	3	1.2	1	0.5	2	3.3	2	1.1	2	2.2	0	0.0
Sharps injury	1	0.4	1	0.5	0	0.0	3	1.7	0	0.0	3	3.3
MSM/IDU	0	0.0	0	0.0	0	0.0	2	1.1	1	1.1	1	1.1
Origin												
Europe	260	53.0	199	54.1	61	50.0	197	54.6	87	53.0	110	56.3
Africa	200	40.7	143	38.9	57	46.7	143	39.6	66	40.2	77	39.1
North America	11	2.2	10	2.7	1	0.8	5	1.4	2	1.2	3	1.5
Asia	10	2.0	8	2.2	2	1.6	7	1.9	5	3.0	2	1.0
South America	4	0.8	4	1.1	0	0.0	3	0.8	0	0.0	3	1.5
Carribean	3	0.6	3	0.8	0	0.0	0	0.0	0	0.0	0	0.0
Australasia	2	0.4	1	0.3	1	0.8	4	1.1	4	2.4	0	0.0
Middle East	0	0.0	0	0.0	0	0.0	1	0.3	0	0.0	1	0.5

Key:
CF currently failing on non-nucleoside reverse transcriptase inhibitors (NNRTI) -containing regimen, PE previous experience of a NNRTI-containing regimen.
Risk group: Hetero heterosexual, MSM men who have sex with men, IDU injecting drug users.

Of the 967 NNRTI treatment-experienced patients, 600 (62.1%) presented with at least one NNRTI related mutation, 367/600 (61.2%) had specific ETV related mutations (CF patients, 74.9% (412/550) NNRTI related mutations, 252/412 (61.2%) ETV related mutations; PE patients, 45.1% (188/417) NNRTI related mutations, 115/188 (61.2%) ETV related mutations).

Table 6.2: The number of non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations that were identified in the cohort and the breakdown of these by the number of etravirine (ETV) specific mutations that were seen in all patients; those who were currently failing (CF) on a NNRTI regimen (n=550) and those with previous experience (PE) of NNRTI usage (n=417).

Number of NNRTI mutations	Number of ETV specific mutations																	
	0			1			2			3*			4*			Total		
	All	CF	PE	All	CF	PE	All	CF	PE	All	CF	PE	All	CF	PE	All	CF	PE
0	367	138	229	0	0	0	0	0	0	0	0	0	0	0	0	367	138	229
1	183	119	64	102	57	45	0	0	0	0	0	0	0	0	0	285	176	109
2	42	34	8	120	89	31	49	33	16	0	0	0	0	0	0	211	156	55
3	8	7	1	19	15	4	34	27	7	13	9	4	0	0	0	74	58	16
4	0	0	0	4	3	1	5	4	1	10	7	3	3	2	1	22	16	6
5	0	0	0	1	1	0	2	1	1	2	1	1	3	3	0	8	6	2
Total	600	298	302	246	165	81	90	65	25	25	17	8	6	5	1	967	550	417

Key:
CF currently failing on non-nucleoside reverse transcriptase inhibitors (NNRTI)-containing regimen, PE previous experience of a NNRTI-containing regimen**. * Patients highlighted in the blue box had ≥3 ETV related mutations**

- Overall, 91.6% of all patients (91.3% CF and 92.2% PE) in this NNRTI treatment-experienced clinical cohort failing first-generation NNRTIs that were evaluated in the “theoretical susceptibility” model would be susceptible to the new NNRTI: ETV.
- Thirty-one patients (highlighted in the blue box) had ≥3 ETV related mutations: 8.4% (31/367) of all patients, 8.7% (22/252) CF patients, 7.8% (9/115) PE patients, and may not benefit from taking ETV (see **Table 6.4** for further analyses).

Table 6.3: The prevalence of specific etravirine (ETV) related mutations seen in all non-nucleoside reverse transcriptase inhibitors (NNRTI) experienced patients; currently failing (CF) and with previous experience (PE).

ETV related mutations	All pts		CF pts		PE pts	
	n	%	n	%	n	%
V90I	0	0.0	0	0.0	0	0.0
A98G	43	4.5	32	5.8	11	2.6
L100I	28	2.9	20	3.6	8	1.9
K101E	61	6.3	45	8.2	16	3.8
K101H	0	0.0	0	0.0	0	0.0
K101P	0	0.0	0	0.0	0	0.0
V106I	1	0.1	1	0.2	0	0.0
E138A	0	0.0	0	0.0	0	0.0
V179D	26	2.7	17	3.1	9	2.2
V179F	0	0.0	0	0.0	0	0.0
V179T	0	0.0	0	0.0	0	0.0
Y181C*	194	20.1	131	23.8	63	15.1
Y181I	4	0.4	4	0.7	0	0.0
Y181V	0	0.0	0	0.0	0	0.0
G190A**	145	15.0	98	17.8	47	11.3
G190S	19	2.0	16	2.9	3	0.7
M230L	4	0.4	3	0.6	1	0.2

Key:
CF currently failing on non-nucleoside reverse transcriptase inhibitors (NNRTI)-containing regimen, PE previous experience of a NNRTI-containing regimen**.**

*Y181C, a potent mutation associated with ETV resistance, had a prevalence of 20.1% in all patients; 23.8% of CF patients and 15.1% of PE patients.

**G190A, a mutation associated with minimal ETV resistance, was the next most prevalent mutation, seen in 15.0% of all patients; 17.8% of the CF patients and 11.3% of the PE patients.

All other ETV related mutations were seen at a prevalence of <10.0% or not at all.

Table 6.4: The weighted score of 31 patients (22 were currently failing (CF) on a non-nucleoside reverse transcriptase inhibitor (NNRTI) containing regimen, nine had previous experience (PE) with a NNRTI-containing regimen) identified with ≥3 etravirine (ETV) related mutations, and their likely clinical response to ETV (Vingerhoets et al 2008; Vingerhoets et al 2010). Thirty out of the 31 patients (96.8%) would have a reduced response to ETV.

Combinations of NNRTI mutations: ETV specific mutations highlighted in blue	CF pts (n=22)	PE pts (n=9)	Weighted score of ETV mutation combinations*	Response to ETV$
5 NNRTI muts:				
L100I+Y181C+G190A+A98G+V108I	2	-	7	Reduced
K103N+Y181C+G190A+A98G+K101E	1	-	5.5	Reduced
K103N+Y181C+G190A+K101E+K101Q	1	-	4.5	Reduced
K103N+Y181C+G190A+K101E+V108I	-	1	4.5	Reduced
4 NNRTI muts:				
K103N+Y181C+G190A+A98G	1	-	4.5	Reduced
K103N+Y181C+G190A+K101E	2	-	4.5	Reduced
Y181C+G190A+K101E+V108I	1	1	4.5	Reduced
Y181C+G190A+A98G+K101E	-	1	5.5	Reduced
Y181C+G190A+K101E+V108I	2	1	4.5	Reduced
Y181C+G190A+A98G+V108I	1	1	4.5	Reduced
Y181C+G190S+A98G+K101E	2	-	6	Reduced
3 NNRTI muts:				
Y181C+G190A+A98G	4	-	4.5	Reduced
Y181C+G190A+K101E	4	4	4.5	Reduced
G190A+A98G+K101E	1	-	3	Intermediate

Key:
* **Weighted scores:** L100I 2.5, Y181C 2.5, G190S 1.5, G190A 1, A98G 1, K101E 1
$ **Response to ETV:** ETV score ≥4 reduced response, ETV score 2.5 to 3.5 intermediate response, ETV score 0 to 2 highest response (Vingerhoets et al 2008, 2010).

Chapter 6: The "theoretical susceptibility" of the cohort to new ARVs

The second-generation PI: Darunavir (DRV)

For resistance to the PIs to occur, an accumulation of major mutations and several minor mutations are required to decrease efficacy, with some specific mutations resulting in cross-resistance to the whole PI class. Today, all PIs prescribed in UK clinics are boosted with RTV to enhance PI drug levels and provide a higher genetic barrier. This strategy has helped to reduce failure to the PI class of drugs as witnessed in the previous chapter with the significant decline in major PI resistance mutations from the EC to the LC (39.6% EC vs 15.6% LC, $p<0.001$).

Substantial resistance to the 'first-generation' unboosted PIs resulted in significant PI resistance in patients in the community. 'Second-generation' PIs (all boosted with RTV) had increased efficacy with less resistance, but toxicity became an issue. The development of newer PIs designed against resistant viruses were required.

The new second-generation PI: DRV was developed against a drug resistant PI target and had a strong binding affinity for the HIV-1 PR (De Meyer et al 2005). The major 'POWER' clinical trials (De Meyer et al 2005; De Meyer et al 2006; Katlama et al 2007b; Haubrich et al 2007) demonstrated the benefits of using boosted-DRV in treatment-experienced patients (and later with treatment-naïve patients (Ortiz et al 2008)) and highlighted new mutational profiles associated with a diminished response to DRV, including:

V11I; V32I; L33F; I47V; I50V; I54L/M; G73S; L76V; I84V and L89V.

If ≥3 DRV specific mutations were present at baseline, this significantly reduced the virological response (De Meyer et al 2008).

DRV research aims

The aim of the first DRV study undertaken was to define the frequency and type of PI resistance mutations present in our PI treatment-experienced

clinical cohort and determine the "theoretical susceptibility" of this cohort to the future use of DRV (using genotype analyses).

A second study was undertaken to evaluate the predictive value of using virtual phenotype analyses relative to genotype analyses alone, to determine susceptibility to DRV. Did either the genotype or virtual phenotype analyses provide a more effective tool than the other, or were they comparable?

Method

Patient selection

Using the treatment-experienced clinical cohort, all patients whose clinicians indicated on the ICVC resistance request form that their patient had treatment experience with the PIs SQV, IDV, RTV, NFV, APV, LPV, LPV/r, TPV, ATV, FPV (whether currently failing (CF) on their PI-containing regimen; with previous experience (PE) of a PI-containing regimen but not currently taking PIs or currently not on any treatment but with previous PI experience), were identified and included in the analyses (1996-2006).

Determining resistance

The mutational profile for each PI treatment-experienced patient was established, including the prevalence of first-generation PIs and second-generation PIs (all boosted with RTV) mutations, and the specific DRV related mutations, as identified by the POWER clinical trials:

V11I, **V32I**, **L33F**, **I47V**, I50V, **I54L**, I54M, G73S, L76V, I84V, **L89V**

Key:
mutations in **bold** were initially defined as high impact (HI) mutations in the POWER studies, developed in ≥10.0% of virological failures

and as per the IAS-USA drug resistance mutations list (Johnson et al 2009):

Darunavir/ ritonavir[s]	V		V	L				I		I		I				T	L		I		L
	11		32	33				**47**		**50**		**54**				74	**76**		**84**		89
	I		I	F				V		V		M L				P	V		V		V

NB. Assignment of high impact/major mutations by the POWER and IAS-USA lists differed somewhat (mutations in bold); the POWER studies identified G73S as a relevant mutation but this was not confirmed by the IAS-USA list who had T74P on the list which was not indicated by the POWER studies.

Chapter 6: The “theoretical susceptibility” of the cohort to new ARVs

The “theoretical susceptibility” of our cohort to the use of DRV was determined as: for patients with ≤2 DRV related mutations, they should be susceptible to DRV. For those identified with ≥3 DRV related mutations, their mutational profiles were further evaluated using virtual phenotype analyses to determine the likely clinical response (maximal response, reduced response, minimal response) against the specific DRV mutations that were identified.

Results of the DRV genotype study

Eight hundred and eighty-five PI treatment-experienced patients were identified in the cohort:

- 532 (60.2%) were CF on a PI-containing regimen (72.0% one PI; 27.0% two PIs; 1.0% ≥ three PIs)
- 188 (21.0%) with PE but not currently on a PI-containing regimen (57.0% one PI; 27.0% two PIs; 6.0% ≥ three PIs)
- 165 (19.0%) currently not on any treatment but with PE of PIs (53.0% one PI; 31.0% two PIs; 16.0% ≥ three PIs)

Table 6.5: Demographics of patients currently failing (CF) or with previous experience (PE) of protease inhibitors (PIs) containing regimens.

	Currently failing (CF) pts (n=532)						Previous experience (PE) pts (n=353)					
	All CF		CF with no DRV muts (n=428)		CF with DRV muts (n=104)		All PE		PE with no DRV muts (n=331)		PE with DRV muts (n=22)	
	n	%	n	%	n	%	n	%	n	%	n	%
Sex Male	386	76.3	308	75.1	78	81.3	262	76.4	241	74.8	21	100.0
Female	120	23.7	102	24.9	18	18.8	81	23.6	81	25.2	0	0.0
Age Min	20		20		30		23		23		37	
Max	71		69		71		74		74		59	
Mean	44.5		44.2		45.7		43.9		43.7		45.7	
Risk group												
Hetero	191	65.4	160	51.5	31	55.4	104	58.4	103	59.5	1	20.0
MSM	87	29.8	62	37.1	25	44.6	156	31.5	52	30.1	4	80.0
IDU	4	1.4	4	4.1	0	0.0	5	2.8	5	2.9	0	0.0
Blood/tissue transfer	7	2.4	7	6.2	0	0.0	8	4.5	8	4.6	0	0.0
Mother-to-child	2	0.7	2	0.5	0	0.0	1	0.6	1	0.6	0	0.0
Sharps injury	0	0.0	0	0.5	0	0.0	3	1.7	3	1.7	0	0.0
MSM/IDU	0	0.0	0	0.0	0	0.0	1	0.6	1	0.6	0	0.0
Origin												
Europe	234	52.5	186	52.0	61	50.0	189	64.1	177	63.0	12	85.7
Africa	176	39.5	149	41.6	57	46.7	95	32.2	94	33.5	1	7.1
North America	12	2.7	5	1.4	1	0.8	5	1.7	4	1.4	1	7.1
Asia	11	2.5	10	2.8	2	1.6	2	0.7	2	0.7	0	0.0
South America	8	1.8	6	1.7	0	0.0	0	0.0	0	0.0	0	0.0
Carribean	1	0.2	1	0.3	0	0.0	0	0.0	0	0.0	0	0.0
Australasia	3	0.7	1	0.3	1	0.8	3	1.0	3	1.1	0	0.0
South Africa	1	0.2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0

Of the 885 PI treatment-experienced patients, 759 (86.0%) presented with at least one PI related mutation, 126/759 (16.6%) had specific DRV related mutations (CF patients, 80.3% (427/532) PI related mutations, 104/427 (24.6%) DRV related mutations; PE patients, 94.1% (332/353) PI related mutations, 22/332 (6.3%) DRV related mutations).

Table 6.6: The number of protease inhibitors (PI) mutations that were identified in the cohort and the breakdown of these by the number of darunavir (DRV) specific mutations that were seen in all patients; those who were currently failing (CF) on a PI regimen (n=532) and those with previous experience (PE) of PI usage (n=353).

Number of PI mutations	Number of DRV specific mutations																	
	0			1			2			3*			≥4*			Total		
	All	CF	PE	All	CF	PE	All	CF	PE	All	CF	PE	All	CF	PE	All	CF	PE
0	80	32	48	0	0	0	0	0	0	0	0	0	0	0	0	80	32	48
1	239	118	121	2	2	0	0	0	0	0	0	0	0	0	0	241	120	120
2	214	126	88	1	0	1	0	0	0	0	0	0	0	0	0	215	126	89
3	101	60	41	6	5	1	0	0	0	0	0	0	0	0	0	107	65	42
4	55	37	18	13	9	4	1	1	0	0	0	0	0	0	0	69	47	22
5	27	20	7	19	16	3	1	1	0	2	1	1	0	0	0	49	38	11
≥6	43	34	9	57	51	6	13	11	2	7	5	2	4	4	0	124	104	21
Total	759	427	332	98	83	15	15	13	2	9	6	3	4	4	0	885	532	353

Key:
CF currently failing on protease inhibitors (PI)-containing regimen, PE previous experience of a PI-containing regimen**.**
*** Patients highlighted in the blue box had ≥3 DRV related mutations**

- Overall, 89.7% of all patients (91.3% CF and 92.2% PE) in this PI treatment-experienced clinical cohort failing PIs that were evaluated in the “theoretical susceptibility” model would be susceptible to the new PI: DRV.
- Thirteen patients (highlighted in the blue box) had ≥3 DRV related mutations: 1.5% (13/885) of all patients, 1.9% (10/532) CF patients, 0.8% (3/353) PE patients, and may not benefit from taking ETV.

Table 6.7: Prevalence of specific darunavir (DRV) related mutations and the combinations of mutations in those patients with more than one related mutation.

DRV related mutations	n=126	
	n	%
V32I	5	4.0
L33F	16	12.7
I47V	2	1.6
I50V	4	3.2
I54L	4	3.2
G73S	21	16.7
I84V	46	36.5
V32I + L33F	1	0.8
V32I + I47V	2	1.6
L33F + I50V	1	0.8
L33F + I54L	1	0.8
L33F + G73S	2	1.6
L33F + I84V	3	2.4
I54L + I84V	2	1.6
G73S + I84V	3	2.4
V32I + L33F + I47V	1	0.8
V32I + L33F + I84V	2	1.6
V32I + I47V + I50V	1	0.8
V32I + I47V + I54M	1	0.8
V32I + I47V + I84V	1	0.8
L33F + I47V + I84V	1	0.8
L33F + G73S + I84V	1	0.8
I54L + G73S + I84V	1	0.8
V32I + I47V + I54L + I84V	2	1.6
V32I + L33F + I47V + I54L + I84V	2	1.6

- Of the 126 patients with DRV related mutations, 98 (77.8%) had only one DRV mutation, 15 (11.9%) had two, nine (7.1%) had three, two (1.6%) had four and two (1.6%) had five DRV related mutations.

Patient selection for the virtual phenotype and genotype study

Using the same PI treatment-experienced clinical cohort as in the genotype study, a case-control design was utilised whereby patients who were

identified in the first study as having DRV-related mutations (the cases) were paired with sequential controls (i.e. those patients with no DRV-related mutations) and matched by sex, age and number of PI (non-DRV related) mutations.

The genotype FASTA sequences generated using the TRUGENE® system were sent to VircoLab Inc. to be analysed by their virtual phenotype tool.

Results of the DRV virtual phenotype and genotype study

A total of 194 virtual phenotype analyses were conducted: 97 patients were in both the case (DRV related mutations) and control (no DRV related mutations) groups.

Table 6.8: Background prevalence of protease mutations in the case and control groups. The case group on average had more protease inhibitor (PI) mutations (6.45 PI mutations) than the control group (2.07 PI mutations), ($p<0.001$).

	No. PI muts		**No. RT muts**		**No. PI polys**		**No. RT polys**	
	Case	**Control**	Case	Control	Case	Control	Case	Control
Mean	**6.45**	**2.07**	5.54	2.27	7.27	6.29	9.64	9.66
Minimum	**1**	**0**	0	0	1	1	2	2
Maximum	**12**	**7**	11	11	15	13	19	20

Review of the case group (n=97)

Using the genotype analyses; in the case group, 88.7% of the patients had two or less DRV related mutations and would therefore be susceptible to this new drug; with 11.3% presenting with ≥3 DRV related mutations. The specific DRV mutations prevalent included: V32I (14.4%), L33F (24.7%), I47V (11.3%), I50V (5.2%), I54L (10.3%), I54M (1.0%), G73S (20.6%) and I84V (53.6%). The V11I, T74P, L76V and L89V DRV related mutations were not seen in the case group.

Using the virtual phenotype analyses, 86.6% of the case groups' sequences were interpreted as having 'maximal response' to DRV, confirming good agreement between the two systems used.

Table 6.9: Virtual phenotype analyses of the case group - the ‘maximal response’ finding of 86.6% was comparable with the genotype finding of 88.7% susceptibility (not significant (NS)).

	Frequency	%	Fold change			
			Minimum	Maximum	Mean	Standard Deviation
Maximal response	84	86.6	0.4	9.8	2.31	1.93
Reduced response	12	12.4	10.6	70.9	30.38	16.31
Minimal response	1	1.0	163.8	163.8	163.8	-

Key:
Virtual phenotype clinical cut-off vales:
Low clinical cut-off (Maximal virologic response (CCO1)) = 10.0
High clinical cut-off (Minimal virologic response (CCO2)) = 106.9

Table 6.10: Review of the 12 case group patients with a ‘reduced response’ as per the virtual phenotype analyses; in relation to the darunavir (DRV) related mutations and combinations, and genotype interpretation.

DRV mutation combinations as determined by genotype			Genotype interpretation	Virtual phenotype interpretation
No. of muts	Combination	n		
1	I50V	3	Susceptible	Reduced response
2	V32I+I47V I54L+I84V	1 1	Susceptible Susceptible	Reduced response
3	V32I+L33F+I84V V32I+I47V+I50V V32I+I47V+I84V L33F+I47V+I84V	2 1 1 1	Possible resistance	Reduced response
4	V32I+I47V+I54L+I84V	1	Resistance	Reduced response
5	V32I+L33F+I47V+I54L+I84V	1	Resistance	Reduced response

Mutations for the one patient reported as having a minimal response by the virtual phenotype tool were the same as the mutations seen with the reduced response patient who also had five DRV related mutations: V32I+L33F+I47V+I54L+I84V. The fold change for the virtual phenotype minimal response patient was 163.8 whilst the fold change for the virtual phenotype reduced response patient was 70.9. Table 6.12 compares the two patients’ demographics, treatment history and mutational profiles.

Table 6.11: Comparison of the demographics, treatment history and mutation profiles of the minimal response patient and the reduced response patient who both presented with five darunavir (DRV) related mutations: V32I+L33F+I47V+I54L+I84V.

	Virtual phenotype minimal response patient	**Virtual phenotype reduced response patient**
Sample date	19/03/2004	29/08/2003
Reason for test	Viral rebound on salvage therapy	Viral load raised
Origin of infection	European	European
Ethnicity	White non-hispanic	White non-hispanic
Risk group	MSM	MSM
Current VL (c/mL)	215,000	21,100
CD4 (cells//µL)	104	284
Current treatment	SQV, LPV/r	LPV/r, APV, TDF, 3TC
Previous treatment	IDV, NFV, EFV, ddI, ABC, d4T, TDF, 3TC, ZDV, APV (3 previous PIs)	d4T, ddI, EFV, SQV, ABC (1 previous PI)
Subtype	B	B
Protease (PR) mutations	K20R, **V32I**, **L33F**, M36I, M46I, **I47V**, F53L, **I54L**, L63P, A71V, **I84V**, L90M (n=12)	L10F, **V32I**, **L33F**, M46I, **I47V**, **I54L**, L63P, A71T, **I84V** (n=9)
Reverse transcriptase (RT) mutations	V75I, F77L, Y115F, F116Y, V118I, Q151M, M184V, G190A (n=8)	A62V, D67N, L74V, V75T, L100I/M, K103N, M184V, K219Q (n=8)
PR polymorphisms	G16A, E34V, E35D, S37D, K55R, Q58E, D60E, I62V, G68E, L89I, I93L (n=11)	I13V, G16A, L19P, E35D, I64V, P79S, T91A, F99L (n=8)
RT polymorphisms	S68G, D121Y/C, S162C, I178L, Q207E, R211K (n=6)	I50N, F61S, S68N, I178M, Q207K, R211S (n=6)
Virtual phenotype fold change	163.8	70.9

Key:
MSM men who have sex with men
DRV related mutations highlighted in bold

Of other (not DRV related) PR mutations, the patients had two in common: M46I and L63P. Different PR mutations prevalent included: K20R, M36I, F53L, A71V and L90M for the virtual phenotype minimal response patient and L10F and A71T for the virtual phenotype reduced response patient.

Chapter 6: The "theoretical susceptibility" of the cohort to new ARVs

Review of the control group (n=97)

Using the genotype analyses; no DRV related mutations were identified in the control group. Using the virtual phenotype analyses, all control group patients would have maximal response to DRV.

Table 6.12: Virtual phenotype analyses of the control group: the maximal response finding of 100.0% supported the genotype analyses findings of no darunavir (DRV) related mutations.

	Frequency	%	Fold change			
			Minimum	Maximum	Mean	Standard Deviation
Maximal response	97	100.0	0.3	7.4	0.67	0.71

Discussion

In this study, I evaluated a "theoretical susceptibility" model using published resistance profiles of new ARVs including the second-generation NNRTI: ETV; and the second-generation PI: DRV.

Of the NNRTI treatment-experienced clinical cohort, 91.6% were "theoretical susceptible" to ETV as they had ≤2 ETV related mutations. Of the 31 patients with ≥3 ETV related mutations, on further analysis using the weighted genotype score proposed by Vingerhoets et al (2008; Vingerhoets et al 2010); 30/31 (96.8%) would have a reduced response to ETV, with one patient having an intermediate response to ETV use.

Vingerhoets et al (2010) determined that the Y181I and Y181V mutations would have the highest weight factor, therefore impact on ETV and in our cohort, <1.0% presented with the Y181I mutation and no patients had the Y181V mutation. Apart from a prevalence of 20.1% of the Y181C mutation and 15.0% of the G190A mutation, our cohort did not harbour DRV related mutations.

Of the PI treatment-experienced clinical cohort, 89.7% were "theoretical susceptible" to DRV as they had ≤2 DRV related mutations. Of the 13

patients with ≥3 DRV related mutations, on further analysis using virtual phenotype outputs, 12 patients would have a reduced response to DRV, with one patient having a minimal response.

Two patients had five DRV related mutations V32I+L33F+I47V+I54L+I84V, with the virtual phenotype tool interpreting this as minimal response for one patient and reduced response for the other. There were no clear indications as to why these two patients, with the same DRV related mutations, had been characterised as such, but possible explanations for this interpretation (using the data presented in Table 6.12) may include:

- the minimal response patient was more treatment-experienced (10 ARVs) compared to the reduced response patient (five ARVs): the patient had treatment experience with APV; the V32I and I47V mutations are associated with APV resistance
- the minimal response patient presented with a larger number of PR mutations and the following were different to the reduced response patient: K20R, M36I, F53L, A71V and L90M. Although none of these mutations have been reported in the research literature as having an impact on DRV, De Meyer et al (2008) did report that when ≥3 DRV related mutations were present, alongside a high number of other PR mutations, then a significantly reduced virological response was observed
- the reduced response patient had L10F and A71T PR mutations, again, the research literature does not report that these mutations have an impact on DRV
- the minimal response patient had 11 PR polymorphisms whilst the reduced response patient had eight.

To conclude, availability of new second-generation drugs like ETV and DRV provide new treatment options for patients failing older ARVs and as shown by our “theoretical susceptibility” model, a large majority of our treatment-experienced clinical cohort would benefit from these new drugs.

Chapter 7: The evolution of subtype profiles in the clinical cohort

Historically, the HIV-1 research conducted and the literature published has focussed on subtype B populations. This has occurred, due to the fact that treatment for HIV-1 patients was originally only available in the Western, developed countries, therefore sequences were only available from these countries and when analysed, subtype B viruses were predominant. With the increase in treatment worldwide and with the 'migration of viruses' from endemic regions to Western countries and having access to treatment, there has been an increase in the identification of non-B subtypes.

Earlier research work conducted at the ICVC identified the Roche AMPLICOR HIV-1 MONITOR Test version 1.0 (v1.0) assay was not detecting, or was suboptimally detecting, the VL of HIV-1 viruses with a non-B subtype (Arnold et al 1995). The primers used in the Roche AMPLICOR HIV-1 MONITOR Test v1.0 assay were based on subtype B and on identification of these discordant VL results, new primers were added which were susceptible to non-B subtypes (Roche AMPLICOR HIV-1 MONITOR Test v1.5). The ICVC collaborated with Roche in the SENTRY Study to ensure that the performance of the Roche AMPLICOR HIV-1 MONITOR Test v1.5 and future assays, maintained their reliability over time as evolution of HIV-1 non-B viruses were identified further (http://www.roche.com/media/store/releases/med_dia_2002-07-03.htm).

Subtype B only accounts for 11.0% of the global epidemic (Hemelaar et al 2011), therefore a focus on the other subtypes is warranted. The genotype resistance test kits and the rules-based algorithms used to interpret mutational profiles are based on a reference subtype B virus (De Luca and Perno 2003; Youree and D'Aquila 2002). Until recently, the majority of the published resistance data has been based on subtype B sequences. The relevance of PR and RT sequence differences among non-B viruses, as compared to subtype B viruses, and the relevance this may have for therapeutic success is not known (Clavel et al 2004).

Chapter 7: The evolution of subtype profiles in the clinical cohort

Research aims

Using available FASTA '*pol*' (PR and RT) sequences generated by the TRUGENE® GeneLibrarian™ archive for the treatment-naïve and treatment-experienced patients, I investigated:

- The utility and concordance of results amongst five freely available online analytical tools to determine subtype.

On completion of this characterisation and assignment of a subtype I then investigated:

- the prevalence of subtype B, pure non-B subtypes and recombinant forms circulating in the treatment-naïve and treatment-experienced populations
- whether PR and RT mutation and polymorphic profiles emerged and evolved differently in patients infected with subtype B or non-B/CRF subtypes.

Method

Characterising subtype profiles in the ICVC Clinical Cohort Resistance Database: The Quad Study

A total of 1,642 complete FASTA '*pol*' sequences (i.e. full PR and RT sequences; partial PR only or RT only sequences were excluded) were manually entered into five online analytical tools to determine subtype including:

- the Stanford subtyping tool: //hivdb.Stanford.edu/
- the NCBI subtyping tool: //www.ncbi.nlm.nih.gov/
- the Los Alamos RIP 2.0 subtyping tool: //hiv-web.lanl.gov/content/hiv-db/RIPPER/RIP.html/
- the STAR subtyping tool: //www.vgb.ucl.ac.uk/starn.shtml/
- the REGA subtyping tool: //dbpartners.stanford.edu/RegaSubtyping/

The Stanford, NCBI and Los Alamos RIP 2.0 subtyping tools used a similarity search tool whereby a 'window' slid along the query FASTA sequence, and each 'window' was compared by BLAST (a protein database search programme) to the reference sequences for different virus subtypes

(Rozanov et al 2004; Gifford et al 2006). The STAR subtyping tool used a distance-based protocol and an underlying statistical model to determine subtype (Myers et al 2005). The REGA subtyping tool used phylogenetic analyses to determine subtype (de Oliveira 2005). Please see Appendix 2 for examples of the subtype outputs from each of the subtyping tools employed.

In order to assign an overall subtype for each patient sequence where there were discordant outputs from the five tools, the following rules were applied:

- if REGA assigned a subtype, used REGA (due to its use of phylogenetic analyses and therefore employing a tool closer to the 'gold standard' interpretation of full-length genome sequences (Robertson et al 1999))
- if REGA was 'unassigned', the NCBI subtyping graphical output allowed thorough examination of the sequence to determine subtype/recombination (please see Appendix 2)
- if the NCBI graphical output was indeterminable, the Los Alamos RIP 2.0 subtyping tool output was reviewed for confirmation, followed by the Stanford and STAR outputs.

The Stanford, NCBI and Los Alamos subtyping tools assigned a subtype to all 1,642 sequences submitted. REGA (n=252, 15.3%) and STAR (n=145, 8.8%) were unable to assign subtype for a significant number of sequences ($p<0.05$). Reasons for REGA non-assignment included 'no cluster with pure subtype, no detection of recombination' (n=65, 25.8%); 'cluster with pure subtype, detection of recombination but failure to classify as CRF' (n=85, 33.7%); 'cluster with CRF, detection of recombination in pure subtype but failure to classify as CRF' (n=33, 13.1%); 'cannot explain' (n=84, 33.3%).

The five online tools produced concordant subtype results for 1,186 (72.2%) of the sequences submitted. The remaining 456 sequence analyses (27.8%) resulted in 57 combinations of concordant/discordant/unassigned results across the five tools. Using the rules described above, an overall subtype

was however, assigned for each sequence. Complete discordance across all five tools (including unassignment) was only found in 51 (5.1%) of the sequences submitted.

The use of one subtyping tool alone, compared with any of the other tools, would have resulted in the misclassification of >27.0% or more of patients' subtype. Discordant outputs across the tools probably reflects the content of the comparative databases used in each tool, and the difficulty in keeping these tools up-to-date for determination of new and recombinant viruses.

Patient selection

All patients identified on the ICVC Clinical Cohort Resistance Database who had a consensus subtype available, were eligible for inclusion in the study. For any patients who had more than one resistance entry available on the ICVC Clinical Cohort Resistance Database and therefore had more than one FASTA sequence available; the earliest sample date/FASTA sequence/subtype result, was included.

Determining resistance

The IAS-USA Update of the Drug Resistance Mutations in HIV-1: December 2009 list (Johnson et al 2009) was used to determine major resistance mutations prevalent in this study.

NRTI mutations included: M41L, A62V, K65R, D67N, 69Insert, K70R, L74V, V75I, F77L, Y115F, F116Y, Q151M, M184I/V, L210W, T215F/Y, K219E/Q.

NNRTI mutations included: V90I, A98G, L**100**I, K**101**E/H/P, K**103**N, V**106**A/M, V**108**I, E138A, V179D/F/T, Y**181**C/I/V, Y**188**C/L/H, G**190**A/S, P**225**H, M230L.

PI mutations included: L10C/F/I/R/V, V11I, I13V, G16E, K20I/M/R/T/V, L24I, D**30**N, V**32**I, L33F/I/V, E34Q, E35G, M36I/L/V, K43T, M**46**I/L, I**47**A/V, G**48**V, I**50**L/V, F53L/Y, I**54**A/L/M/S/T/V, Q**58**E, D60E, I62V, L63P, I64L/M/V, H69K, A71I/L/T/V, G73A/C/S/T, T**74**P, L**76**V, V77I, V**82**A/F/L/S/T, N83D, I**84**V, I85V, N**88**D/S, L89V, L**90**M, I93L/M.

Key:

For those NNRTI and PR amino acid positions highlighted in bold, they were considered major (primary) mutations which occur first and the non-bold were minor (secondary mutations) that are supportive to the molecular changes which are associated with the primary mutations.

Statistical analyses were conducted using SPSS (version 22). Chi^2 (χ^2) analyses were used to test for any significant differences in the distribution of subtypes between the EC and the LC; and to test for any significant differences in the distribution of mutations between the non-B/CRF subtypes cohort and the subtype B cohort. Significance was assigned at the 0.05 level ($p<0.05$).

Results

A total of 1,642 patients' consensus subtype data were included in this study.

Of 518 treatment-naïve patient sequences submitted for subtype analyses, 283 (54.6%) had a subtype B virus, 200 (38.6%) were pure non-B subtypes and 35 (6.8%) CRFs.

Of 1,124 treatment-experienced patient sequences analysed, 584 (52.0%) were subtype B, 424 (37.7%) pure non-B subtypes and 116 (10.3%) CRFs.

Table 7.1: Prevalence of specific pure subtypes and circulating recombinant forms (CRFs) within the treatment-naïve and treatment-experienced clinical cohort populations. Overall, 52.8% of patients were subtype B, 38.0% of patients had a pure non-B subtype and 9.2% of patients had a CRF.

Consensus subtype	Treatment-naive		Treatment-experienced		Total	
	n	%	n	%	n	%
A1	19	3.7	87	7.7	106	6.5
A1; B	1	0.2	1	0.1	2	0.1
A1; CRF01_AE	1	0.2	0	0.0	1	0.1
A1; D	0	0.0	2	0.2	2	0.1
A1; G	0	0.0	2	0.2	2	0.1
A1; J	0	0.0	1	0.1	1	0.1
A2	0	0.0	3	0.3	3	0.2
A2; D	0	0.0	1	0.1	1	0.1
B	283	54.6	584	52.0	867	52.8
B; A1	0	0.0	1	0.1	1	0.1
C	160	30.9	240	21.4	400	24.4
C; B	0	0.0	3	0.3	3	0.2
CRF01_AE	5	1.0	12	1.1	17	1.0
CRF01_AE; A1	0	0.0	2	0.2	2	0.1
CRF02_AG	14	2.7	59	5.2	73	4.4
CRF02_AG; B	0	0.0	1	0.1	1	0.1
CRF02_AG; G	0	0.0	1	0.1	1	0.1
CRF06_cpx	1	0.2	5	0.4	6	0.4
CRF10_CD	6	1.2	5	0.4	11	0.7
CRF13_cpx	0	0.0	2	0.2	2	0.1
D	13	2.5	57	5.1	70	4.3
D; A1	2	0.4	2	0.2	4	0.2
D; B	1	0.2	3	0.3	4	0.2
D; C	0	0.0	2	0.2	2	0.1
D; G	0	0.0	5	0.4	5	0.3
F1	0	0.0	10	0.9	10	0.6
F2	0	0.0	1	0.1	1	0.1
G	8	1.5	21	1.9	29	1.8
G; A1	1	0.2	0	0.0	1	0.1
G; B	1	0.2	1	0.1	2	0.1
H	0	0.0	1	0.1	1	0.1
H; A1	0	0.0	2	0.2	2	0.1
J	0	0.0	4	0.4	4	0.2
J; B	0	0.0	1	0.1	1	0.1
J; C	1	0.2	0	0.0	1	0.1
J; F	1	0.2	0	0.0	1	0.1
J; K	0	0.0	2	0.2	2	0.1
TOTAL	518	100.0	1,124	100.0	1,642	100.0

Key: consensus subtypes with a ';' between the letters indicates PR subtype followed by RT subtype e.g. A1; B = A1 subtype characterised in PR and B subtype in RT.

Gender and subtype

Of the treatment-naïve patients (n=505/518 with corresponding gender data), of those with a subtype B virus, 263/278 (94.6%) were male, whilst 143/227 (63.0%) of those characterised with a non-B/CRF subtype were female.

Of the treatment-experienced cohort (n=876/1,124 with corresponding gender data), 414/476 (87.0%) were male with a subtype B virus whist 218/400 (54.5%) were female and characterised with a non-B/CRF subtype.

There was a significant difference observed in both the treatment-naïve and treatment-experienced patient groups with subtype B associated with males and subtype non-B/CRF significantly associated with females ($p<0.05$).

Risk exposure group and subtype

Heterosexual risk exposure was associated with non-B/CRF subtype viruses: treatment-naïve n=161/189 (85.2%) classified as heterosexual and non-B; treatment-experienced n=187/218 (90.3%) classified as heterosexual and non-B. In this non-B heterosexual population, subtype C was the most predominant: treatment-naïve n=118/161 (73.3%); treatment-experienced n=96/187 (51.3%). MSM was associated with a subtype B diagnosis: treatment-naïve n=148/162 (91.4%) classified as MSM and subtype B; treatment-experienced n=137/144 (95.1%) classified as MSM and subtype B.

Chapter 7: The evolution of subtype profiles in the clinical cohort

Hospital area and subtype

Of the treatment-naïve subtype non-B/CRF patients analysed, 104/235 (44.3%) were seen in hospitals in the Greater London area; 78/235 (33.2%) in the South East and low frequencies elsewhere. Of the treatment-experienced subtype non-B/CRF patients, the majority 428/540 (79.3%) were seen in hospitals in the Greater London area.

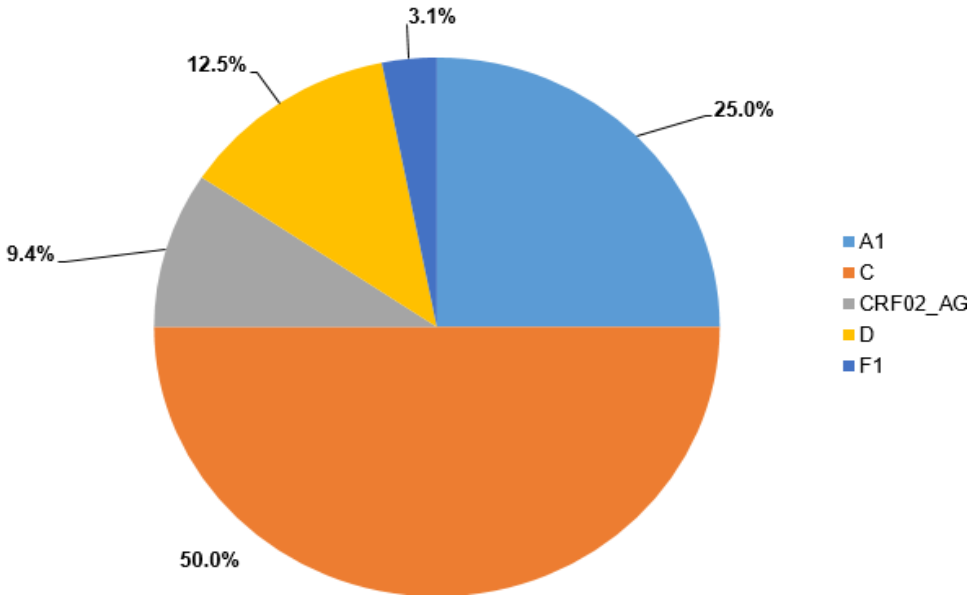
Table 7.2: Overall, the majority of patients in the North West, Wales and East Midlands geographical regions were characterised with subtype B viruses; a greater number of patients in the South West and Eastern regions were characterised with non-B subtypes/circulating recombinant forms (CRFs); whilst the prevalence of subtype B/non-B/CRFs were in total, comparable across the South East and Greater London regions.

Geographical region	Treatment-naïve				Treatment-experienced				Total			
	Subtype B		Subtype non-B/CRF		Subtype B		Subtype non-B/CRF		Subtype B		Subtype non-B/CRF	
	n	%	n	%	n	%	n	%	n	%	n	%
Scotland	0	0.0	0	0.0	1	100.0	0	0.0	1	100.0	0	0.0
North West	5	71.4	2	28.6	7	87.5	1	12.5	12	80.0	3	20.0
Wales	6	85.7	1	14.3	49	92.5	4	7.5	55	91.7	5	8.3
East Midlands	2	50.0	2	50.0	9	64.3	5	35.7	11	61.1	7	38.9
Eastern	22	37.9	36	62.1	40	44.4	50	55.6	62	41.9	86	58.1
Greater London	187	64.3	104	35.7	398	48.2	428	51.8	585	52.4	532	47.6
South East	50	39.1	78	60.9	67	67.0	33	33.0	117	51.3	111	48.7
South West	11	47.8	12	52.2	13	40.6	19	59.4	24	43.6	31	56.4
Total	283	54.6	235	45.4	584	52.0	540	48.0	867	52.8	775	47.2

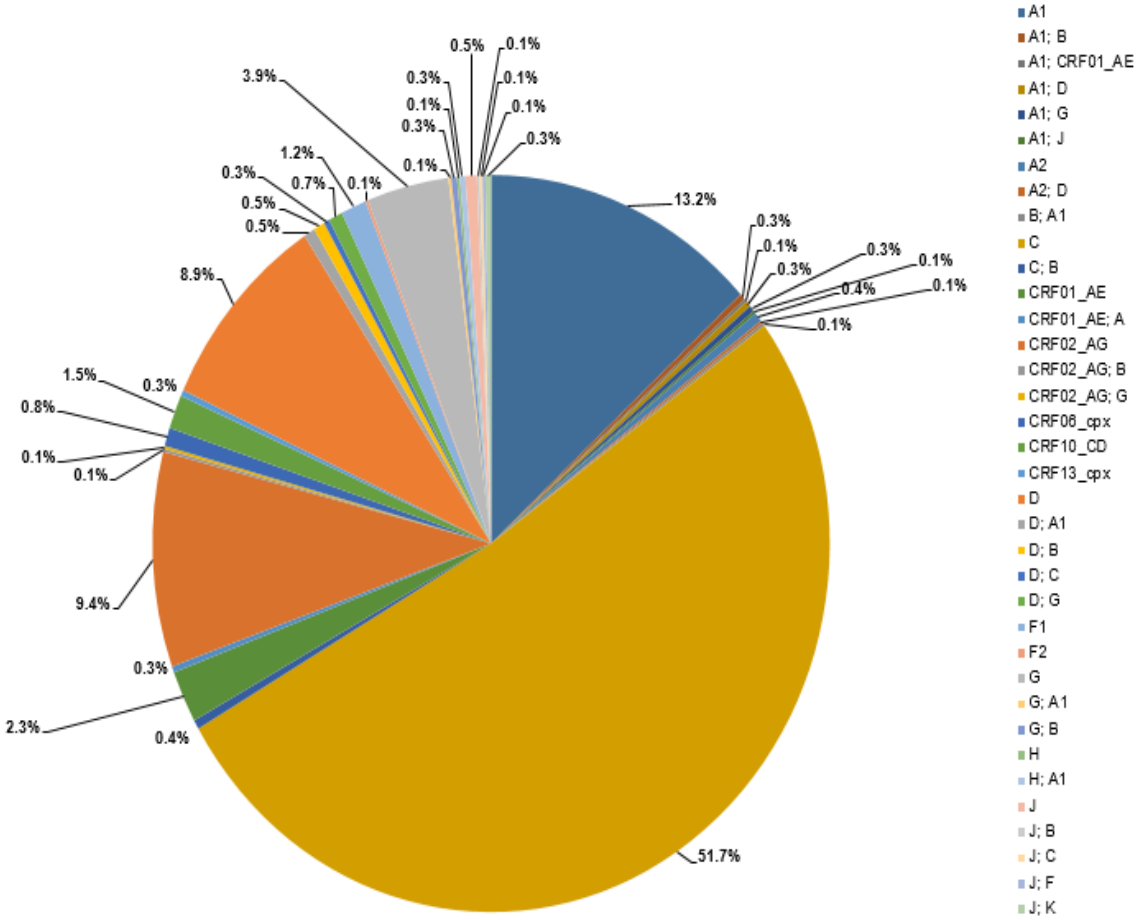
Non-B subtypes and CRFs characterised in the early cohort (EC: 1996-2000) versus the late cohort (LC: 2001-2006)

There was a significant increase in non-B subtypes and CRFs characterised over time with 32/132 (24.2%) of the EC and 743/1,510 (49.2%) in the LC, $p<0.05$ (illustrated in Graphs 7.1 and 7.2 below).

Graph 7.1: Non-B subtypes and circulating recombinant forms (CRFs) characterised in the early cohort (1996-2000): subtype C was the predominant non-B subtype (50.0%).



Graph 7.2: Non-B subtypes and circulating recombinant forms (CRFs) characterised in the late cohort (2001-2006): there was a significant expansion in non-B subtypes and CRFs compared with the early cohort.



Prevalence of PR and RT resistance mutations/polymorphisms in subtype non-B and subtype B patient sequences

Combining the distinct non-B/CRF subtypes into an overall non-B treatment-naïve group and treatment-experienced group (due to low frequencies of distinct non-B/CRF subtypes in the cohort), statistical analyses were performed to determine the differences in prevalence of resistance mutations and polymorphisms identified in subtype non-B/CRF and subtype B patient sequences.

Table 7.3: Significant protease (PR) mutations associated with non-B subtypes compared to subtype B in the treatment-naïve and treatment-experienced patients ($p<0.05$).

PR mutations	Treatment-naïve				Treatment-experienced			
	Subtype B		Subtype non-B/CRF		Subtype B		Subtype non-B/CRF	
	n	%	n	%	n	%	n	%
I13V	55	19.4	79	33.6	137	23.5	247	45.7
G16E	13	4.6	24	10.2	15	2.6	67	12.4
K20R	9	3.2	50	21.3	33	5.7	105	19.4
M36I	54	19.1	209	88.9	139	23.8	494	91.5
D60E	20	7.1	55	23.4	43	7.4	74	13.7
H69K	5	1.8	208	88.5	10	1.7	421	78.0
I93L	144	50.9	185	78.7	236	40.4	265	49.1

There were no significant differences in the prevalence of RT mutations in the treatment-naïve and treatment-experienced subtype non-B/CRF and subtype B groups.

Table 7.4: Significant protease (PR) mutations associated with subtype B compared to subtype non-B/CRF in the treatment-naïve and treatment-experienced patients ($p<0.05$). (* Major PR mutations)

PR mutations	Treatment-naïve				Treatment-experienced			
	Subtype B		Subtype non-B/CRF		Subtype B		Subtype non-B/CRF	
	n	%	n	%	n	%	n	%
L10I	41	14.5	17	7.2	114	19.5	47	8.7
L10V	29	10.2	12	5.1	0	0.0	0	0.0
D30N*	0	0.0	0	0.0	24	4.1	6	1.1
M46I*	0	0.0	0	0.0	54	9.2	27	5.0
L63P	190	67.1	74	31.5	374	64.0	166	30.7
A71T	27	9.5	5	2.1	60	10.3	11	2.0
A71V	0	0.0	0	0.0	81	13.9	14	2.6
I84V*	0	0.0	0	0.0	39	6.7	11	2.0

Table 7.4 (contd.): Significant protease (PR) mutations associated with subtype B compared to subtype non-B/CRF in the treatment-naïve and treatment-experienced patients (p<0.05). (* Major PR mutations)

PR mutations	Treatment-naïve				Treatment-experienced			
	Subtype B		Subtype non-B/CRF		Subtype B		Subtype non-B/CRF	
	n	%	n	%	n	%	n	%
N88D*	0	0.0	0	0.0	18	3.1	6	1.1
L90M*	0	0.0	0	0.0	70	12.0	35	6.5

There were no significant differences in the prevalence of RT mutations amongst the treatment-naïve subtype B and non-B/CRF groups: Table 7.5 highlights the significant RT mutations observed in the treatment-experienced group.

Table 7.5: Significant reverse transcriptase (RT) mutations associated with subtype B compared to subtype non-B/CRF in the treatment-experienced patients (p<0.05).

RT mutations	Treatment-experienced			
	Subtype B		Subtype non-B/CRF	
	n	%	n	%
M41L	117	20.0	48	8.9
A62V	18	3.1	5	0.9
T69D	16	2.7	6	1.1
L210W	79	13.5	27	5.0
T215Y	115	19.7	55	10.2

Within the treatment-naïve cohort, 14 significant PR polymorphisms associated with the non-B subtypes compared to subtype B were identified including: T12S, I15V, L19I/T/V, S37K/N, R41K/N, K45R, Q61E, K70R, T74S, L89M. Twenty-two RT polymorphisms were statistically different in the non-B treatment-naïve cohort compared to subtype B including: T39D/E, E40D, S48T, K49R, E53D, V60I, D121H/Y, D123G/N/S, I135V, E138A, K173A/T, Q174K, D177E, T200A, Q207E, R211S, V245Q.

Within the treatment-experienced cohort, 15 significant PR polymorphisms associated with the non-B subtypes compared to subtype B were identified including: T12S, K14R, I15V, L19I/T/V, S37K/N, R41K, K45R, R57K, Q61E, K70R, T74S, L89M. Twenty-seven RT polymorphisms were statistically

different in the non-B treatment-experienced cohort compared to subtype B including: T39D/E, E40D, S48T, K49R, V60I, D121H/Y, D123G/N/S, E138A, T139A, K173A/T, Q174K, D177E, I178M, T200A/E, I202V, E204K, Q207E, R211K/S, V245K/Q.

Discussion

Of 1,642 FASTA patient sequences submitted to five online analytical tools for subtype determination, there was 72.2% concordance across all five tools. A subtype could be determined for the 27.8% of sequences submitted whereby there were discordant/unassigned outputs across the five tools, but this required a knowledge and understanding of the tools and their outputs to ensure appropriate subtype characterisation. Such online tools were evaluated as it was deemed that in the future, clinicians could use such tools, on provision of their patient's FASTA sequence, to determine subtype and assist in the clinical management of their patient.

Overall, 52.8% of the patients were classed as having a subtype B virus; 38.0% had pure non-B subtypes, of which, the majority were subtype C (24.4%); with 9.2% CRFs. There was a significant increase in the prevalence of non-B subtypes and CRFs from the EC (24.2%) to the LC (49.2%). In the EC, 4 pure non-B subtypes were characterised (A1, C, D, F1) alongside CRF02_AG whilst in the LC, 9 pure non-B subtypes were characterised (A1, A2, C, D, F1, F2, G, H, J) alongside 27 CRFs, illustrating the increasing genetic diversity and expansion of viruses and evolution over time.

Subtype B characterisation was significantly associated with males whose risk group was MSM, conversely, subtype non-B subtypes were associated with the heterosexual risk group.

Non-B subtypes and CRFs were evident in all geographical regions included in this study apart from Scotland, where only one subtype B patient was characterised. The non-B/CRF subtypes were more prevalent in the larger

populated regions, in particular Greater London, where 532/775 (68.7%) of the non-B/CRF subtypes were characterised.

Analyses indicated some significant differences in the prevalence of PR mutations amongst the non-B and the B subtypes in the treatment-naïve and treatment-experienced populations including I13V, G16E, K20R, M36I, D60E, H69K and I93L. These are all minor PR mutations as per the IAS-USA mutation list (Johnson et al 2009). The mutation I13V is associated with subtypes A, G and CRF02_AG and this was evident here where 87.7% of subtype A had the mutation, 96.6% of subtype G and 82.2% of the CRF02_AG subtypes. The mutations M36I and I93L are both associated with subtype C (Grossman et al 2001) with 44.0% of all subtype C viruses harbouring the M36I mutation and 88.7% with the I93L mutation.

Of the significant PR mutations seen in the treatment-experienced patients, there were three major PR mutations as per the IAS-USA list (Johnson et al 2009) with the N88D mutation facilitating the appearance of D30N and L90M after NFV treatment failure (Mitsuya et al 2006).

A number of significant PR and RT polymorphisms were associated with the non-B subtypes compared with the subtype B viruses in the treatment-naïve cohort and alongside the minor PR mutations, warrant further investigation to determine whether these mutations/polymorphisms lead to different mutational pathways in the non-B subtypes. At the ICVC, work has started to determine the ‘genetic fingerprints’ of the treatment-naïve patients by individual subtype to define whether different mutational pathways are evident (please see Figure 7.1 below), and work in this field will continue based on the initial findings from this study.

Figure 7.1: Attempts to define the 'genetic fingerprint' of the non-B subtype treatment-naïve patients with >20.0% prevalence of protease (PR) mutations/polymorphisms compared with the subtype B treatment-naïve patients.

Treatment-naïve cohort - PR Mutations and Polymorphisms >20% frequency by subtype																									
	L10	**T12**	**I13**	**K14**	**I15**	**G16**	**L19**	**K20**	**E35**	**M36**	**S37**	**R41**	**K45**	**R57**	**D60**	**Q61**	**I62**	**L63**	**I64**	**C67**	**H69**	**K70**	**V77**	**L89**	**I93**	
A	I		V		V			R	D	I	D or N	K		K							K			M		**A**
(n=19)	30.8%		87.7%		23.1%			53.8%	84.6%	100.0%	23.1% or 76.9%	84.6%		76.9%							76.9%			100.0%		(n=19)
B					V				D		N	K					V	P					I		L	**B**
(n=283)					21.7%				43.1%		47.1%	22.5%					31.6%	58.5%					25.7%		44.9%	(n=283)
C		S			V		I	R	D	I	N	K			E			P			K			M	L	**C**
(n=160)		69.3%			80.2%		60.4%	20.8%	24.8%	90.0%	74.3%	82.2%			25.7%			22.8%			95.1%			70.3%	97.5%	(n=160)
CRF01_AE	V		V			E			D	I	N	K	R	K							K			M		**CRF01_AE**
(n=5)	28.6%		100.0%			57.1%			100.0%	100.0%	100.0%	100.0%	28.6%	28.6%							100.0%			100.0%		(n=5)
CRF02_AG		K	V	R					D	I	N	K						P			K	R		M		**CRF02_AG**
(n=14)		20.0%	82.2%	76.9%					26.7%	100.0%	86.7%	93.3%						26.7%			93.3%	40.0%		100.0%		(n=14)
D			V							I	N	K					V		V							**D**
(n=13)			56.3%							46.2%	75.0%	93.8%					25.0%		62.5%							(n=13)
G		A	V	R	V				D or Q	I	N	K								E	K			M		**G**
(n=8)		28.6%	96.6%	100.0%	42.9%				28.6%	100.0%	85.7%	100.0%								42.9%	100.0%			85.7%		(n=8)
	L10	**T12**	**I13**	**K14**	**I15**	**G16**	**L19**	**K20**	**E35**	**M36**	**S37**	**R41**	**K45**	**R57**	**D60**	**Q61**	**I62**	**L63**	**I64**	**C67**	**H69**	**K70**	**V77**	**L89**	**I93**	

Key:

boxes highlighted in red indicate significant PR polymorphisms in the treatment-naïve non-B subtype patients relative to the subtype B patients.

Chapter 8: Final discussion

The ICVC was established to provide VL and genotypic resistance testing for clinical centres across the UK to ensure the best possible care for their patients with HIV-1. The ICVC was a small, functional unit, which prided itself on its high quality of sample management: patient samples were transported and separated on the same day of arrival at the laboratory and stored appropriately to maintain the quality of the sample to ensure successful VL/resistance testing outcomes. At the time, this was not common practice in laboratories, although the National Health Service (NHS) has now caught-up with these standards and have set-up their own laboratories to test all HIV-1 VL/resistance samples, so the ICVC no longer provides this service.

The over-riding aim of each of the studies conducted and presented in this thesis, was to provide data and evidence of current issues impacting on the clinical care of patients living with HIV-1 infection in the UK, and consider how best to manage and improve their care. Using a bespoke clinical cohort resistance database, the clinical and resistance mutations data were interrogated to determine how the clinical care of HIV-1 patients, whether treatment-naïve or treatment-experienced; of subtype B or non-B origin; could be managed and improved.

Limitations encountered

The clinicians who requested genotypic tests were conducted on their patients' samples were asked to complete a corresponding ICVC resistance request form. We were reliant on the clinicians/clinical centres completing these forms appropriately, although it is evident this did not always occur. From the data analyses, there were some clear discrepancies e.g. in the treatment-experienced clinical cohort analyses, it was stated the majority of the cohort were male (n=968), although the risk exposure group data indicated 250 (52.1%) were heterosexual and 170 (35.4%) were MSM. One would expect the majority of male cases to be related to the MSM risk

exposure group, but if the clinician did not specify this on the resistance request form, then it was not assumed, and the field was left blank on the database.

Another example which suggested the clinical data provided did not always tally completely with the resistance data, related to the persistence of the M184I/V NRTI mutation which by and large, disappears within 12 days of treatment cessation (Zaccarelli et al 2003). In the treatment-experienced clinical cohort analyses, based on the clinical data provided, 37.5% of patients classed as 'not on ART but previous experience' and 31.3% of patients classed as 'treatment-experienced but not indicated whether current or previous ART experience' had evidence of this M184I/V mutation suggesting their treatment classification was suspect, but, if it was not clearly indicated on the resistance request form, then nothing was assumed.

Other limitations included the oversimplification of some of the analyses, in particular with the treatment-experienced data where patient analyses included numerous drugs/drug classes; the frequency of taking these drugs; whether the patient was currently on or off treatment and any previous treatment history. With more than 50 mutations associated with the NRTIs, 40 mutations associated with the NNRTIs and more than 60 mutations associated with PI resistance (Shafer and Schapiro 2008), the complexity of interpreting these mutations and the effect of single and multiple mutations on treatment analyses were likely oversimplified also.

Resistance in a UK HIV-1 treatment-naïve clinical cohort

This study identified 380 treatment-naïve patients who had genotypic resistance tests conducted between 2001 and 2004, and determined an overall resistance prevalence rate of 16.5%. From 2001 to 2002, resistance prevalence rates increased (10.8% to 16.9%), declined in 2003 (7.2%) and then rose again, up until the end of June 2004 (14.9%). The overall

prevalence rate for patients grouped as PHI was 14.5%, for those grouped as CN it was 11.1%, and for those who were pregnant and treatment-naïve it was 8.0%.

Table 8.1 presents the findings of other research groups who conducted analyses into the prevalence of resistance mutations in treatment-naïve populations. All research groups who presented an overall resistance prevalence rate reported a finding of >10.0%, although the ICVC's overall rate of 16.5% was the highest. Our pattern of resistance prevalence rates over the years: increase between 2001 and 2002; decrease between 2002 and 2003; increase between 2003 and 2004; were generally supported by the other research groups' findings although there was some variability in the actual percentage rates.

Table 8.1: Comparison of the ICVC clinical cohort treatment-naïve resistance prevalence rates with other research.

Reference	Time-scale	Treatment-naïve population	Resistance mutation interpretation tool/algorithm used	Main Findings											
				Overall resistance (%)	Resistance by year				Resistance by drug class				Resistance by grouping		
					2001 (%)	2002 (%)	2003 (%)	2004 (%)		NRTI (%)	NNRTI (%)	PI (%)	PHI (%)	CN (%)	Preg (%)
ICVC Clinical Cohort Resistance Database	2001-2004 (end of June 2004)	n=380 20.0% PHI UK	TRUGENE® GuideLines	16.5	10.8	16.9	7.2	14.9	Overall	1.8	9.2	1.6	14.5	11.1	8.0
									2001	2.7	8.1	0.0			
									2002	2.2	14.6	2.2			
									2003	0.6	7.2	0.0			
									2004	3.4	8.0	4.6			
UK HIV Drug Resistance Database (HPA 2006)	2002-2004	n=2,469 UK	IAS-USA Guidelines (version 2005)	11.4	-	16.0	12.0	9.0	2004	4.5	4.5	2.1	-	-	-
Figures above since updated on the UK HIV Drug Resistance Database (http://www.hivrdb.org.uk/)	*2002-2004*	*n=4,148 UK*	+ additional mutations agreed by virologists who were members of UK Collaborative Group on HIV Drug Resistance	*11.6*	-	*13.6*	*11.4*	*11.0*	*2002*	*9.4*	*5.0*	*4.2*	-	-	-
									2003	*7.1*	*5.1*	*2.7*			
									2004	*6.5*	*4.3*	*2.6*			
UK Group on Transmitted HIV Drug Resistance (Cane et al 2005)	1996-2003 (Feb 1996 to May 2003)	n=2,357 7.0% PHI UK	Stanford HIVdb algorithm (version 2004.04)	14.2	2000-2001 16.2		2002-2003 19.2		Overall	9.9	4.5	4.6	22.0	14.0	-
									2002-2003	12.4	8.1	6.6			
CATCH (Wensing et al 2005)	1996-2002	n=2,208 35.2% PHI *European cohort including 19 countries*	IAS-USA Guidelines (version 2002) + substitutions at codon 215	10.4	-	-	-	-	Overall	7.6	2.9	2.5	13.5	8.7	-
									1996-1998	13.4	2.3	2.3			
									1999-2000	9.8	3.1	3.1			
									2001-2002	6.3	9.2	9.2			

The most striking difference between the ICVC's prevalence rates and those reported by the other research groups, involved the resistance by drug class findings. Overall, the ICVC identified a prevalence rate of resistance mutations impacting on the NRTI drug class as 1.5%, the NNRTI class as 9.2% and the PI class as 1.6%. However, the other groups with comparable findings reported much higher NRTI resistance rates: 9.9% (Cane et al 2005), 7.6% (Wensing et al 2005); lower NNRTI rates: 4.5% (Cane et al 2005), 2.9% (Wensing et al 2005) and higher PI rates: 4.6% (Cane et al 2005), 2.5% (Wensing et al 2005). On review and re-grouping of the yearly findings, our NNRTI resistance rates were comparable: 2001-2002, 12.7% (ICVC) 9.2% (Wensing et al 2005); 2002-2003, 9.8% (ICVC) 8.1% (Cane et al 2005); however our NRTI and PI resistance rates remained low compared with the other findings.

This variability in the findings likely reflects the design and methodology of the different research studies. For example, the TRUGENE® GuideLines Rules were used in this study to interpret the resistance mutations that were identified in the ICVC cohort as this reflected the resistance report returned to the clinicians. The other studies presented here employed a range of interpretation tools including the IAS-USA mutation list, the Stanford database, and supplemented these lists with mutations that they deemed as important.

The impact of these studies on the guidelines and recommendations for resistance testing in treatment-naïve patients with HIV-1

Collectively, all of these studies which determined an increase in the prevalence of resistance mutations in the treatment-naïve population, particularly with rates above 10.0%, provided evidence that genotypic resistance testing for all treatment-naïve patients with HIV-1 infection, should be conducted before the commencement of HAART. This would ensure the patients were starting on the optimal first-line treatment regimen to suppress their virus, and afford them the best clinical care. By 2003, the ICVC had

highlighted the significant level of resistance in treatment-naïve patients and was promoting the genotypic testing of all treatment-naïve patients (Loveday at al 2003, MacRae et al 2003). National, European, and International guidelines, were subsequently amended to reflect this recommendation (Vandamme et al for the European HIV Drug Resistance Panel 2004, Gazzard on behalf of the BHIVA Writing Committee 2005, Hammer et al 2006).

An update on the prevalence of resistance in the UK HIV-1 treatment-naïve population

The UK HIV Drug Resistance Database has continued surveillance into the prevalence of drug resistance mutations in the treatment-naïve population in the UK. Since 2005, the prevalence of resistance to any drug class has remained below 10.0% (~8.0%) and for 2013, it was reported to be 6.6% (http://www.hivrdb.org.uk/). There appears to be an association in baseline resistance testing strategies as advocated at the ICVC and which were finally integrated into treatment guidelines with lower levels of resistance now reported in treatment-naive patients. The prevalence of drug resistance mutations in each of the drugs classes in 2013 was 3.4% for the NRTIs, 3.0% for the NNRTIs and 1.6% for the PIs. With the development of more potent and tolerable drugs and combinations to treat patients with HIV-1 and ensure virus suppression; this has led to a decrease in transmitted drug resistance mutations (Kouyos and Günthard 2015).

Contribution of this study to contemporary/scientific knowledge

The ICVC collaborated with and shared this treatment-naïve clinical cohort data with the CATCH Study (Combined Analysis of Resistance Transmission Over Time of Chronically and Acute Infected HIV Patients, Wensing et al 2005). The CATCH study pooled treatment-naïve data from 19 European countries (n=2,208) and analysed the data set with uniformed definitions: 10.4% of treatment-naïve patients had evidence of ≥1 drug resistance mutations. By sharing our data and collaborating with 18 other European

clinical cohort research groups, a larger “supercohort” was developed (Lau et al 2007); with the CATCH study producing a definitive report of the prevalence of resistance in a European-wide study.

In conclusion, using the ICVC Clinical Cohort Resistance Database, a significant increase in the prevalence of resistance mutations in a UK treatment-naïve clinical cohort was identified. On presentation of this work at International and European conferences, alongside other research groups’ work, guidelines were updated to recommend resistance testing was conducted for all treatment-naïve patients, including those who were PHI and CN.

The overall impact of conducting this study has led to an improvement in the clinical care of treatment-naïve patients: by mandating the use of genotypic resistance tests for treatment-naïve patients before the commencement of ART, it can be ensured that they start on the optimum treatment regimen to suppress their virus.

Resistance in a UK HIV-1 treatment-experienced clinical cohort

Of 1,786 treatment-experienced patients who had genotypic resistance tests conducted between 1996 and 2006, an overall resistance prevalence rate of 68.1% was determined. Other clinical cohort research studies determined prevalence rates of between 70.0-80.0% (Gallego et al 2001, Tamalet et al 2003, Gonzales et al 2003, Pillay et al 2005, De Mendoza et al 2007). This study identified a decrease in resistance to the two drug class combination of NRTIs+PIs from the EC to the LC; and an increase in NNRTI mutations and resistance to NRTI+NNRTI combinations in the LC, compared to the EC (these findings were supported by Pillay et al 2005, Mendoza et al 2007).

Application of the treatment-experienced clinical cohort findings to the use of ART in the developing World

Identification of mutations and mutational patterns from the treatment-experienced clinical cohort resistance study can be applied to the developing countries, where the majority of the 35.3 million people infected with HIV-1 live, with 70.8% residing in sub-Saharan Africa (Joint United Nations Programme on HIV/AIDS (UNAIDS) 2013). Due to the failure of vaccine studies to control and protect against HIV-1 transmission (The rgp120 HIV Vaccine Study Group 2005; Buchbinder et al 2008; Rerks-Ngarm et al 2009), 'treatment as prevention' has been introduced to try and contain the main global HIV-1 pandemic (Barnighausen et al 2014), until a vaccine is developed. Access to ART has expanded in recent years in these countries after the World Health Organization (WHO) launched and updated numerous goals and guidelines to expand global ART coverage: 8 million people (54.0%) of those with a CD4 count of ≤350 cells/μL (the level at which the WHO recommended initiation of treatment) were on ART by the end of 2011, with 9.7 million people on ART by the end of 2012 (WHO 2013a).

In order to reach treatment targets and expand access to ART, simple and affordable regimens which could be prescribed to large numbers of people were required and the WHO initially recommended treatment with a fixed-dose combination of two NRTIs and a NNRTI (d4T+3TC+NVP) for first-line treatment. Recommendations have since been updated with TDF+3TC+EFV (or TDF+FTC) preferred, with ZDV+3TC+EFV (or NVP) suggested as an alternative first-line regimen (WHO 2013b). For those clinically failing their first-line treatment regimen, the WHO recommended second-line ART should include a boosted PI (LPV/r or ATV/r) and two NRTIs (ZDV+3TC or TDF+3TC (FTC)), dependent on what was used previously. The WHO have adopted the use of these fixed-dose combinations as they are efficacious, tolerable, robust, convenient (no food/drink restrictions), affordable, and compatible with other drugs e.g. co-infection with TB/hepatitis (Vitoria et al 2014).

Monitoring in these settings is often by clinical deterioration alone, resistance tests are rarely available for clinical care in the developing countries: before initiation of ART, a CD4 test is recommended. To determine treatment success, clinical observations are used; with further CD4 tests recommended every six months if possible; a VL test at six months after initiating ART if possible, and every 12 months thereafter if possible (WHO 2013b). The WHO have recognised the value of VL testing to determine treatment failure but it is not routinely used in all cases due to cost; the WHO aim to increase the capacity of developing countries to incorporate its use into clinical care which will likely lead to an increased demand for second and third-line treatment regimens (Vitoria et al 2014).

Applying the findings from the treatment-experienced clinical cohort resistance study, Table 8.2 highlights the prevalence of the resistance mutations identified in this cohort, related to the drugs recommended by the WHO for use in first-line treatment in the developing countries.

Table 8.2: Prevalence of resistance mutations identified in the ICVC treatment-experienced clinical cohort in relation to the first-line treatment regimens recommended by the World Health Organisation (WHO) for use in developing countries. ***Key***: * Thymidine analogue mutations (TAMs)

	Resistance mutations and the drug class impacted as per the WHO's first-line treatment recommendations		Currently on ART (n=680)		All treatment-experienced patients (n=1,488)	
			n	**%**	**n**	**%**
NRTI mutations and drug impacted	M41L*	d4T, ZDV	189	27.8	299	20.1
	K65R	d4T, 3TC, TDF	12	1.8	43	2.9
	D67N*	d4T, ZDV	185	27.2	284	19.1
	K70R*	d4T, ZDV	125	18.4	216	14.5
	M184I	3TC	15	2.2	36	2.4
	M184V	3TC	305	44.9	550	37.0
	L210W*	d4T, ZDV	128	18.8	189	12.7
	T215F*	d4T, ZDV	51	7.5	78	5.2
	T215Y*	d4T, ZDV	205	30.1	310	20.8
	K219E*	d4T, ZDV	37	5.4	74	5.0
	K219Q*	d4T, ZDV	59	8.7	93	6.3

Table 8.2 (contd.): Prevalence of resistance mutations identified in the ICVC treatment-experienced clinical cohort in relation to the first-line treatment regimens recommended by the World Health Organisation (WHO) for use in developing countries.

	Resistance mutations and the drug class impacted as per the WHO's first-line treatment recommendations		Currently on ART (n=680)		All treatment-experienced patients (n=1,488)	
			n	**%**	**n**	**%**
NNRTI mutations and drug impacted	L100I	NVP, EFV	8	1.2	21	1.4
	K103N	NVP, EFV	163	24.0	316	21.2
	V106A	NVP, EFV	17	2.5	26	1.7
	V106M	NVP, EFV	0	0.0	4	0.3
	V108I	NVP, EFV	27	4.0	66	4.4
	Y181C	NVP, EFV	108	15.9	204	13.7
	Y181I	NVP, EFV	5	0.7	9	0.6
	Y188C	NVP, EFV	3	0.4	4	0.3
	Y188H	NVP, EFV	1	0.1	3	0.2
	Y188L	NVP, EFV	8	1.2	25	1.7
	G190A	NVP, EFV	86	12.6	146	9.8
	G190S	NVP, EFV	6	0.9	14	0.9

Of the NRTI treatment-experienced clinical cohort, 39.1% had three or more TAMs which impacted d4T and ZDV usage with 72.6% of the EC presenting with one or more major NRTI mutations. The prevalence of the M184V mutation which impacts 3TC usage was 37.0% in this cohort and these data likely reflects the resistance patterns that are circulating within the developing countries who are using the older first-generation NRTI drugs as first-line treatment. Ominously, a study newly published which conducted resistance testing in patients on long-term antiretroviral treatment in routine HIV clinics in Togo (n=164 patients on first-line ART), reported 99.4% of those patients as having drug resistance mutations (Konou et al 2015). Prevalence of the M184V mutation was reported as 99.4%, with TAMs reported in 71.0% of patients.

As was indicated, K103N, Y181C and G190A were the dominant major NNRTI mutations in this treatment-experienced clinical cohort and as established in the treatment-naïve clinical cohort analyses, these NNRTI

mutations were transmitted and persisted in the treatment-naive cohort. Due to the NNRTIs low genetic barrier, the development of one of these mutations precludes the usage of any of the first-generation NNRTIs (WHO initially recommended NVP then EFV for first-line treatment). Again, troublingly, the research findings from Togo confirmed our findings and reported the most frequent NNRTI mutations seen were indeed the K103N, Y181C and G190A mutations, although specific prevalence rates were not reported (Konou et al 2015).

Contribution of this study to contemporary/scientific knowledge

Initially, this study was conducted to determine at a population level, the prevalence and trends of resistance mutations in a UK treatment-experienced clinical cohort to ensure the clinicians were provided with evidence to determine the best treatment options for their patients and ensure the best possible clinical care for them.

An important facet of the study was also to provide evidence of the key mutations circulating in the treatment-experienced population and therefore the source of the potential mutations that may be transmitted to newly infected persons.

Finally, applying the treatment-experienced clinical cohort findings to the developing world where access to treatment has expanded greatly but resistance testing is not conducted, one can foresee the resistance mutations and patterns that are likely to arise.

In conclusion, using the ICVC Clinical Cohort Resistance Database, the prevalence of resistance mutations in a UK treatment-experienced clinical cohort were established and showed the success of new ART treatments by their impact on the EC and LC and the reduction in mutations/resistance. Resistance testing is one important tool that can be used to define the next

best treatment options and ensure the best possible care for patients with HIV-1.

The "theoretical susceptibility" of the clinical cohort to new ARVs

A model was developed to determine the "theoretical susceptibility" of the treatment-experienced clinical cohort's potential to use two new second-generation drugs. Using published mutation profiles, the prevalence of these resistance mutations within the clinical cohort were reviewed and the patients' "theoretical susceptibility" to these new drugs was determined.

Using the mutational profile for the second-generation NNRTI, ETV, it was established that 91.3% of the NNRTI treatment-experienced patients in the clinical cohort who were currently failing treatment with their first-generation NNRTIs would be "theoretically susceptible" to treatment with ETV. Only 8.7% presented with ≥3 ETV mutations and would not be susceptible to its use. This theoretical approach for the determination of the utility of new drugs in different communities/populations is a useful way of predicting new drug usage and in this case, the ETV findings were supported by the data of other researchers including Llibre et al (2008) who determined 8.1% of their clinical cohort had ≥3 ETV mutations; Picchio et al (2008) determined a rate of 7.5% and Scott et al (2008) 10.5%. Of interest, the resistance study from Togo (Konou et al 2015) noted 21.5% of their patients were resistant to ETV and it would be interesting to review the specific ETV related mutations prevalent and apply our "theoretical susceptibility" model to their findings.

The model was also applied to the new PI DRV to determine how many of the PI treatment-experienced patients within the cohort would be "theoretically susceptible" to its future use. Again, using the published resistance mutation profiles, it was established that 89.7% of the PI treatment-experienced patients would be "theoretically susceptible" to treatment with DRV. The model's findings were supported by the virtual

phenotype analyses which were conducted on our behalf by Virco, and comparable levels of susceptibility to the new PI DRV were determined. Also, other research groups' findings generally supported this work including Rusconi et al (2007) who reported 94.8% of their cohort had ≤2 DRV related mutations and therefore would be susceptible to its use; Mitsuya (2007) reported 95.9% of their cohort would be susceptible with Lathouwers (2015) reporting a rate of 92.5%.

Contribution of this study to contemporary/scientific knowledge

This "theoretical susceptibility" model has since been imitated by another research group (Hofstra et al 2014) to determine the prevalence of resistance mutations to the second-generation NNRTI rilpivirine in treatment-naïve patients from Europe. As per our model, they used a list of NNRTI mutations and determined the prevalence of these mutations in their cohort, and then predicted susceptibility to rilpivirine using the Stanford algorithm to score levels of resistance and proposed the number of patients who would be susceptible to the use of rilpivirine.

This research by Hofstra et al (2014) displayed the utility of our model and that the "theoretical susceptibility" model can easily be used in the future when new PI/NRTI/NNRTI drugs are launched, to determine the likely success of these new drugs within treatment-experienced and treatment-naïve cohorts. Ultimately, the model provides a simple tool which can be used to translate the resistance mutations prevalent in the clinical cohort and inform the potential use and susceptibility of the cohort to these new drugs.

The evolution of subtype profiles in the clinical cohort

The subtype profiles of 1,642 patients in the clinical cohort were determined by manually submitting their FASTA sequences to five, online subtyping tools. Overall, these tools provided a concordant subtype result for 72.2% of the sequences submitted. For the other 27.8% of subtype results, an overall

subtype result could be assigned by the researcher based on the outputs of the tools and applying specific rules to assign subtype. The study highlighted the complexity of subtype characterisation using such tools and that the operator needs some knowledge of how the tools work and the requirement that these online tools are kept up-to-date, as HIV-1 viruses continue to evolve and recombine over time.

A significant increase in the prevalence of pure and recombinant non-B subtypes in the LC (49.2%) compared with the EC (24.2%) was determined and this genetic expansion over time was confirmed by the findings of the UK Collaborative Group on HIV Drug Resistance, 2014.

Some PR mutations and polymorphisms specific to the non-B subtypes compared to subtype B were identified, which may have an impact on the resistance pathways of non-B subtypes. As illustrated (Figure 7.1), a 'genetic fingerprint' for each of the individual non-B subtypes has started to be developed at the ICVC. Due to the small frequencies of individual non-B subtypes seen, studies frequently combine these subtypes altogether into one non-B subtype group but future work and collaboration is required to pool the data of other clinical cohort research groups and develop a larger "supercohort" (Lau et al 2007), to consider the individual non-B subtypes. This would produce a powerful tool which could be utilised to determine the relevance of subtype characterisation on patients' clinical care and whether subtype diversity and recombination may pose potential problems for patient care in relation to HIV-1 pathogenesis, clinical response, natural drug mutations and molecular assay efficacy.

Contribution of this study to contemporary/scientific knowledge

Through collaboration with the CATCH research group, the FASTA sequences submitted to them were shared (with permission from the ICVC Collaborative Research Group), with the researchers who developed the

REGA subtyping tool and the sequences were used to validate this subtype tool (de Oliveira et al 2005). As determined in our study, the REGA subtyping tool provided a powerful tool to determine subtype and through this collaboration, we contributed to its development. Since these analyses were conducted, the REGA subtyping tool's algorithm and reference dataset has been updated (version 3.0) and on evaluation against other online subtyping tools, showed a sensitivity and specificity of more than 96.0% in the *pol* region (Pineda-Peña et al 2013).

Final conclusions

The engagement of the clinical centres who used the ICVC to establish the ICVC Collaborative Research Group and support the pooling of the clinical and resistance data to form the ICVC Clinical Cohort Resistance Database; allowed the development of a powerful tool to conduct analyses to ultimately, try and improve the clinical care of patients with HIV-1. That is, with the recommendation of genotype resistance testing in treatment-naïve patients with HIV-1; the surveillance of treatment-experienced patients to ensure they were on the optimal treatment regimen and the development of new, more potent therapies/drug classes; the surveillance of subtype non-B and CRF forms and their mutational pathways: the treatment and clinical care of patients with HIV-1 in the UK has improved significantly.

As indicated, these issues are now of importance in the developing world and with the introduction of 'treatment as prevention' to try and control the global pandemic; the patients living with HIV-1 need to be supported to ensure treatment continuity and high adherence levels to the ART; and importantly, early diagnosis of new HIV-1 cases to suppress further transmission. With increased travel and movement between countries; vigilance is required, as the biggest threat to effective ART treatment remains the virus' ability to replicate and recombine and mutate and evolve within an individual host and within the larger community.

Appendices

Appendix 1: Protocol F10: ROCHE COBAS AMPLICOR HIV-1 MONITOR v1.5 ASSAY (STANDARD)

PROTOCOL F10

ROCHE COBAS AMPLICOR HIV-1 MONITOR v1.5 ASSAY (STANDARD)

DOCUMENT REVISION HISTORY			
Version number	**Issue Date**	**Revision date**	**Sections changed from previous version**
1	24/8/04	24/8/05	N/a
2	21/12/04	21/12/05	footer
3	5/7/05	5/7/06	Front page, step 17
4	20/6/06	20/6/07	Removal of reference to Amplilink protocol.

AUTHORISED BY: **K. ZENAD**

COPY NUMBER:

LOCATION OF COPIES:

1. **Quality Manager**
2. **Laboratory Manager**
3. **Lab 2**

International Clinical Virology Centre (ICVC), Central Register of Charities, number 1091046, Companies House registration No. 4300212

Reagents

Absolute alcohol *
Distilled water
Isopropanol
Roche Amplicor HIV-1 Monitor v1.5 kit **
Milton

*** Flammable (Store at room temperature in a fireproof container).**
**** Toxic, harmful, corrosive (Store at 2-8^{o}C, DO NOT FREEZE).**

Equipment

100µl -1000µl pipette
20µl-200µl pipette
2ml unskirted Sarstedt tubes
600ml beaker
A-rings
10ml graduated pipettes
Cobas Amplicor equipment and accessories
100ml Clear glass screw neck bottle.
Centrifuge
5µl-50µl pipette
Plugged tips
Vortex mixer
Fine tipped pastettes
D-cups
Pipetboy
Class 2 safety cabinet
37°C Water bath

Procedure

NOTE: all **VORTEX** steps should last a minimum of 15 seconds unless otherwise stated.

a) Sample Preparation

1. **Thaw plasma samples** in a tray of water at room temperature
2. Complete Q31, Cobas Amplicor Audit Sheet.
3. Remove the following items from the Cobas Amplicor kit:
    - lysis reagent,
    - QS,
    - NHP,
    - negative control,
    - Low +ve control.
    - High +ve control.

4. Place lysis reagent in 37°C water bath. (do not leave it in the water bath for longer than 10-15 minutes)

5. Date, number and place orientation marks on 2ml unskirted Sarstedt tubes:
   Use 24 tubes if there are 21 samples (+ 3 controls).
   Use 12 tubes if there are 9 samples (+ 3 controls).

b) Preparation of Standard Working Lysis Reagent

1. Check lysis reagent is fully dissolved, **VORTEX** until no crystals are visible. **VORTEX QS** tubes (at least 10 seconds) Then VORTEX QS tubes upside down for 10 seconds. Add 100µl QS to each lysis reagent bottle and VORTEX.

International Clinical Virology Centre (ICVC), Central Register of Charities, number 1091046, Companies House registration No. 4300212

2. Add 600µl of lysis reagent to each sample and control tube tube.
3. VORTEX plasma tube, transfer 200µl of plasma to the appropriately labeled tube containing lysis. Repeat for each sample.
4. For kit controls VORTEX negative human plasma (NHP) and transfer 200µl to each control lysis tube (tube # 22,23 and 24 if 21 samples, tube # 10,11and 12 if 9 samples) and VORTEX.
5. VORTEX kit control tube and transfer 50µl to the correct control lysis tube. Repeat for each control.
6. VORTEX all lysis tubes for 20 seconds.
7. Incubate lysis tubes for 10 minutes at room temperature in the Class II Microbiological Safety Cabinet.
8. During incubation return plasma samples to freezer and prepare fresh 70% ethanol (21ml absolute ethanol + 9ml distilled water).
9. Add 800µl of isopropanol to each tube and VORTEX for 20 seconds.
10. Spin at maximum speed (12500-16000g) for 15 minutes room temperature. (place orientation marks on the outside). If the COBAS is available, prepare the A-rings during this spin.
11. Using a fine tip pastette carefully remove the supernatant and discard into the waste beaker. **Take care not to disturb the pellet.**
12. Add 1ml of 70% ethanol to each tube and VORTEX.
13. Spin at maximum speed (12500-16000g) for 5 minutes (place orientation marks facing out).
14. Using fine tip pastette completely remove supernatant and check lid.
15. Add 400µl of diluent (HIV-1 Dil) to each tube and VORTEX.
16. Transfer 50µl of extract to each A-ring tube. Load extracts 1-12 into A-ring A and extracts 13-24 into A-ring B.
17. If COBAS is unavailable, store extracts in the freezer between –60°C and -80°C.
18. Transfer A-rings to post PCR laboratory and follow COBAS instructions. Amplification **MUST** be started within 45 minutes after adding extract to A-ring. (Refer to Protocol F9, Operation of COBAS)

c) A-Ring Preparation (to be started during 15 min centrifugation step if COBAS is available)

1. Label A-rings A and B and make a note of the serial numbers on the working list.
2. VORTEX manganese (Mn^{++}) solution (pink). Mix MMX by inverting 10-12 times.
3. Add 100µl of Mn^{++} solution to each tube of master mix (MMX).
4. Mix by inversion (10-12 times).
5. Add 50µl of MMX to each tube in two A-rings (24 tubes).
6. Place A-rings in bags in fridge (can be kept for a maximum of 3 hrs).

International Clinical Virology Centre (ICVC), Central Register of Charities, number 1091046, Companies House registration No. 4300212

Documentation

Q31 Cobas Amplicor Audit sheet

International Clinical Virology Centre (ICVC), Central Register of Charities, number 1091046, Companies House registration No. 4300212

Appendices

Appendix 2: Frequencies of specific protease (PR) and reverse transcriptase (RT) mutations found in the clinical cohort, using the TRUGENE® resistance guidelines (Genelibrarian version current at time of testing).

Appendix 2: PR and RT mutations in the clinical cohort

Appendix 2_Table 9.1a: Frequencies of specific protease (PR) mutations found in the cohort, using the TRUGENE® resistance guidelines (Genelibrarian version current at time of testing) showed a broad range of mutations.

Protease (PR) mutations	n	%
L10I	483	15.6
L10R	5	0.2
L10V	194	6.3
K20M	59	1.9
K20R	348	11.3
L23I	3	0.1
L24I	35	1.1
D30N	78	2.5
V32I	28	0.9
L33F	60	1.9
M36I	1,545	50.0
M36L	16	0.5
M36TIPL	1	0.03
M36V	11	0.4
M46I	197	6.4
M46L	64	2.1
I47V	20	0.6
G48V	42	1.4
I50V	16	0.5
F53L	32	1.0
I54A	2	0.1
I54L	20	0.6
I54M	2	0.1
I54T	3	0.1
I54V	171	5.5
L63A	4	0.5
L63HPYS	3	0.1
L63P	1,519	49.1
L63PLSF	8	0.3
L63S	34	1.1
L63T	26	0.8
L63TIPL	8	0.3
L63TRSP	1	0.03
A71T	231	7.5
A71V	271	8.8
G73S	63	2.0
G73T	5	0.2
V77I	265	8.6
V82A	167	5.4
V82F	16	0.5
V82S	5	0.2
V82T	23	0.7
I84V	99	3.2
N88D	56	1.8
N88S	5	0.2
L90M	298	9.6

Key: mutations in **bold** are classed as major resistance mutations as per the IAS-USA Update of the Drug Resistance Mutations in HIV-1: December 2009 list (Johnson et al 2009), and reduce ART susceptibility.

Appendix 2: PR and RT mutations in the clinical cohort

Appendix 2_Table 9.1b: Frequencies of specific reverse transcriptase (RT) mutations found in the cohort, using the TRUGENE® resistance guidelines (Genelibrarian version current at time of testing) showed a broad range of mutations.

Reverse transcriptase (RT) mutations	n	%
M41L	496	16.1
E44D	59	1.9
A62V	46	1.5
K65R	73	2.4
D67N	499	16.1
T69D	73	2.4
T69N	34	1.1
T69S	4	0.1
K70E	1	0.03
K70G	1	0.03
K70R	372	12.1
L74I	5	0.2
L74V	139	4.5
V75I	30	1.0
V75M	6	0.2
V75T	22	0.7
F77L	31	1.0
W88G	2	0.1
A98G	84	2.7
A98S	42	1.4
L100I**	52	1.7
K101E**	108	3.5
K101P**	2	0.1
K101Q**	40	1.3
K103N**	560	18.1
K103R**	10	0.3
K103S**	10	0.3
V106A**	44	1.4
V106I**	3	0.1
V106M**	7	0.2
V108I	116	3.8
Y115F	40	1.3
F116Y	23	0.7
V118I	160	5.2
Q151M	28	0.9
V179D	54	1.7
V179E	18	0.6
Y181C**	345	11.2
Y181I**	10	0.3
M184I	61	2.0
M184V	871	28.2
Y188C**	7	0.2
Y188H**	7	0.2
Y188L**	37	1.2
G190A**	254	8.2
G190E**	3	0.1

Appendix 2_Table 9.1b (contd.): Frequencies of specific reverse transcriptase (RT) mutations found in the cohort, using the TRUGENE® resistance guidelines (Genelibrarian version current at time of testing) showed a broad range of mutations.

Reverse transcriptase (RT) mutations	n	%
G190S**	28	0.9
H208Y	44	1.4
L210W	311	10.1
T215C	10	0.3
T215D	13	0.4
T215F	139	4.5
T215NTYS	23	0.7
T215S	26	0.8
T215Y	504	16.3
K219E	125	4.0
K219Q	168	5.4
P225H**	39	1.3
F227L**	25	0.8
M230L**	9	0.3
P236L**	1	0.03

Key: mutations in **bold** are classed as major resistance mutations as per the IAS-USA Update of the Drug Resistance Mutations in HIV-1: December 2009 list (Johnson et al 2009), and reduce ART susceptibility.

** are specific NNRTI RT mutations.

Appendices

Appendix 3: Demographics, protease (PR) and/or reverse transcriptase (RT) mutations and drug class impacted (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs)) in the treatment-naïve patients

Appendix 3: Resistance in a UK HIV-1 treatment-naïve clinical cohort

Appendix 3_Table 10.1a: Demographics, protease (PR) and/or reverse transcriptase (RT) mutations and drug class impacted (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs)) in treatment-naïve patients in 2001: three out of the four patients had evidence of resistance (R) to the NNRTI drug class.

Pt	Sex[+]	Age	Reas[^]	Origin	Risk[#]	VL (c/mL)	CD4 (cells/µL)	Hospital Area[$]	Subtype	Mutations	Drug Class Impacted
1	M	45	CN	Africa	Hetero	-	-	EA	C	**PR:** K20R, M36I, L63P/S **RT:** A98S	A98S possible R to NNRTIs
2	M	33	CN	Europe	MSM	178,000	-	GL	B	**PR**: M36I, L63P **RT**: V179E	V179E possible R to NNRTIs
3	M	68	PHI	Europe	MSM	830,000	580	SE	B	**PR**: L63P **RT**: K103N	K103N R to NNRTIs
4	M	35	CN	Europe	MSM	>75,000	50	SE	B	**PR:** A71T, V77I **RT:** T69N	T69N R to ddC, possible R to ddI (NRTIs)

Key:
[+]Sex: M male, F female
^Reason for test: CN chronic-naïve, PHI primary HIV-1 infection
[#]Risk group: MSM men who have sex with men, Hetero heterosexual
[$]Hospital area: EA Eastern, GL Greater London, SE South East

Appendix 3_Table 10.1b: Demographics, protease (PR) and/or reverse transcriptase (RT) mutations and drug class impacted (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs)) in treatment-naïve patients in 2002: the majority of the patients identified with drug resistance mutations were from the Greater London area (14/15 patients); presented with mutations to the NNRTI drug class (12/15 patients) and one patient had resistance mutations which impacted all three drugs classes.

Pt	Sex[+]	Age	Reas[^]	Origin	Risk[#]	VL (c/mL)	CD4 (cells/µL)	Hosp Area[$]	Subtype	Mutations	Drug Class Impacted
1	M	28	PHI	Europe	MSM	131,000	446	GL	B	**PR**: L10I **RT**: K103N	K103N R to NNRTIs
2	M	28	PHI	Europe	MSM	459,000	422	GL	B	**PR**: L10I **RT**: K103N	K103N R to NNRTIs
3	M	42	CN	Europe	MSM	24,700	-	GL	B	**PR**: L10I **RT**:K103N/T	K103N R to NNRTIs
4	M	32	PHI	Europe	MSM	100,000	538	NW	B	**PR**: L10I **RT**: V179D	V179D possible R to NNRTIs
5	M	41	CN	Europe	MSM	413,000	315	GL	B	**PR**: K20M, M36I, L63P **RT**: V179D	V179D possible R to NNRTIs
6	M	36	CN	Europe	MSM	211,000	139	GL	B	**PR**: D30N, L63P, A71V, N88D **RT**: D67N, K101E, K219Q	D30N R to NFV. N88D+A71V possible R to NFV (PIs). D67N+/or K219Q possible R to ZDV+ABC (NRTIs). K101E, possible R to NNRTIs
7	M	49	PHI	Europe	MSM	156,000	550	GL	B	**PR**: M36I, L63P **RT**: A98S	A98S possible R to NNRTIs

Appendix 3_Table 10.1b (contd.): Demographics, protease (PR) and/or reverse transcriptase (RT) mutations and drug class impacted (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs)) in treatment-naïve patients in 2002: the majority of the patients identified with drug resistance mutations were from the Greater London area (14/15 patients); presented with mutations to the NNRTI drug class (12/15 patients) and one patient had resistance mutations which impacted all three drugs classes.

Pt	Sex[+]	Age	Reas[^]	Origin	Risk[#]	VL (c/mL)	CD4 (cells/µL)	Hosp Area[$]	Subtype	Mutations	Drug Class Impacted
8	M	44	CN	Europe	MSM	>100,000	75	GL	C	**PR**: M36I, L63# **RT**: V179D	V179D possible R to NNRTIs
9	M	39	CN	Africa	Hetero	282,000	34	GL	C	**PR**: M36I **RT**: Y181C	Y181C R to NVP+DLV, possible R to EFV (NNRTIs)
10	M	43	CN	Europe	MSM	203,000	135	GL	B	**PR**: L63P, A71T **RT**: M41L	M41L possible R to ZDV (NRTI)
11	M	36	PHI	Europe	MSM	86,800	880	GL	B	**PR**: L63P, A71V **RT**: V179D	V179D possible R to NNRTIs
12	M	47	CN	-	-	62,600	63	GL	B	**PR**: L63P **RT**: K103N	K103N R to NNRTIs
13	M	28	PHI	Europe	MSM	29,800	517	GL	B	**PR**: L63P **RT**: K103N, Y181C	K103N+Y181C R to NNRTIs
14	M	31	PHI	Europe	MSM	368,000	444	GL	B	**PR:** A71T, L90M	R to SQV+NFV, poss R to IDV+RTV (PIs)
15	M	38	CN	Europe	MSM	>750,000	18	GL	B	**RT**: K103N	K103N R to NNRTIs

Key:
[+]Sex: M male, F female [^]Reason for test: CN chronic-naïve, PHI primary HIV-1 infection
[#]Risk group: MSM men who have sex with men, Hetero heterosexual [$]Hospital area: GL Greater London, NW North West

Appendix 3: Resistance in a UK HIV-1 treatment-naïve clinical cohort

Appendix 3_Table 10.1c: Demographics, protease (PR) and/or reverse transcriptase (RT) mutations and drug class impacted (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs)) in treatment-naïve patients in 2003: the majority of the patients exhibited resistance mutations to the NNRTI drug class (11/12); one patient had resistance mutations which impacted two drugs classes (NRTI and NNRTI).

Pt	Sex[+]	Age	Reas[^]	Origin	Risk[#]	VL (c/mL)	CD4 (cells/µL)	Hospital Area[$]	Subtype	Mutations	Drug Class Impacted
1	M	35	CN	-	MSM	13,300	-	GL	B	**PR**: L10I **RT**: K103N	K103N, R to NNRTIs
2	M	34	CN	Europe	MSM	279,000	206	GL	B	**PR**: L10I **RT**: K103N, V118I*	K103N, R to NNRTIs
3	M	36	CN	S. Africa	MSM	>10,000	69	NW	B	**PR**: L10I, M36I **RT**: V179D	V179D, poss R to NNRTIs
4	M	41	CN	Europe	-	60,500	-	GL	B	**PR**: L10I, L63P **RT**: V179D	V179D, poss R to NNRTIs
5	F	-	Preg	Africa	Hetero	892,000	-	GL	A	**PR**: K20R, M36I **RT**: K103N	K103N, R to NNRTIs
6	F	30	Preg	Africa	Hetero	80,700	-	GL	C	**PR**: K20R, M36I, A71T **RT**: K65R, Y115F, Y181C	K65R, R to ddI+ddC, poss R to TDF (NRTIs). K65R+Y115F poss R to ABC (NRTI). Y181C R to NVP+DLV, poss R to EFV (NNRTIs).

Appendix 3_Table 10.1c (contd.): Demographics, protease (PR) and/or reverse transcriptase (RT) mutations and drug class impacted (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs)) in treatment-naïve patients in 2003: the majority of the patients exhibited resistance mutations to the NNRTI drug class (11/12); one patient had resistance mutations which impacted two drugs classes (NRTI and NNRTI).

Pt	Sex[+]	Age	Reas[^]	Origin	Risk[#]	VL (c/mL)	CD4 (cells/µL)	Hospital Area[$]	Subtype	Mutations	Drug Class Impacted
7	M	36	CN	Asian	Hetero	200,000	-	EA	C	**PR**: M36I **RT**:K101Q	K101Q, poss R to NNRTIs
8	F	42	CN	Africa	Hetero	-	-	SE	C	**PR**: M36I, L63P **RT**: K103N	K103N, R to NNRTIs
9	M	42	CN	Europe	-	277,000	258	GL	B	**PR**: M36I, L63P **RT**: V179D	V179D, poss R to NNRTIs
10	M	32	CN	Europe	MSM	359,000	329	GL	B	**PR**: L63P **RT**: K103N	K103N, R to NNRTIs
11	M	37	CN	-	-	230,000	180	GL	B	**PR**: L63P **RT**: K103N	K103N, R to NNRTIs
12	M	43	PHI	Europe	MSM	113,000	203	EA	B	**RT**: K101Q	K101Q, poss R to NNRTIs

Key:

[+]Sex: M male, F female

^Reason for test: CN chronic-naïve, PHI primary HIV-1 infection

[#]Risk group: MSM men who have sex with men, Hetero heterosexual

[$]Hospital area: EA Eastern, GL Greater London, NW North West, SE South East

[*]Presence of specific mutation reflects horizontal transmission although mutation alone does not affect any of the drug classes

Appendix 3

Appendix 3_Table 10.1d: Demographics, protease (PR) and/or reverse transcriptase (RT) mutations and drug class impacted (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs)) in treatment-naïve patients in 2004: compared with the previous years where resistance mutations to the NNRTIs were identified in the majority of the cases; 2004 saw an expansion in the number of cases with resistance mutations to the other drugs classes (4/13 had NRTI resistance mutations, 4/13 had PI mutations and 5/13 had NNRTI mutations).

Pt	Sex[+]	Age	Reas[^]	Origin	Risk[#]	VL (c/mL)	CD4 (cells/µL)	Hospital Area[$]	Subtype	Mutations	Drug Class Impacted
1	M	35	PHI	Europe	-	329,000	-	GL	B	**PR**: L10I, L63P **RT**: V118I*, K219Q	K219Q, poss R to ZDV (NRTI)
2	M	39	CN	Europe	MSM	27,945	321	WA	B	**PR**: L10V, K20M, L63TRSP **RT**: M184T/I	M184I, R to 3TC, poss R to ddC (NRTIs)
3	M	31	CN	-	-	5,910	-	GL	B	**PR**: L10V, M36I **RT**: K101E	K101E, poss R to NNRTIs
4	F	45	CN	-	-	7,570	-	GL	A; B	**PR**: K20R, M36I, L63P **RT**: K70R, M184V, T215NTYS, K219E	K70R, M184V, T215Y, K219E R to ZDV, ddI, ddC, 3TC, d4T, ABC (NRTIs)
5	F	40	CN	Africa	Hetero	291,000	100	GL	C	**PR**: M36I **RT**: K103N	K103N, R to NNRTIs
6	M	39	CN	-	-	161,000	97	GL	C	**PR**: M36I, L63P/A **RT**: K103N	K103N, R to NNRTIs

Appendix 3_Table 10.1d (contd.): Demographics, protease (PR) and/or reverse transcriptase (RT) mutations and drug class impacted (non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs)) in treatment-naïve patients in 2004: compared with the previous years where resistance mutations to the NNRTIs were identified in the majority of the cases; 2004 saw an expansion in the number of cases with resistance mutations to the other drugs classes (4/13 had NRTI resistance mutations, 4/13 had PI mutations and 5/13 had NNRTI mutations).

Pt	Sex[+]	Age	Reas[^]	Origin	Risk[#]	VL (c/mL)	CD4 (cells/µL)	Hospital Area[$]	Subtype	Mutations	Drug Class Impacted
7	M	35	CN	Europe	MSM	650,000	450	GL	B	**PR**: M36I, L63P **RT**: F227L	F227L, poss R to NNRTIs
8	M	51	PHI	-	-	107,000	-	GL	B	**PR**: M36I, L63P/S **RT**: M41L, T215N/D*	M41L, poss R to ZDV. T215D reflects prior NRTI usage.
9	M	58	CN	Europe	MSM	7,940	423	EA	B	**PR**: M46I/L, L63P	M46I/L, poss R to IDV, RTV, APV, ATAZ (PIs)
10	M	35	CN	-	-	83,600	-	GL	B	**PR**: L63P, A71V	A71V, poss R to ATAZ (PI)
11	M	29	CN	-	-	274,000	-	GL	B	**PR**: L63P, A71V	A71V, poss R to ATAZ (PI)
12	M	41	CN	-	-	63,800	98	GL	B	**PR**: L63P **RT**: K101Q	K101Q, poss R to NNRTIs
13	M	41	CN	Europe	-	>750,000	155	GL	B	**PR:** A71T, L90M	L90M, R to SQV+NFV, poss R to IDV+RTV (PIs)

Key:
[+]Sex: M male, F female ^Reason for test: CN chronic-naïve, PHI primary HIV-1 infection
[#]Risk group: MSM men who have sex with men, Hetero heterosexual [$]Hospital area: EA Eastern, GL Greater London, WA Wales
[*]Presence of specific mutation reflects horizontal transmission although mutation alone does not affect any of the drug classes

Appendices

Appendix 4: Characterising subtype profiles

Appendix 4: Characterising subtype profiles

FASTA '*pol*' (PR and RT) sequences generated by the TRUGENE® system were manually entered into five online analytical tools to determine subtype including:

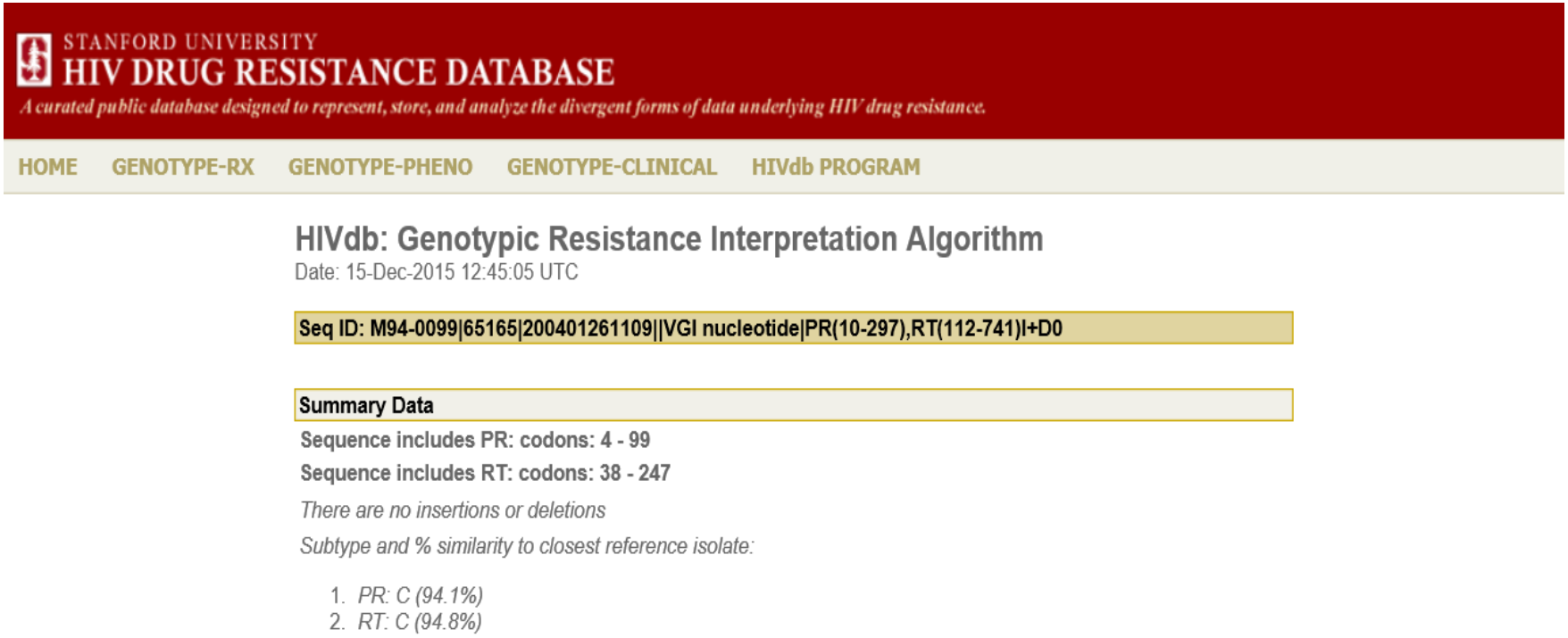
- the Stanford subtyping tool: //hivdb.Stanford.edu/
- the NCBI subtyping tool: //www.ncbi.nlm.nih.gov/
- the Los Alamos RIP 2.0 subtyping tool: //hiv-web.lanl.gov/content/hiv-db/RIPPER/RIP.html/
- the STAR subtyping tool: //www.vgb.ucl.ac.uk/starn.shtml/
- the REGA subtyping tool: //dbpartners.stanford.edu/RegaSubtyping/

An example of a FASTA sequence

>M94-0099|65165|200401261109||VGI nucleotide|PR(10-297),RT(112-741)I+D0
NNNNNNNNNACTCTTTGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGcCAgATAAAGGAGG
CTCTCTTAGACACAGGAGCAGATGATACAGTATTAGARGArATAAATTTRCCAGGAAAATGGAAAC
CAAAAATGATAGGRGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGATCAAATACTCATAGAAA
TTTGTGGAAAAAAGGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGGAAGA
AAyATGTTGACTCAGCTTGGATGCACACTAAACTTYNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNTGTGAAGAAATGGAGAAGGAAGGAAAAATTACAAAAATWGGG
CCTgAAAatCCATATAACACTCCAGTGTTTGCCATAAAAAAGAAGGACAGTACTAARTGGAGAAAA
TTAGTAGATTTCAGGGARCTTAATAARAGAACTCAAGACTTYTGGGAAGTTCAATTAGGAATACCC
CACCCAGCAGGGTTAAAAAAGAAAAAATCAGTGACAGTACTAGAYGTGGGAGATGCATATTTTTC
AGTTCCTTTAGATAAAGACTTCAGGAARTATACTGCATTcACcATACCTAGTATAAACAATGAAACA
CcAGGGATTAGATATCAATAYAATGTRCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCA
GAGTAGCATGACAAAAATCTTAGAGCCCTTTAGGGCACAAAATCCAGGAATAGTCATCTATCAAT
ATATGGATGACTTGTATGTAGGATCTGACTTAGAAATAGGGCAACATAGAGCAAAAATAGAAGAG
TTAAGAGAACATCTATTGAAGTGGGGATTTACCACACCAGATAAGAAACATCAGAAAGAACCCCC
ATTTCTTTGGATGGGGTATGAACTCCATCCTGACAAATGGACAGTACAGCCTATAGAGCTGCCA

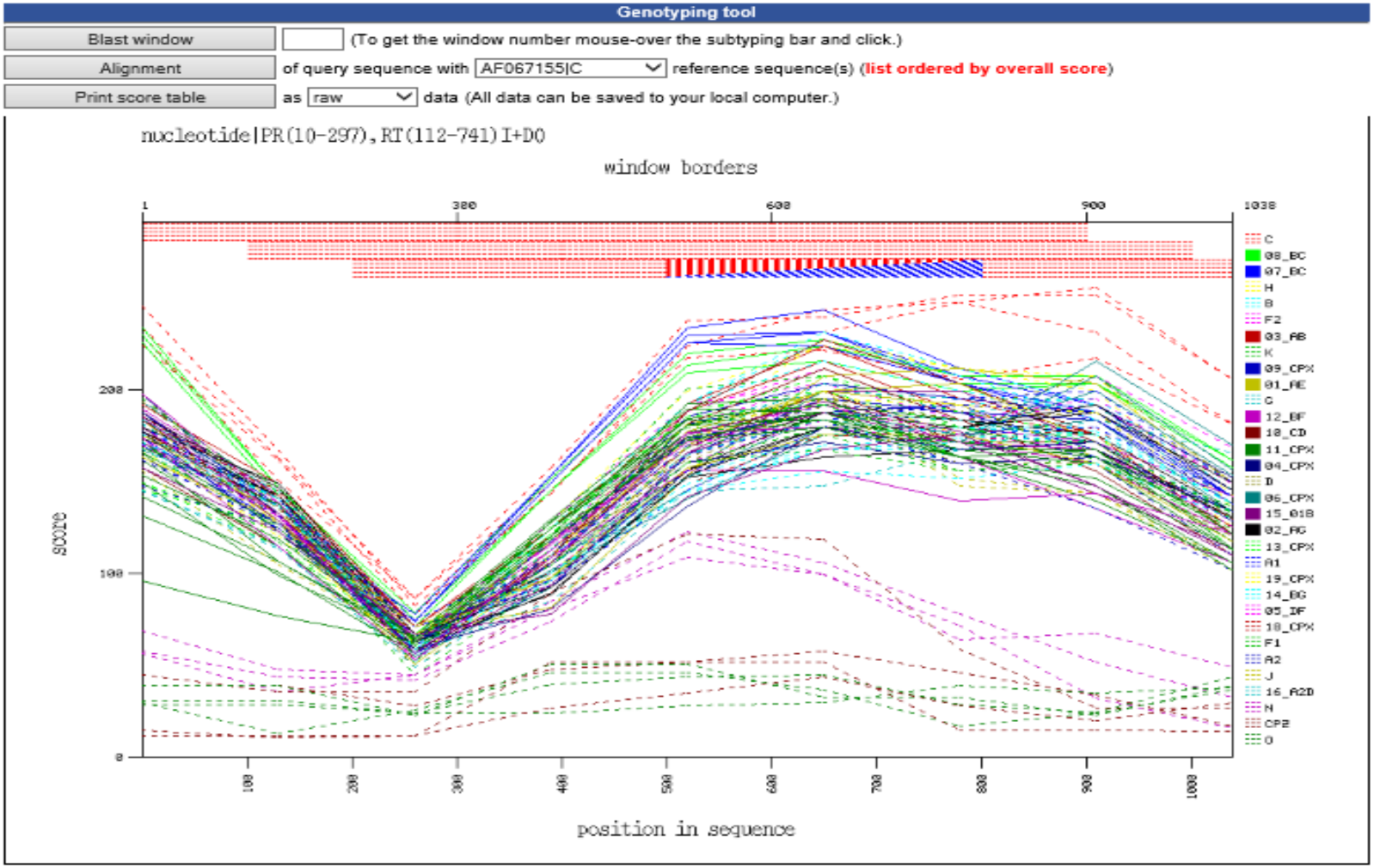
Appendix 4: Characterising subtype profiles

The Stanford subtyping tool output



Appendix 4: Characterising subtype profiles

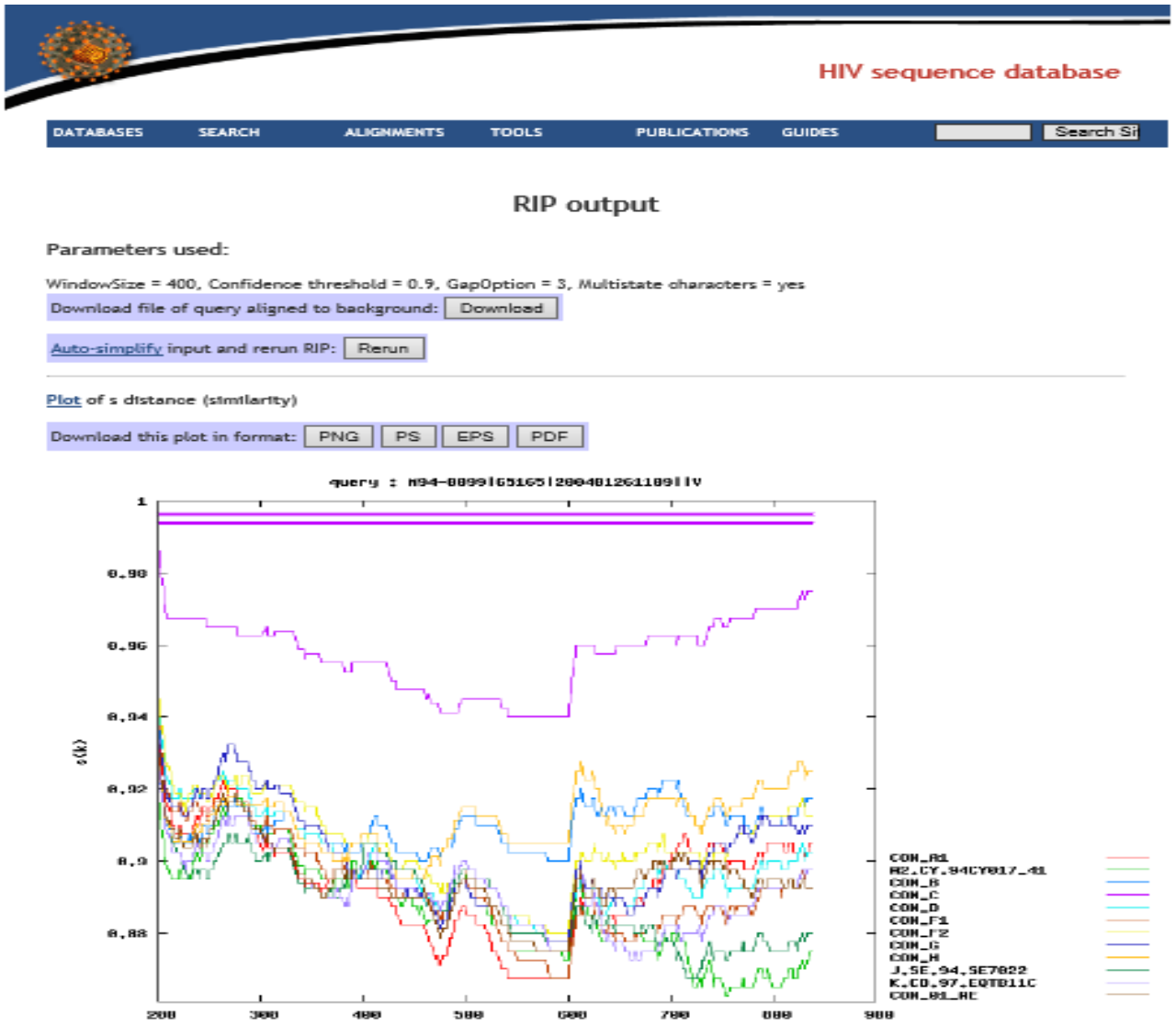
The NCBI subtyping tool output



Plot description:
The top part of the graph shows for each window the color code for the reference sequence with the highest score. If there are 2 identical scores for a specific window with different subtypes the window bar is divided diagonally.

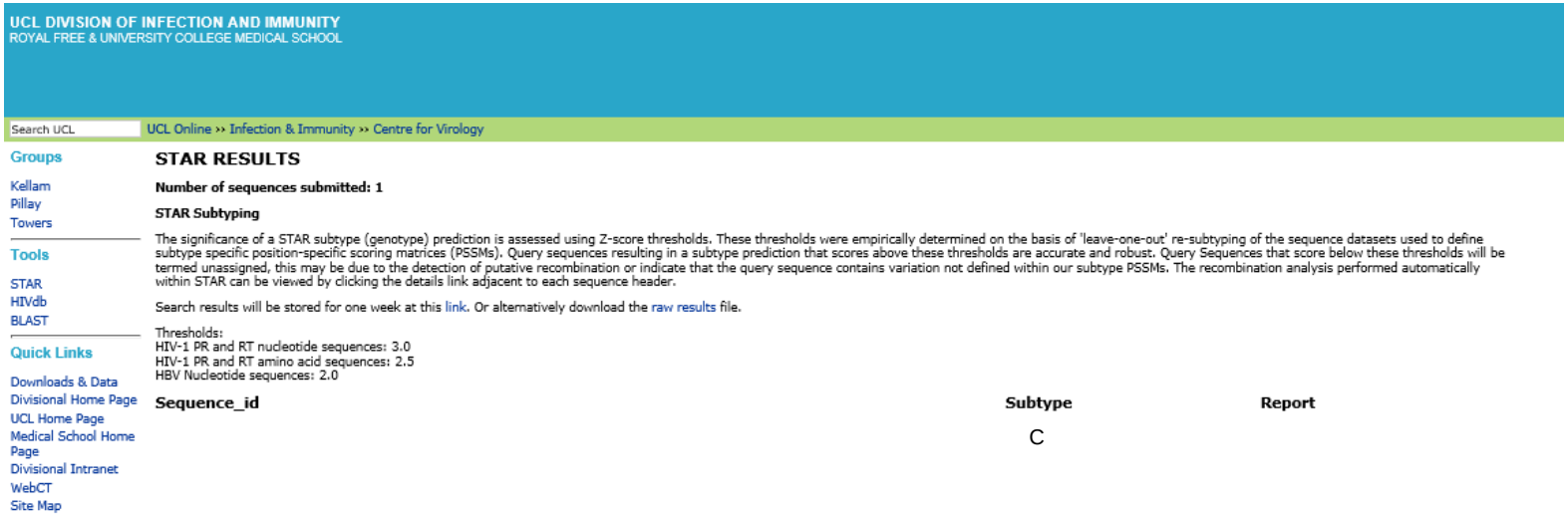
Appendix 4: Characterising subtype profiles

The Los Alamos RIP 2.0 subtyping tool output



Appendix 4: Characterising subtype profiles

The STAR subtyping tool output



UCL DIVISION OF INFECTION AND IMMUNITY
ROYAL FREE & UNIVERSITY COLLEGE MEDICAL SCHOOL

Search UCL | UCL Online ›› Infection & Immunity ›› Centre for Virology

Groups
Kellam
Pillay
Towers

Tools
STAR
HIVdb
BLAST

Quick Links
Downloads & Data
Divisional Home Page
UCL Home Page
Medical School Home Page
Divisional Intranet
WebCT
Site Map

STAR RESULTS

Number of sequences submitted: 1

STAR Subtyping

The significance of a STAR subtype (genotype) prediction is assessed using Z-score thresholds. These thresholds were empirically determined on the basis of 'leave-one-out' re-subtyping of the sequence datasets used to define subtype specific position-specific scoring matrices (PSSMs). Query sequences resulting in a subtype prediction that scores above these thresholds are accurate and robust. Query Sequences that score below these thresholds will be termed unassigned, this may be due to the detection of putative recombination or indicate that the query sequence contains variation not defined within our subtype PSSMs. The recombination analysis performed automatically within STAR can be viewed by clicking the details link adjacent to each sequence header.

Search results will be stored for one week at this link. Or alternatively download the raw results file.

Thresholds:
HIV-1 PR and RT nucleotide sequences: 3.0
HIV-1 PR and RT amino acid sequences: 2.5
HBV Nucleotide sequences: 2.0

Sequence_id	Subtype	Report
	C	

Appendix 4: Characterising subtype profiles

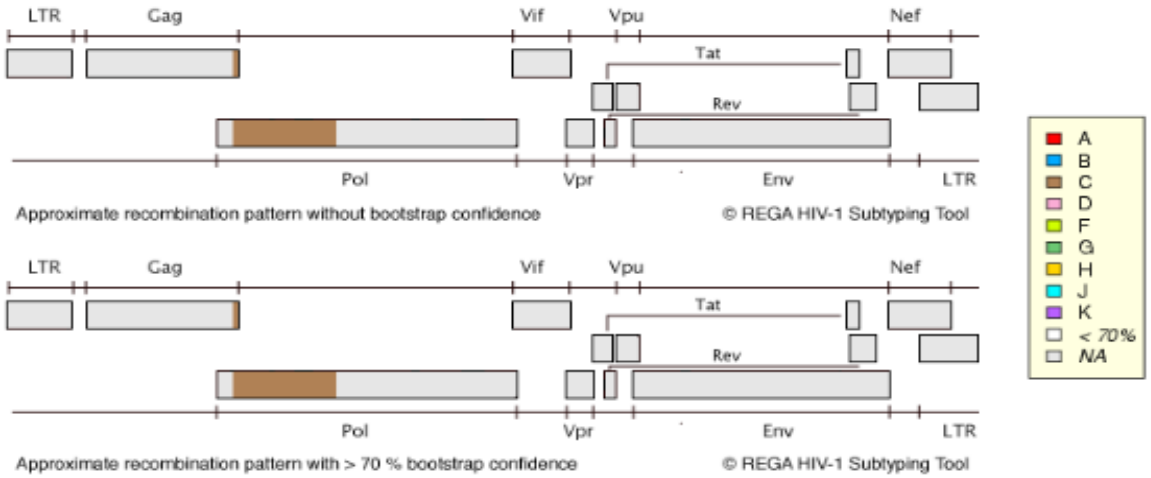
The REGA subtyping tool output

KATHOLIEKE UNIVERSITEIT LEUVEN — REGA HIV Subtyping Tool — HIV BIOINFORMATICS BIOAFRICA

Sequence Assignment

Sequence name : M94-0099|65165|, length: 1038 bps

Assignment: HIV-1 Subtype C, Bootstrap: 100.0%



Motivation: *Subtype assigned based on sequence > 800 bps clustering with a pure subtype with bootstrap > 70% without recombination in the bootscan.*

Appendix 5: List of publications arising from this thesis

Appendix 5: Publications arising from this thesis

2011

C Loveday, **E MacRae**. The prevalence of E138K mutation and other polymorphisms prior to ETV usage in a drug-experienced cohort failing on M184I/V-inducing NRTI regimens. In XV International HIV Drug Resistance Workshop: 2011 June 7-11; Los Cabos, Mexico. Abstract number 49

Background: The E138K mutation confers 3-4-fold resistance to etravirine (ETV) and other second-generation NNRTIs in tissue culture and in clinical trials. Examination of recombinant viruses and RT enzymes containing E138K with M184I/V showed reversal of low processivity and low fitness normally associated with the latter. Here, we have examined the prevalence and frequency of polymorphisms at codon 138 of RT in the ICVC Cohort failing on 3TC, FTC, ABC and/or ddI prior to introduction of ETV, to estimate the background changes and drug impact at codon 138 relative to a drug-naïve population.
Methods: The ICVC database contains over 5,000 RT/PR sequences with demographics from drug-naïve (DN) and drug-experienced (DE) UK patients. We selected consecutive patients, prior to the ETV era, failing on selected NRTIs (±M184I/V) and examined the frequency and nature of polymorphic changes at codon 138. Statistical analysis was by SPSS (version 17).
Results: Sequences from 3,071 patients (DE: 2,356, DN: 715) were included with ~40% currently having M184I/V. Polymorphisms (A, D, G, K, Q, X) were seen at codon 138 in 130/2,356 (5.5%) of DE and 43/715 (6.0%) of DN (not significant [NS]). A higher frequency of E138K was found in DE (13/2,356; 0.55%) than DN (1/715; 0.1%; NS). All 13 of these DE patients were currently failing with M184I/V. Polymorphism E138A had significantly higher prevalence in the DN (39/715; 5.5%) than the DE (82/2,356; 3.5%; $p<0.01$). Polymorphisms at E138D/G/Q/X all had <0.5% prevalence with no differences between DE and DN patients.
Conclusions: In the pre-ETV era, codon 138 was highly polymorphic but exhibits a low frequency of change from WT (<5.0%) in patients failing with M184I/V or on therapies driving these mutations. The E138A frequency significantly declined in DE patients and E138K had a tantalizing, but non-significant, increase in the DE patients. It would appear that prior to ETV, there was no major pathway to enhance replication of M184I/V-bearing viruses via codon 138. However, this study defines a baseline for further exploration of recent databases in the ETV era, and directs future *in vitro* recombinant studies.

E MacRae, C Loveday. A retrospective cross-sectional comparison of fluid-phase inflammatory markers including: immunoglobulin (Ig) G, M and A, associated circulating immune complexes (CIC) and total complement activation markers in AIDS, HIV-1 antibody positive and negative patient cohorts. In 6th IAS Conference on HIV Pathogenesis, Treatment and Prevention, 2011 July 17-20, Rome, Italy. Abstract number CDA059.

C Loveday, **E MacRae**. An audit of HIV/AIDS clinic care in a small rural UK clinic showed Caucasian (C) versus black African (BA) late presenters had equally good responses to therapy. In 6th IAS Conference on HIV Pathogenesis, Treatment and Prevention, 2011 July 17-20, Rome, Italy. Abstract number CDB348.

Appendix 5: Publications arising from this thesis

Background: Small rural HIV/AIDS services (<300 patients) can be under-resourced in terms of staff, expertise and networks, and their patient populations skewed by patient self-selection, overseas asylum seekers and late presenters. To address strategies for optimizing patient care at our centre we carried out a retrospective clinical notes audit to determine population demographics, disease stage and current responses to therapy.
Method: Our care model used European and BHIVA guidelines, 3 monthly patient visits and weekly review of patient issues by 1 doctor, HIV nurse, HCA, pharmacist, social worker and a community worker, all with part-time contracts. We had an external network to clinics and nurse networks regionally. Daily telephone access for advice and additional care was available through the HIV nurse.
The audit collected anonymous, consecutive, retrospective, demographic, virological and therapeutic data from patients over a 2 month period. These data were analyzed by SSPS version 17.
Results: The patients (n=33) included C(39%, male:10, female:3) and BA (55%, male:6,female:12) with a mean age 42.6 years (range:17-60) and 79% >38 yrs. Comparison of C v BA showed mean ages 44.1 v 41 yrs, symptomatic/AIDS 46 v 83%, highest VL 5.11 v 5.2 log copies/ml, lowest CD4 228 v 183/mm^3, on therapy 85 v 89%, current VL>40:100 v 100%, current CD4 687 v 476/mm^3 ,time undetectable 44.5 v 36.1 months, on 1st therapy 45 v 75%, respectively. There was low level co-infection with HBV and no HCV, and patients allowing GP shared care was significantly higher in C (62%) versus BA (37%, p<0.01).
Conclusion: There was 100% undetectable VL, a mean CD4 rise of 371/mm^3 over a mean 44 months in C and BA late presenters in our rural UK clinic setting. Our care model adhered closely to guidelines, had weekly virtual ward rounds and took advantage of regional networks.

C Loveday, **E MacRae**, D Human, RM Lloyd Jr. Evaluation of a strategy to deliver low-technology laboratory (LTL) support to provide cost-effective, real-time and contemporary clinical care to patients in resource-limited settings (R-LS) – phase 1: molecular tests. In 6th IAS Conference on HIV Pathogenesis, Treatment and Prevention, 2011 July 17-20, Rome, Italy. Abstract number MOPE421.

C Loveday, R Lloyd, R Diaz, **E MacRae**, Z Grossman, R Mathis, D Burns and M Holodney. Evaluation of a dry plasma matrix transport device for genotyping of HIV-1, HBV and HCV, and quantification of HIV-1 VL to provide an economic approach for real-tie clinical care in resource-limited settings. In BHIVA 17th Annual Conference: 2011 April 6-8, Bournemouth, UK. Oral abstract number O10.

2010

C Loveday, RM Lloyd Jr, **E MacRae**, Z Grossman, R Mathis, D Burns, J Cooper and M Holodniy. **A simple solid matrix transport device SampleTanker for economic collection, storage and transport of patient plasma between clinical sites for HIV-1 molecular and antibody testing.** In Second Joint Conference of the British HIV Association and the British Association for Sexual Health and HIV; 2010 April 20-23; Manchester, UK. Abstract number P55

Appendix 5: Publications arising from this thesis

C Loveday, **E MacRae** and RM Lloyd Jr. **Evaluation of an economic solid matrix dry plasma transportation device (SampleTanker: Vive ST^{IM}) for qualitative and quantitative HIV-1 serology using patient plasma posted between different clinical sites.** In XVIII International AIDS Conference; 2010 July 18-23; Vienna, Austria. Abstract

2009

Loveday C, **MacRae E**, Holodniy M, Mathis R, Burns D, Cooper J, Lloyd Jr RM, Grossman Z. **Evaluation of SampleTanker® for Collection, Storage and Transport of Dried Plasma from a Resource-Limited Setting (R-LS) to a Resource-Rich Setting (R-RS) for HIV-1 Genotypic Analysis.** In XVIII International HIV Drug Resistance Workshop; 2009 June 9-13; Fort Myers, Florida. Abstract

---

Dimitrios Paraskevis, Oliver Pybus, Gkikas Magiorkinis, Angelos Hatzakis, Annemarie MJ Wensing, David A van de Vijver, Jan Albert, Guiseppe Angarano, Birgitta Äsjö, Claudia Balotta, Enzo Boeri, Ricardo Camacho, Marie-Laure Chaix, Suzie Coughlan, Dominique Costagliola, Andrea DeLuca, Carlos de Mendoza, Inge Derdelinckx, Zehava Grossman, Osama Hamouda, I M Hoepelman, Andrzej Horban, Klaus Korn, Claudia Kuecherer, Thomas Leitner, Clive Loveday, **Eilidh MacRae**, I Maljkovic, Laurence Meyer, Claus Nielsen, Eline LM Op de Coul, Vidar Ormaasen, Luc Perrin, Elisabeth Puchhammer-Stöckl, Lidia Ruiz, Mika Salminen, Jean-Claude Schmit, Rob Schuurman, Vincent Soriano, J Stanczak, Maja Stanojevic, Daniel Struck, Kristel Van Laethem, M Violin, Sabine Yerly, Maurizio Zazzi, Charles A Boucher and Anne-Mieke Vandamme. **Tracing the HIV-1 subtype B mobility in Europe: a phylogeographic approach.** Retrovirology 2009; 6: 49

2008

MacRae E, Loveday C and on behalf of the ICVC Clinical Collaborative Research Group. **Significant Increase in Prevalence of HIV-1 Recombinant Forms in a UK Non-B Subtype Clinical Cohort over 10 Years**. In 15th Conference on Retroviruses and Opportunistic Infections (CROI); 2008 February 3-6; Boston, USA. Abstract number 511

Background: To determine the prevalence and characterisation of HIV-1 non-B (NB) subtypes and recombinant forms in a UK clinical cohort between 1996 and 2005.
Methods: As part of clinical care, patient sequences derived from resistance testing at baseline were submitted for subtype characterisation using online tools. Sequential clinical patient sequences characterised with a NB subtype were included in the analyses. The prevalence of NB subtypes and recombinant forms in the early cohort (EC: 1996-2000); and the late cohort (LC: 2001-2005) was determined. Statistical analyses were performed using SPSS (v14).
Results: Total patients characterised with a NB subtype remained unaltered (EC n=632; LC n=661). However, there was a significant increase in recombinant forms

from the EC: 44/632 (6.96%) presented with a recombinant form versus the LC: 141/661 (21.33%); p<0.001. The EC revealed five distinct recombinant forms: CRF01_AE (38.64%), unclassifiable recombinant forms (34.09%), A/F (15.90%), A/C (9.09%), A/D (2.27%); whilst the LC showed 30 distinct recombinant forms: including CRF02_AG (43.97%), CRF01_AE (9.93%), CRF10_CD (7.09%), CRF06_cpx (4.26%), D/A1, D/G (both 3.55%) CRF13_cpx, D/C, G/B, H/A1, J/K (all 1.42%). Of eight geographical regions tested in the EC, 88.64% of recombinant forms were circulating in Greater London, with patients in three other geographical regions presenting with a recombinant form (all CRF01_AE). Of eight geographical regions tested in the LC, all regions had evidence of recombinant forms with the greatest prevalence in Greater London (83.69%) with 25 distinct recombinant forms identified in this region. Demographic data in the EC and LC were not significantly different.
Conclusions: These data reveal a significant increase in numbers and geographical distribution of recombinant forms in a UK clinical cohort over the last ten years. Once NB subtypes are established in a community it appears that in a short time recombination may occur to give new biologically successful viruses. These findings have implications for the clinical / virological management of patients using PCR in the future.

2007

MacRae E, Loveday C and on behalf of the ICVC Clinical Collaborative Research Group. **Susceptibility of a first generation NNRTI treatment experienced UK clinical cohort to TMC-125.** In 11th European AIDS Conference (EACS); 2007 October 24-27; Madrid, Spain. Abstract number P3.1/05

2006

Loveday C, **MacRae E**, on behalf of the ICVC Clinical Collaborative Research Group. **Susceptibility of a protease inhibitor (PI) treatment-experienced UK clinical cohort to TMC-114.** Eighth Congress on Drug Therapy in HIV Infection, November 12-16, 2006, Glasgow, UK. Abstract PL2-2.

Purpose of the study: The new PI TMC-114 has approval for use in the USA and is likely in the EU later this year. TMC-114 is expected to be effective for many patients with extensive PI-treatment experience. The aim of this study was to determine the susceptibility of a UK PI treatment-experienced clinical cohort to TMC-114.
Methods: PI-experienced patients who had genotypic resistance tests performed at failure of their current regimen as part of their clinical care were included (1996-2006). Mutations were derived from the Power 1, 2 and 3 trials including PR: V11I, **V32I**, **L33F**, **I47V**, I50V, **I54L**, I54M, G73S, L76V, I84V, **L89V** (**bold**=high impact mutations (HI) developed in ≥10% virologic failures). Statistical analyses were performed using SPSS (v14).
Summary of results: 885 patients were included: 532(60.2%) currently failing with PI containing regimens; 188(21.2%) on non-PI treatment but with PI experience; 165(18.6%) off treatment, but with PI experience.
104(19.5%) patients currently on PI treatment presented with TMC-114 related mutations including: **V32I**=15, **L33F**=23, **I47V**=13, I50V=6, **I54L**=10, I54M=1,

G73S=23, I84V=49. 83 patients had one related mutation (of which, 22 were HI), 11 had two (13 HI), 6 had three (12 HI), 2 had four (**32**+**47**+**54L**+84) and 2 had five (**32**+**33**+**47**+**54L**+84).
8 patients on non-PI treatment had **L33F**=1, **I54L**=1, G73S=1, I84V=5 (no patient had >1 mutation); 14 patients off treatment had: **V32I**=3, **L33F**=7, **I54L**=1, G73S=4, I84V=9 (7 with one mutation (3 HI), 4 with two (3 HI), 3 with three (5 HI)).
Conclusion: In this PI treatment-experienced clinical cohort, the majority had very low frequency of TMC-114 related mutations. Only 10 patients had ≥10 PR mutations with 2 patients including 4 HI mutations. Based on these data TMC-114 should be of benefit to PI treatment-experienced patients in a UK clinical cohort.

Loveday C, **MacRae E** and on behalf of the ICVC Clinical Collaborative Research group [Abstract]. **Limitations in using online tools to determine HIV-1 subtype in clinical practice: a comparison of 5 tools**. In XV International HIV Drug Resistance Workshop: 2006 June 13-17; Sitges, Spain. Abstract number 116

Background: HIV-1 subtype characterisation is becoming an important aspect of clinical management of infection. Currently, patients' HIV-1 subtype is defined by submitting *pol* sequences to one online clinical database. The aim of this study is to compare accuracy of subtype results derived from 5 popular online analytical tools using 1002 consecutive clinical samples.
Methods: Subtype analyses were performed using PR and RT gene sequences from clinical samples. The 5 clinical databases used were:
Stanford://hivdb.Stanford.edu/,
NCBI://www.ncbi.nlm.nih.gov/,
REGA://dbpartners.stanford.edu/RegaSubtyping/,
STAR: //www.vgb.ucl.ac.uk/starn.shtml/,
LosAlamos RIP 2.0: //hiv-web.lanl.gov/content/hiv-db/RIPPER/RIP.html/.
Comparative and statistical analyses were performed using SPSS.
Results: 1002 sequences were submitted for subtyping. Stanford, NCBI, LosAlamos assigned a subtype to all sequences. REGA (237, 23.7%) and STAR (135,13.5%) were unable to assign subtype for a significant number of sequences ($p<0.001$).
Concordant results across all 5 tools=585 (58.4%), including subtypes A=22(3.8%), B=388(66.3%), C=164(28%), D=6(1%), F=2(0.3%), G=3(0.5%). The remaining 417 (41.6%) analyses resulted in 40 combinations of concordant / discordant / unassigned results across the 5 tools.
Concordance across 4 tools with 1 tool providing a discordant or unassigned result =158(15.8%), including:
Stanford+NCBI+REGA+STAR=68 (LosAlamos discordance associated with subtype B=46, CRF02_AG=22). Stanford+NCBI+STAR+LosAlamos=62 (REGA unassigned=61, REGA discordance associated with CRF01_AE).
NCBI+REGA+STAR+LosAlamos=14 (Stanford discordance associated with A=5, C=9). Stanford+NCBI+REGA+LosAlamos=8 (STAR unassigned=8).
Stanford+REGA+STAR+LosAlamos=6 (NCBI discordance associated with A=5, G=1).
Concordance across 3 tools with the other 2 tools concordant or providing discordant / unassigned results=134(13.4%). Discordance / unassigned results across all 5 tools=51(5.1%), including:

Stanford+NCBI+LosAlamos discordant, REGA+STAR unassigned=39
Stanford+NCBI+REGA+LosAlamos discordant, STAR unassigned=11
Stanford+NCBI+STAR+LosAlamos discordant, REGA unassigned=1
Overall concordance across the individual tools: Stanford+STAR=80.7%, Stanford+NCBI=79.2%, Stanford+LosAlamos=77%, STAR+LosAlamos=76.6%, STAR+NCBI=74.7%, NCBI+LosAlamos=70%, REGA+STAR=69.9%, REGA+NCBI=69.5%, REGA+Stanford=67.8%, REGA+LosAlamos=63.5%.
Conclusion: There was 58.4% concordance across all 5 tools. Use of one tool alone, compared with any of the other tools will result in misclassification of 20% or more of patients' subtype. Unassignment of sequences by REGA and STAR probably reflects the stringency of the tools. Discordance across the tools reflects the difficulty in keeping these tools up-to-date to determine new and recombinant viruses. The evolution of new, complex recombinant viruses at a community level will result in subtyping becoming increasingly difficult to interpret.

---

van de Vijver DAMC, Wensing AMJ, Angarano G, Asjo B, Balotta C, Boeri E, Camacho R, Chaix ML, Costagliola D, De Luca A, Derdelinck I, Grossman Z, Hamouda O, Hatzakis A, Hemmer R, Hoepelman A, Horban A, Korn K, Kucherer C, Leitner T, Loveday C, **MacRae E**, Maljkovic I, de Mendoza C, Meyer L, Nielsen C, Op de Coul EL, Ormaasen V, Paraskevis D, Perrin L, Puchhammer-Stockl E, Ruiz L, Salminen M, Schmit JC, Schneider F, Schuurman R, Soriano V, Stanczak G, Stanojevic M, Vandamme AM, Van Laethem K, Violin M, Wilbe K, Yerly S, Zazzi M, and Boucher CAB for the SPREAD Programme. **The Calculated Genetic Barrier for Antiretroviral Drug Resistance Substitutions is Largely Similar for Different HIV-1 Subtypes.** Journal of Acquired Immune Deficiency Syndrome 2006; 41 (3): 352-360

Loveday C, **MacRae E** and on behalf of the ICVC Clinical Collaborative Research group [Abstract]. **Comparison of online clinical database HIV-1 subtyping tools.** In 13th Conference on Retroviruses and Opportunistic Infections: 2006 February 5-8; Denver, Colorado, USA. Poster number 660 *[*Awarded Young Investigator Award]*

Background: Sequences used to define HIV-1 resistance are often applied to online databases to define subtype to improve patient care. This study aims to compare 3 common analytical tools using sequential clinical samples.
Methods: Subtype analyses were performed using PR and RT gene sequences from samples with epidemiological evidence of genetic diversity (2002 - end Aug 2005). Three clinical comparative tools were used: **Stanford**://hivdb.Stanford.edu/, **NCBI**://www.ncbi.nlm.nih.gov/, **REGA**://dbpartners.stanford.edu/RegaSubtyping/. Comparative and statistical analyses were performed using SPSS.
Results: 1646 sequences were submitted for subtyping. Subtype B=827(50.2%); non-B (NB)=800(48.6%); indeterminate=19(1.2%). All three analytical tools provided concordant results for 1206(73.3%) of sequences submitted, B=742, NB=464, including A=41, C=337, D=9, F=7, G=19, H=2, CRF02_AG=49.
REGA was unable to assign a significant number of samples n=311,18.9% relative to Stanford and NCBI ($p<0.001$). Of the 311, Stanford and NCBI provided concordant subtype analyses for 146(46.9%) and discordant for 165(53.1%). Reasons for non-assignment by REGA: no cluster with pure subtype, no detection of recombination (n=65:20.9%); cluster with pure subtype, detection of

recombination but failure to classify as CRF (n=115:37%); cluster with CRF, detection of recombination in pure subtype but failure to classify as CRF (n=39:12.5%); cannot explain (n=92:29.6%).
Of the remaining 129 results, REGA and Stanford were concordant in 12 cases, REGA and NCBI in 30, Stanford and NCBI in 25 and there were 62 discordant results across the 3 tools. Discordant results were associated with subtype A/CRF01_AE (n=48:77.4%), C (n=6:9.7%), F (n=3:4.8%), CRF10_CD (n=2:3.2%), CRF06_cpx, CRF13_cpx, J (all n=1:1.6%).
Conclusions: In 73% of cases there was good agreement between the analytical approaches. Unassignment of 18.9% of sequences in REGA probably reflects the stringency of the process: query sequences submitted to REGA are interrogated by phylogenetic analysis, bootstrap support, bootscanning analysis and phylogenetic signal detection. The NCBI tool was more subjective as the user had to interpret the graphical output produced. Stanford was easy to use but compares query sequences to approx. 94 reference sequences only, and therefore may not detect new and recombinant subtypes. Careful consideration is required when using these tools in isolation for clinical care

2005

MacRae E, Loveday C and on behalf of the ICVC Clinical Collaborative Research Group [Abstract]. **High prevalence of HIV-1 non-B subtype recombinants and diverse polymorphic profiles in a UK clinical cohort - implications for future resistance analysis.** In XIV International HIV Drug Resistance Workshop: 2005 June 7-11; Quebec City, Quebec, Canada. Abstract number 134

Background: Increase in prevalence of non-B subtypes (NB) is associated with clinical issues of detection, sequencing and mutational characterisation. With our development of new approaches to sequence difficult samples, our laboratory has attracted a unique population of UK NB samples. In view of the clinical importance of diverse viruses we have analysed distribution of NB subtypes in naïve and treated patients including the proportion and diversity of recombinants; mutations and polymorphisms associated with NB viruses relative to B.
Methods: Patients who had genotypic resistance tests performed as part of their clinical care between 2003 and February 2005 were included. Mutations were derived from TRUGENE-Genelibrarian (Bayer Diagnostics). Consensus subtype analyses were performed using RT and PR gene sequences with comparative databases (Stanford, NCBI, Los Alamos). Statistical analyses were performed using SPSS.
Results: The last 945 consecutive clinical patient sequences submitted for analysis were included: NB-424(45%), B-521(55%). Naïve: NB-152(36%), B-252(48%); treated: NB-272(64%), B-269(52%).
NB subtypes included: A(7%), C(50%), CRF01_AE(3%), CRF02_AG(9%), D(9%), F(1%), G(4%), H(1%).
17% were recombinants, with 30 combinations exhibiting different subtypes for PR and RT, including: A/CRF01_AE, A/J, J/B, J/C, J/F, J/K, K/C, K/CRF01_AE, K/F.
Significant PR mutations present in NB versus B using the IAS-USA definition found: **K20R** (naïveNB-20%, B-2.4%; treatedNB-21%, B-7.8%) **M36I** (naïveNB-99%, B-19.1%; treatedNB-85%, B-20.8%) more frequently in NB viruses ($p<0.05$ (Chi^2)).

L63P was more prevalent in B viruses (naïveNB-23%, B-58.7%; treatedNB-28%, B-61.3%) ($p<0.05$).
15 PR polymorphisms were significantly more prevalent in NB versus B naïve sequences: T12S, I13V, I15V, G16E, L19I/T/T;I, S37N, R41K, K45R, D60E, Q61E, H69K, K70R, T74S, L89M, I93L ($p<0.001$). Applying the Tipranavir resistance score, six of these polymorphisms have clinical implications for viral resistance.
No RT mutations were significantly more prevalent in NB versus B but 16 RT polymorphisms were: T39D/E/K, E40D, K43E, S48T, K49R, V60I, D123G/N/S, I135V, E138A, S162A, K173A/T, Q174K, D177E, T200A, Q207A/E, V245Q ($p<0.001$).
Conclusions: In a UK NB population, there was a high prevalence of new diverse recombinant viruses. Although few resistance mutations were significantly different between NB and B, there was disproportionate representation of polymorphisms including some currently described as resistance mutations for newer PIs. These data imply the need for specific NB mutational charts for analysis of resistance.

---

van de Vijver DAMC, Wensing AMJ, Angarano G, Asjo B, Balotta C, Boeri E, Camacho R, Chaix ML, Costagliola D, Op de Coul ELM, de Luca A, Maljkovic I, de Mendoza C, Derdelinck I, Grossman Z, Hamouda O, Hatzakis A, Hoepelman IM, Hemmer R, Horban A, Korn K, Kucherer C, Leitner T, Loveday C, **MacRae E**, Meyer L, Nielsen C, Ormaasen V, Perrin L, Paraskevis D, Puchhammer-Stockl E, Ruiz L, Salminen M, Schmit JCC, Schneider F, Schurrmann R, Soriano V, Stanczak G, Stanojevic M, Vandamme AM, Van Laethem K, Violin M, Wilbe K, Yerly S, Zazzi M, and Boucher CAB on behalf of the SPREAD Programme [Abstract]. **Differences in the frequency of minor substitutions between HIV-1 subtypes and their potential impact on the genetic barrier for resistance to protease inhibitors.** In XIV International HIV Drug Resistance Workshop: 2005 June 7-11; Quebec City, Quebec, Canada. Abstract number 132

---

van de Vijver DAMC, Wensing AMJ, Asjo B, Bruckova M, Brunn Jorgensen L, Horban A, Linka M, Lazanas M, Loveday C, **MacRae E**, Nielsen C, Paraskevis D, Poljak M, Puchhammer-Stockl E, Ruiz L, Schmit JCC, Stanczak G, Stanojevic M, Vandamme AM, Vercauteren J, and Boucher CAB on behalf of the SPREAD Programme [Abstract]. **Selective transmission of drug resistance mutations.** In XIV International HIV Drug Resistance Workshop: 2005 June 7-11; Quebec City, Quebec, Canada. Abstract number 113

Loveday C, Grant P, Goodall R, Pillay D, **MacRae E**, Asboe D, Williams I, Stoehr W, Babiker A, on behalf of the Forte Virology Group and Trial Steering Committee. **Adding a PI for 6 months to a Standard NNRTI-based Regimen Reduces the Risk of Virological Failure without Inducing Resistance to the PI: The Forte Virology Analysis** [Abstract]. In 12th Conference on Retroviruses and Opportunistic Infections: 2005 February 22-25; Boston, Massachusetts, USA. Poster number 575.

Wensing AMJ, van de Vijver DAMC, Angarano G, Asjo B, Balotta C, Boeri E, Camacho R, Chaix ML, Costagliola D, de Luca A, Derdelinck I, Grossman Z, Hamouda O, Hatzakis A, Hemmer R, Hoepelman A, Horban A, Korn K, Kucherer

C, Leitner T, Loveday C, **MacRae E**, Maljkovic I, de Mendoza C, Meyer L, Nielsen C, Op de Coul EL, Ormaasen V, Paraskevis D, Perrin L, Puchhammer-Stockl E, Ruiz L, Salminen M, Schmit JC, Schneider F, Schuurman R, Soriano V, Stanczak G, Stanojevic M, Vandamme AM, Van Laethem K, Violin M, Wilbe K, Yerly S, Zazzi M, and Boucher CAB for the SPREAD Programme. **Prevalence of Drug-Resistant HIV-1 Variants in Untreated Individuals in Europe: Implications for Clinical Management.** Journal of Infectious Diseases 2005; 192 (6): 958-966

2004

Loveday C, **MacRae E**, Johnson M and on behalf of the ICVC Clinical Collaborative Research Group. **The Changing Prevalence of HIV-1 Protease (PR) and Reverse Transcriptase (RT) Polymorphisms in Primary HIV Infection (PHI), Chronic-Naïve, and following Exposure to Antiretroviral Therapy (ART)** [Abstract]. In XIII International HIV Drug Resistance Workshop: 2004 June 8-12; Canary Islands, Tenerife, Spain. Abstract number 82

Background: The significance of commonly occurring polymorphisms in PR and RT in early infection and before and after ART is poorly understood. In previous smaller studies using 251 selected polymorphisms with a prevalence of greater than 1% derived from the ICVC database we have seen homogeneity at infection with increasing polymorphic diversity in chronic-naïve infection, and evidence of selection under pressure of ART. Using the database with over 3000 patients we explore these questions in detail.
Methods: The frequency of 251 polymorphisms in PR and RT were analysed relative to patient disease stage and therapy, using the ICVC database. Sequence data were derived from VGI TRUGENE HIV-1 (Bayer Diagnostics). Chi-square statistical analyses were performed using SPSS (version 12 for Windows).
Results: Using 646 patient sequences (PHI (n=70), chronic-naïve (n=253) and post-ART (n=323)), significant homogeneity was exhibited in PHI relative to chronic-naïve (PR: p=0.007, RT: p=0.001). Polymorphisms commonly present at PHI were PR: codons 37 (87.14%), 41 (45.71%), 93 (54.29%) and RT: codons 122 (58.57%), 211 (68.57%), 214 (84.29%) and 245 (58.57%).
In chronic-naive patients, the polymorphic repertoire expanded significantly (PR: p=0.007, RT: p=0.001) with the prevalence of polymorphisms at PR: codon 69 (p=0.008) and RT: codons 39 (p=0.019), 173 (p=0.028), 174 (p=0.023), 177 (p=0.017) and 200 (p=0.006) significantly increased compared to PHI.
Following ART, polymorphisms at PR: codons 13, 16, 55, 64, 70, 72, 74, 93 (all p<0.05) and RT: codons 43, 90, 123, 135, 138, 203, 218, 221, 228, 238 (all p<0.05) had a significant increase in prevalence relative to chronic-naïve patients. Prevalence of PR: codons 19 (p=0.024), 45 (p=0.005) and RT: codons 48 (p=0.011), 83 (p=0.004), 169 (p=0.011) significantly decreased relative to chronic-naïve patients.
Conclusions: Certain polymorphisms confer biological advantages to HIV during early evolution in the host and following ART. Genetic homogeneity at infection is followed by significant genetic expansion in the polymorphic repertoire of chronic-naïve patients. Drug pressure showed the polymorphic repertoire altered and implies that although these polymorphisms may not confer resistance alone, they provide a background to facilitate resistance in the presence of recognized mutations.

van de Vijver DAMC, Wensing AMJ, Angarano G, Asjo B, Balotta C, Boeri E, Camacho R, Chaix ML, Costagliola D, Op de Coul E, de Luca A, Maljkovic I, de Mendoza C, Derdelinck I, Grossman Z, Hamouda O, Hatzakis A, Hoepelman IM, Hemmer R, Horban A, Korn K, Kucherer C, Leitner T, Loveday C, **MacRae E**, Meyer L, Nielsen C, Ormaasen V, Perrin L, Paraskevis D, Puchhammer-Stockl E, Ruiz L, Salminen M, Schmit JCC, Schneider F, Schurrmann R, Soriano V, Stanczak G, Stanojevic M, Vandamme AM, Van Laethem K, Violin M, Wilbe K, Yerly S, Zazzi M, and Boucher CAB on behalf of the SPREAD Programme [Abstract]. **The Calculated Genetic Barrier For Drug Resistance Mutations In Six Different Non-B Subtypes And Two CRF's In A Large European Dataset Is Largely Similar to Subtype B.** In XIII International HIV Drug Resistance Workshop: 2004 June 8-12; Canary Islands, Tenerife, Spain. Abstract number 87.

Loveday C, **MacRae E**, Johnson M and on behalf of the ICVC Collaborative Research Group. **The rising prevalence of subtype non-B in a UK drug-naïve cohort** [Abstract]. In 2nd European HIV Drug Resistance Workshop: 2004 March 11-13; Rome, Italy. Abstract number 1.17

Background: The ICVC undertakes the virological care of over 7000 patients in 37 clinical centres across the UK. We, and others have reported a temporal rise in the prevalence of subtype non-B and recombinants in the UK and Europe. This retrospective clinical audit aims to investigate drug-naïve resistance profiles in these populations to date.
Methods: All ART-naïve patients who had genotypic resistance tests performed as part of their clinical care between 2001 and 2003 were included (VGI TRUGENE HIV-1 Bayer Diagnostics). Mutations were derived from the rules based algorithm (Genelibrarian report). Consensus subtype analyses were performed using *pol* gene sequences with comparative databases.
(NCBI: http://www.ncbi.nlm.nih.gov/retroviruses/subtype/subtype.html; Stanford: http://hivdb.stanford.edu/ Los Alamos: http://www.hiv.lanl.gov/content/index).
Results: 284 ART-naïve patients presented in the three-year period 2001 to 2003 (n=38; 89; 157). Majority were male (33; 70; 110) with mean age (41.3; 37.4; 35.2). The majority presented as chronic naïve (24; 57; 117) as PHI (11; 27; 28) as pregnant and naïve (3; 5; 12).
In 2001, 10/38 (26.3%) were subtype non-B; 30/89 (33.7%) were in 2002 and 72/157 (45.9%) in 2003. Subtype C was predominant (5 (13.2%); 23 (25.8%); 48 (30.6%)) with increasing diversity and recombinant forms identified too.
Prevalence of subtype non-B has significantly increased ($chi^2 = 6.674$, $p = 0.036$).
Over the years, 33 (9 non-B); 80 (29 non-B); 135 (67 non-B) patients presented with listed mutations associated with ART. Of those, 5 (0 non-Bs); 15 (2 non-Bs); 19 (9 non-Bs) were found to have at least one major mutation and/or evidence of horizontal transmission. The mutations present did not significantly differ from those found in the subtype B cohort. Of note, the prevalence of all PR mutations in non-B patients has significantly increased over time (15/55 = 27.3%; 46/121 = 38%; 106/210 = 50.5% ($chi^2 = 11.537$, $p = 0.003$)), as have RT mutations (0/7 = 0%; 2/19 = 10.5%; 11/22 = 50% ($chi^2 = 11.088$, $p = 0.004$)).
Conclusions: Mutations within subtype non-B viruses found within ART-naïve patients are no different from subtype B populations in the cohort. We have found

an increasing prevalence of subtype non-B within this cohort, predominantly represented by subtype C.

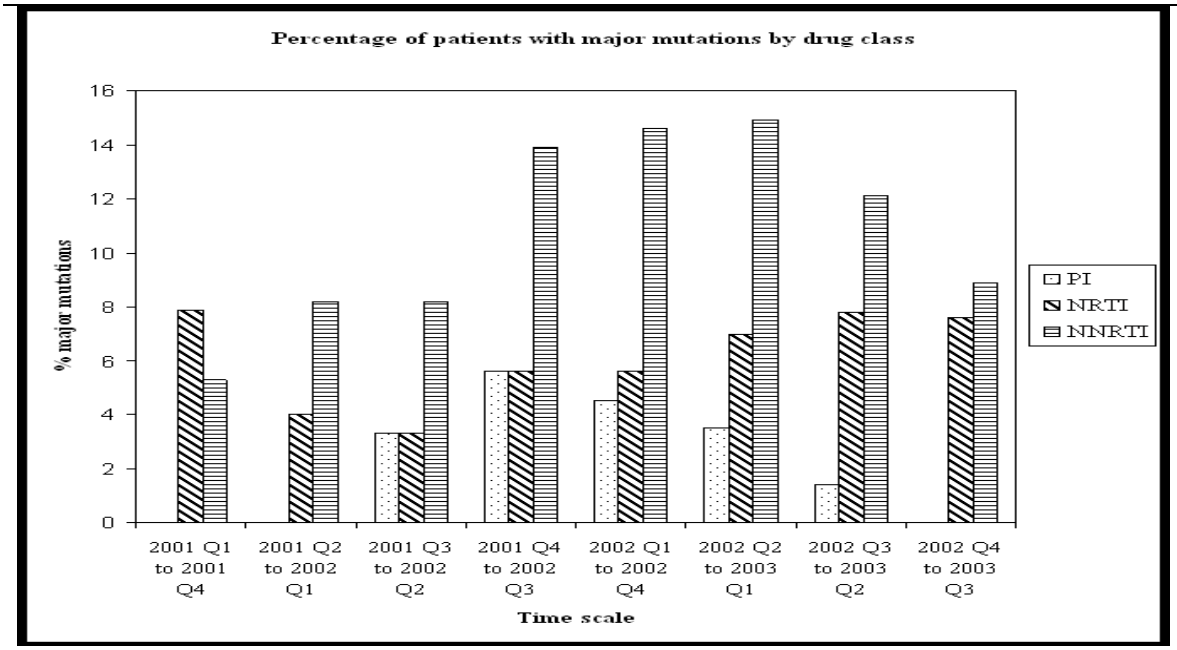
---

van de Vijver DAMC, Wensing AMJ, Op de Coul E, Angarano G, Asjo B, Balotta C, Boeri E, Camacho R, Chaix ML, Costagliola D, de Luca A, Maljkovic I, de Mendoza C, Derdelinck I, Grossman Z, Hamouda O, Hatzakis A, Hoepelman IM, Hemmer R, Horban A, Korn K, Kucherer C, Leitner T, Loveday C, **MacRae E**, Nielsen C, Ormaasen V, Perrin L, Paraskevis D, Puchhammer-Stockl E, Ruiz L, Salminen M, Schmit JCC, Schneider F, Schurrmann R, Soriano V, Stanczak G, Stanojevic M, Vandamme AM, Van Laethem K, Violin M, Wilbe K, Yerly S, Zazzi M, and Boucher CAB on behalf of the SPREAD Programme. **Increasing prevalence of HIV-1 non-B subtypes across Europe from 1996-1999 to 2000-2002; results from the CATCH-study** [Abstract]. In 2nd European HIV Drug Resistance Workshop: 2004 March 11-13; Rome, Italy. Abstract number 1.3

Loveday C, **MacRae E**, Johnson M and on behalf of the ICVC Collaborative Research Group. **A Dynamic Analysis that Allows More Accurate Estimates of the Prevalence of Mutations in ART-Naïve HIV/AIDS Patients in a UK Cohort (January 2001 to Date) [**Abstract]. In 11th Conference on Retroviruses and Opportunistic Infections; 2004 February 8-11; Moscone West, San Francisco, CA, USA. Abstract number 687. *[*Awarded Young Investigator Award]*

Background: The ICVC undertakes the virological care of over 7000 patients in 37 clinical centres across the UK. The annual prevalence of ART mutations in the drug-naïve patients submitted for sequencing has been over 20% from 2001 to 2003. This study uses a sliding dynamic analysis to normalise year on year variations and allow more accurate estimations of resistance in ART-naïve patients from 2001 to date.
Methods: All ART-naïve patients who had genotypic resistance tests performed as part of their clinical care were included (VGI TRUGENE HIV-1 - Bayer Diagnostics). Mutations derived from the rules based algorithm (Genelibrarian report) were analysed. Prevalence points were generated for 4 annual quarters commencing in 2001 and advancing by 3-month intervals to date to provide 8 'annual' comparisons of resistance prevalence. Data were analysed using SPSS 11.5 (**www.spss.com**). Chi-square tests were used to analyse significant differences between major mutations present at the different time-scales.
Results: 252 ART-naïve patients were included. The majority were male (n=184,73%) mean age 36 years (range 18-74). Chronic naïve (n=174,69%), PHI (n=58,23%) and pregnant naïve (n=20,8%) were all represented. Patients derived from Europe (n=133, 57%), Africa (n=84, 33%) and other developing countries (n=35, 10%). 18% (n=45) were subtype NB with representation of most subtypes. 183/252 ART-naïve patients screened had mutations (excluding 40 with L63P only), 29 patients had no mutations. Analysis revealed a gradual and significant rising prevalence (24.7%) of all major mutations to 2002 (Q1-4) but a more recent decline (16.5%) to date.

As presented in the graph, major mutations conferring NNRTI resistance increased up to end 2002 (from 5.3% at 2001 Q1-4 to 14.6% at 2002 Q1-4), with prevalence now declining.



Prevalence of NRTI mutations decreased between 2001 Q2 and 2002 Q2 but has increased and remained at a steady rate (7.5%). PI mutations are evident at low prevalence rates between 2001 Q3 and 2003 Q2 and are absent at the most recent time point. No significant differences in major mutations across the time-scales were observed. Of note, our data showed that NB subtypes were over-represented in patients with major mutations.

Conclusions: A dynamic analysis of resistance data revealed a rising prevalence to 2002 but a more recent decline by end of 2003. This method allows detailed temporal analysis of single mutations and subtypes involved.

2003

MacRae E, Loveday C, Johnson M and on behalf of the ICVC Collaborative Research Group. The rising prevalence of ART resistance mutations in naïve patients with HIV/AIDS in a UK cohort [Abstract]. In 9th European AIDS Conference (EACS); 2003 October 25-29; Warsaw, Poland. Abstract number 3.1/6

Background: ICVC collaborates with 41 clinical centres across Greater London, rural England/Wales (>7000 patients with HIV/AIDS) and recommends the use of ART resistance testing for naïve patients, since establishing a significant increase in mutations in the cohort between 2000 and 2002.

Objectives: To audit the prevalence of ART mutations in naïve patients from June 2002 to June 2003.

Methods: All ART-naïve patients submitted for genotypic sequencing during this period are included. The VGI TRUGENE HIV-1 (Bayer Diagnostics) test was used to determine resistance. Clinical, virological data, mutations (derived from VGI report - GeneLibrarian) and polymorphisms were analysed.

Results: 129 naïve patients were tested (n = 35 PHI, 83 chronic naïve, 11 pregnant). Patient demographics showed majority male (M91:F38); mean age 35.5 years (range 18-65); risk groups: MSM (35%), heterosexual (19%), pregnant (9%),

bisexual (1%), IDU (1%); country of origin: Europe (44%), Africa (28%), Asia, Australasia, North and South America, Middle East (all 1%).
Eighty-eight presented with mutations associated with ART (excluding 23 with L63P only). From the 88, 19 had at least one major mutation, including: RT – K65R (n:1), D67N (n:1), F77L (n:1), A98S (n:1), K101E/Q (n:3), K103N (n:7), Y115F (n:1), V179D/E(n:6), Y181C (n:2), K219Q (n:1); PR – D30N (n:1), N88D (n:1). Prevalence rate – 22%.
Minor mutations included RT – V118I (n:4), T215D (n:1); PR – L10I/V/R (n:26), K20R/M (n:11), M36I/L/T (n:57), L63P/T (n:61) A71V/T (n:15). Sixteen presented with three or more of these minor mutations.
Conclusions: Mutations involving all 3 drug classes prior to ART are evident here. Surveillance is continuing.

Kinloch S, **MacRae E**, Johnson MA and Loveday C. **Horizontal transmission of NNRTI resistance between two patients with recent HIV infection** [Abstract]. In 9th European AIDS Conference (EACS); 2003 October 25-29; Warsaw, Poland. Abstract number 3.1/7

Background: NNRTIs have been used increasingly in first-line therapy in recent years. Resistance to such antiviral drugs is one of the important factors leading to future HAART failure.
Objectives: To describe horizontal transmission of NNRTI resistance between two recently HIV infected patients, naive to HAART.
Methods: Two patients diagnosed at the ICDC were submitted for routine genotypic ART resistance, using the VGI TRUGENE test (Bayer Diagnostics). Clinical, virological data, mutations (derived from VGI report - GeneLibrarian) and polymorphisms were analysed.
Results: Two patients (A and B) with recent HIV infection were diagnosed with a K103N mutation. Both partners had been in a relationship during the six months preceding HIV seroconversion in patient B. Patient A was diagnosed with primary HIV infection (PHI) in June 02 at another London hospital, was naive to HAART, and referred himself to our centre in Aug 02. His genotyping assay showed an isolated K103N. Patient B was first seen on 27.09.02 with a history of a negative HIV test four weeks previously, symptoms consistent with PHI, HIV viraemia > 750.000 c/mL and CD4 count at 422 cells/mm3 (22%). Genotyping showed a similar resistance pattern as Patient A, with only one change observed (polymorphism T165T/I). Sequencing showed there was <1% difference between the two strains suggesting a strong epidemiological link between the two patients.
Conclusions: Horizontal transmission of K103N occurred in these patients naive to HAART. Incidence of NNRTI resistance should continue to be routinely monitored in recently-infected patients as it has major implications in the choice of first-line therapy.

Wensing A, van de Vijver D, Angarano G, Asjo B, Balotta C, Boeri E, Camacho R, De Luca A, de Mendoza C, Derdelinck I, Grossman Z, Hamouda O, Hatzakis A, Hemmer R, Hoepelman A, Horban A, Korn K, Kucherer C, Leitner T, Loveday C, **MacRae E**, Maljkovic I, Nielsen C, Ormaasen V, Perrin L, Paraskevis D, Puchhammer E, Ruiz L, Salminen M, Schmit J, Schneider F, Schurrmann R, Soriano V, Stanczak G, Stanojevic M, Vandamme A, Van Laethem K, Violin M, Wilbe K, Yerly S, Zazzi M, Boucher C. **Drug susceptibility patterns in 195 European**

patients de novo infected with drug resistant virus, implications for Post-Exposure Prophylaxis [Abstract]. In 9th European AIDS Conference (EACS); 2003 October 25-29; Warsaw, Poland. Abstract number LBF 12/1

Loveday C, **MacRae ED**, Johnson M. **A marked increase in prevalence of ART mutations in naïve patients with HIV / AIDS in the UK in 2002 – time to recommend resistance testing prior to therapy**. [Abstract] In 10th Conference on Retroviruses and Opportunistic Infections; 2003 February 10-14; Boston, Massachusetts, USA. Abstract number 632.

References

Arnold, C., Barlow, K.L., Kaye, S., Loveday, C., Balfe, P. and Clewley, J.P. (1995) HIV type 1 sequence subtype G transmission from mother to infant: failure of variant sequence species to amplify in the Roche Amplicor Test. *AIDS Research and Human Retroviruses*, 11(8), p. 999-1001.

Bacheler, L., Jeffrey, S., Hanna, G., D'Aquila, R., Wallace, L., Logue, K., Cordova, B., Hertogs, K., Larder, B., Buckery, R., Baker, D., Gallagher, K., Scarnati, H., Tritch, R. and Rizzo, C. (2001) Genotypic correlates of phenotypic resistance to efavirenz in virus isolates from patients failing nonnucleoside reverse transcriptase inhibitor therapy. *Journal of Virology*, 75(11), p. 4999-5008.

Bärnighausen, T., Eyal, N. and Wikler, D. (2014) HIV treatment-as-prevention research at a crossroads. *PLoS Medicine*, 11(6), e1001654. Available at: doi:10.1371/journal.pmed.1001654 [Accessed 01/12/2015].

Barré-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C. and Axler-Blin, C. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk of acquired immune deficiency syndrome (AIDS). *Science*, 220(4599), p. 868-871.

Bayer HealthCare LLC. (2003) *TRUGENE® HIV-1 Module for Guidelines™ Rules 8.0 (2003-04-15).* Berkeley: Bayer HealthCare LLC.

Berger, E.A., Murphy, P.M. and Farber, J.M. (1999) Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism and disease. *Annual Review of Immunology*, 17, p. 657-700.

Brennan, R.O. and Durack, D.T. (1981) 'Gay compromise syndrome'. *The Lancet*, 318(8259), p. 1338-1339.

Buchbinder, S.P., Mehrotra, D.V., Duerr, A., Fitzgerald, D.W., Mogg, R., Li, D., Gilbert, P.B., Lama, J.R., Marmor, M., del Rio, C., McElrath, M.J., Casimiro, D.R., Gottesdiener, K.M., Chodakewitz, J.A., Corey, L. and Robertson, M.N., the Step Study Protocol Team. (2008) Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *The Lancet*, 372(9653), p. 1881-1893.

Buonaguro, L., Tornesello, M.L. and Buonaguro, F.M. (2007) Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: opathogenetic and therapeutic implications. *Journal of Virology*, 81(19), p. 10209-10219.

Calvez, V. (2001) Resistance. In: Larder, B., Richman, D. and Vella, S. (eds) *HIV Resistance and Implications for Therapy (2nd edition)*. USA: Medicom, p. 2.1.1-2.9.4.

Cameron, D.W., Heath-Chiozzi, M., Danner, S., Cohen, C., Kravcik, S., Maurath, C., Sun, E., Henry, D., Rode, R., Potthoff, A., Leonard, J., for the Advanced HIV Disease Ritonavir Study Group (1998) Randomised placebo-controlled trial of ritonavir in advanced HIV-1 disease. *The Lancet*, 351(9102), p. 543-549.

References

Cane, P., Chrystie, I., Dunn, D., Evans, B., Geretti, A.M., Green, H., Phillips, A., Pillay, D., Porter, K., Pozniak, A., Sabin, C., Smit, E., Weber, J., Zuckerman, M. and Hill, T. (2005) Time trends in primary resistance to HIV drugs in the United Kingdom: multicentre observational study. *British Medical Journal*, 331(7529), p. 1368-1371.

Carr, J.K., Salminen, M.O., Koch, C., Gotte, D., Artenstein, A.W., Hegerich, P.A., St Louis, D., Burke, D.S. and McCutchan, F.E. (1996) Full-length sequence and mosaic structure of a human immunodeficiency virus type 1 isolate from Thailand. *Journal of Virology*, 70(9), p. 5935-5943.

Centers for Disease Control and Prevention. (1981a) Kaposi's sarcoma and *Pneumocystis* pneumonia among homosexual men--New York City and California. *Morbidity and Mortality Weekly Report*, 30(25), p. 305-308.

Centers for Disease Control and Prevention. (1981b) Follow-up on Kaposi's sarcoma and *Pneumocystis* pneumonia. *Morbidity and Mortality Weekly Report*, 30(33), p. 409-410.

Centers for Disease Control and Prevention. (1982) A cluster of Kaposi's Sarcoma and *Pneumocystis carinii* pneumonia among homosexual male residents of Los Angeles and range Counties, California. *Morbidity and Mortality Weekly Report*, June 18, 31(23), p. 305-307.

Centers for Disease Control and Prevention. (1982a) Epidemiologic notes and reports *Pneumocystis carinii* pneumonia among persons with hemophilia A. *Morbidity and Mortality Weekly Report*, July 16, 31(27), p. 365-367.

Centers for Disease Control and Prevention. (1982b) Current trends update on acquired immune deficiency syndrome (AIDS), United States. *Morbidity and Mortality Weekly Report*, September 24, 31(37), p. 507-508, 513-514.

Centers for Disease Control and Prevention. (1982c) Epidemiologic notes and reports possible transfusion-associated acquired immune deficiency syndrome (AIDS), California. *Morbidity and Mortality Weekly Report,* December 10, 31(48), p. 652-654.

Centers for Disease Control and Prevention. (1982d) Unexplained immunodeficiency and opportunistic infections in infants, New York, New Jersey, California. *Morbidity and Mortality Weekly Report,* December 17, 31(49), p. 665-667.

Centers for Disease Control and Prevention. (1983) Epidemiologic notes and reports immunodeficiency among female sexual partners of males with acquired immune deficiency syndrome (AIDS), New York. *Morbidity and Mortality Weekly Report,* January 7, 31(52), p. 697-698.

Centers for Disease Control and Prevention Task Force on Kaposi's Sarcoma and Opportunistic Infections. (1982) Epidemiologic Aspects of the Current Outbreak of Kaposi's Sarcoma and Opportunistic Infections. *The New England Journal of Medicine*, 306(4), p. 248-252.

References

Charneau, P., Borman, A.M., Quillent, C., Guétard, D., Chamaret, S., Cohen, J., Rémy, G., Montagnier, L. and Clavel, F. (1994) Isolation and envelope sequence of a highly divergent HIV-1 isolate: definition of a new HIV-1 group. *Virology*, 205(1), p. 247-253.

Chesney, M.A., Ickovics, J., Hecht, F.M., Sikipa, G. and Rabkin, J. (1999) Adherence: a necessity for successful HIV combination therapy. *AIDS*, 13 (suppl A) p. S271-S278.

Clavel, F., Guétard, D., Brun-Vezinet, F., Chamaret, S., Rey, M.A., Santos-Ferreira, M.O., Laurent, A.G., Dauguet, C., Katlama, C. and Rouzioux, C. (1986) Isolation of a new human retrovirus from West African patients with AIDS. *Science*, 233(4761), p. 343-346.

Clavel, F., Soriano, V. and Zolopa, A.R. (2004) *HIV infection antiretroviral resistance: scientific basis and recommendations for management.* Paris: Bash Medical Publishing.

Coffin, J.M., Haase, A., Levy, J.A., Montagnier, L., Oroszlan, S. and Teich, N. (1986) Human immunodeficiency viruses. *Science*, 232, p. 697.

Cozzi-Lepri, A., Ruiz, L., Loveday, C., Phillips, A.N., Clotet, B., Reiss, P., Ledergerber, B., Holkmann, C., Staszewski, S., Lundgren, J.D., for the EuroSIDA Study Group. (2005) Thymidine analogue mutation profiles: factors associated with acquiring specific profiles and their impact on the virological response to therapy. *Antiviral Therapy*, 10(7), p. 791-802.

Das, K., Clark, A.D. Jr, Lewi, P.J., Heeres, J., De Jonge, M.R., Koymans, L.M., Vinkers, H.M., Daeyaert, F., Ludovici, D.W., Kukla, M.J., De Corte, B., Kavash, R.W., Ho, C.Y., Ye, H., Lichtenstein, M.A., Andries, K., Pauwels, R., De Béthune, M.P., Boyer, P.L., Clark, P., Hughes, S.H., Janssen, P.A. and Arnold, E. (2004) Roles of Conformational and Positional Adaptability in Structure-Based Design of TMC125-R165335 (Etravirine) and Related Non-nucleoside Reverse Transcriptase Inhibitors That Are Highly Potent and Effective against Wild-Type and Drug-Resistant HIV-1 Variants. *Journal of Medicinal Chemistry*, 47(10), p. 2550–2560.

De Leys, R., Vanderborght, B., Vanden Haesevelde, M., Heyndrickx, L., van Geel, A., Wauters, C., Bernaerts, R., Saman, E., Nijs, P. and Willems, B. (1990) Isolation and partial characterization of an unusual human immunodeficiency retrovirus from two persons of west-central African origin. *Journal of Virology*, 64(3), p. 1207-1216.

Delta Coordinating Committee. (1996) Delta: a randomised double-blind controlled trial comparing combinations of zidovudine plus didanosine or zalcitabine with zidovudine alone in HIV-infected individuals. *The Lancet*, 348(9023), p. 283-291.

De Luca, A. and Perno, C.F. (2003) Impact of different HIV resistance interpretation by distinct systems on clinical utility of resistance testing. *Current Opinion in Infectious Diseases*, 16, p. 573-580.

References

de Oliveira, T., Deforche, K., Cassol, S., Salminen, M., Paraskevis, D., Seebregts, C., Snoeck, J., van Rensburg, E.J., Wensing, A.M.J, van de Vijver, D.A., Boucher, C.A., Camacho, R. and Vandamme A-M. (2005) An automated genotyping system for analysis of HIV-1 and other microbial sequences. *Bioinformatics*, 21(19), p. 3797-3800.

De Mendoza, C., Garrido, C., Corral, A., Ramirez-Olivencia, G., Jimenez-Nacher, I., Zahonero, N., Gonzalez-Lahoz, J. and Soriano, V. (2007). Changing rates and patterns of drug resistance mutations in antiretroviral-experienced HIV-infected patients. *AIDS Research and Human Retroviruses*, 23, p. 879-885.

De Meyer, S., Azijn, H., Surleraux, D., Jochmans, D., Tahri, A., Pauwels, R., Wigerinck, P. and de Béthune, M.P. (2005) TMC114, a novel human immunodeficiency virus type 1 protease inhibitor active against protease inhibitor-resistant viruses, including a broad range of clinical isolates. *Antimicrobial Agents Chemotherapy*, 49, p. 2314–21.

De Meyer, S., Vangeneugden, T., Lefebvre, E., Azijn, H., De Baere, I., Van Baelen, B. and de Bethune, M.P. (2006) Phenotypic and genotypic determination of resistance to TMC114: pooled analysis of POWER 1, 2, and 3 [Abstract 73] *Antiviral Therapy*, 11(5), p. S83.

De Meyer, S., Vangeneugden, T., van Baelen, B., de Paepe, E., van Marck, H., Picchio, G., Lefebvre, E. and de Béthune, M.P. (2008) Resistance profile of darunavir: combined 24-week results from the POWER trials. *AIDS Research and Human Retroviruses*, 24(3), p. 379-388.

Dunbar, J.L., Erlen, J.A., Schlenk, E.A., Ryan, C.M., Sereika, S.M. and Doswell, W.M. (2000) Adherence in chronic disease. *Annual Review of Nursing Research*, 18, p.48-90.

Erickson, J. (2001) Virology. In: Larder, B., Richman, D. and Vella, S. (eds) *HIV Resistance and Implications for Therapy (2nd edition)*. USA: Medicom, p. 1.1.1-1.4.10.

European Medicines Agency (EMEA). (2007) Intelence_Etravirine [Online] http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000900/human_med_000844.jsp&mid=WC0b01ac058001d124 [Accessed: 01/12/2015]

European Medicines Agency (EMEA). (2008) Prezista_Darunavir [Online] http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000707/human_med_000988.jsp&mid=WC0b01ac058001d124 [Accessed: 01/12/2015]

European Medicines Agency (EMEA). (2009) Prezista_Darunavir [Online] http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000707/human_med_000988.jsp&mid=WC0b01ac058001d124 [Accessed: 01/12/2015]

References

Gallego, O., Ruiz, L., Vallejo, A., Ferrer, E., Rubio, A., Clotet, B., Manuel, L., Soriano, V. and the ERASE-3 Group (2001) Changes in the rate of genotypic resistance to antiretroviral drugs in Spain. *AIDS*, 15(14), p. 1894-1896.

Gao, F., Robertson, D.L., Morrison, S.G., Hui, H., Craig, S., Decker, J., Fultz, P.N., Girard, M., Shaw, G.M., Hahn, B.H. and Sharp, P.M. (1996) The heterosexual human immunodeficiency virus type 1 epidemic in Thailand is caused by an intersubtype (A/E) recombinant of African origin. *Journal of Virology*, 70(10), p. 7013-7029.

Gallo, R. and Montagnier, L. (1987) The chronology of AIDS research. *Nature*, 326(6112), p. 435-436.

Gallo, R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.J., Redfield, R., Oleske, J. and Safai, B. (1984) Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk from AIDS. *Science*, 224(4648), p. 500-503.

Gallo, R., Wong-Staal, F., Montagnier, L., Haseltine, W.A. and Yoshida, M. (1988) HIV/HTLV gene nomenclature. *Nature*, 333(6173), p. 504.

Gazzard, B. on behalf of the BHIVA Writing Committee. (2005) British HIV Association (BHIVA) guidelines for the treatment of HIV-infected adults with antiretroviral therapy. *HIV Medicine*, 6(Suppl 2), p. 1–61.

Gazzard, B.G. on behalf of the BHIVA Treatment Guidelines Writing Group. (2008) British HIV Association guidelines for the treatment of HIV-1-infected adults with antiretroviral therapy. *HIV Medicine*, 9(8), p. 563–608.

Gifford, R., de Oliveira, T., Rambaut, A., Myers, R.E., Gale, C.V., Dunn, D., Shafer, R., Vandamme, A.M., Kellam, P., Pillay, D., for the UK Collaborative Group on HIV Drug Resistance. (2006) Assessment of automated genotyping protocols as tools for surveillance of HIV-1 genetic diversity. *AIDS*, 20(11), p. 1521-1529.

Gonzales, M.J., Belitskaya, I., Dupnik, K.M., Rhee, S.Y. and Shafer R.W. (2003) Protease and reverse transcriptase mutation patterns in HIV type 1 isolates from heavily treated persons: comparison of isolates from Northern California with isolates from other regions. *AIDS Research and Human Retroviruses*, 19(10), p. 909-915.

Grossman, Z., Vardinon, N., Chemtob, D., Alkan, M.L., Bentwich, Z., Burke, M., Gottesman, G., Istomin, V., Levi, I., Maayan, S., Shahar, E. and Schapiro, J.M. for the Israeli Multi-Center Study Group. (2001) Genotypic variation of HIV-1 reverse transcriptase and protease: comparative analysis of clade C and clade B. *AIDS*, 15(16), p. 1453-1460.

Gu, Z., Fletcher, R.S., Arts, E.J., Wainberg, M.A. and Parniak, M.A. (1994) The K65R mutant reverse transcriptase of HIV-1 cross-resistant to 2', 3'-dideoxycytidine, 2',3'-dideoxy-3'-thiacytidine, and 2',3'-dideoxyinosine shows reduced sensitivity to specific dideoxynucleoside triphosphate inhibitors in vitro. *The Journal of Biological Chemistry*, 269(45), p. 28118-28122.

References

Gulick, R.M., Mellors, J.W., Havlir, D., Eron, J.J., Gonzalez, C., McMahon, D., Richman, D.D., Valentine, F.T., Jonas, L., Meibohm, A., Emini, E.A. and Chodakewitz, J.A. (1997) Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. *The New England Journal of Medicine*, 337(11), p. 734-739.

Hammer, S.M., Katzenstein, D.A., Hughes, M.D., Gundacker, H., Schooley, R.T., Haubrich, R.H., Henry, W.K., Lederman, M.M., Phair, J.P., Niu, M., Hirsch, M.S., Merigan, T.C., for the AIDS Clinical Trials Group Study 175 Study Team. (1996) A trial comparing nucleoside monotherapy with combination therapy in HIV-infected adults with CD4 cell counts from 200 to 500 per cubic millimeter. *The New England Journal of Medicine*, 335(15), p. 1081-1090.

Hammer, S.M., Squires, K.E., Hughes, M.D., Grimes, J.M., Demeter, L.M., Currier, J.S., Eron, J.J. Jr, Feinberg, J.E., Balfour, H.H. Jr, Deyton, L.R., Chodakewitz, J.A., Fischl, M.A., for the AIDS Clinical Trials Group 320 Study Team. (1997) A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. *The New England Journal of Medicine*, 337(11), p. 725-733.

Hammer, S.M., Saag, M.S., Schechter, M., Montaner, J.S., Schooley, R.T., Jacobsen, D.M., Thompson, M.A., Carpenter, C.C., Fischl, M.A., Gazzard, B.G., Gatell, J.M., Hirsch, M.S., Katzenstein, D.A., Richman, D.D., Vella, S., Yeni, P.G., Volberding, P.A., International AIDS Society-USA panel. (2006) Treatment for adult HIV infection: 2006 recommendations of the International AIDS Society-USA panel. *The Journal of the American Medical Association*, 296(7), p. 827-843.

Haubrich, R.H. (2005) HIV resistance: improving interpretation and recent insights [Online]. Available at: http://www.clinicaloptions.com/hiv/ [Accessed 06/12/2005].

Haubrich, R., Berger, D., Chiliade, P., Colson, A., Conant, M., Gallant, J., Wilkin, T., Nadler, J., Pierone, G., Saag, M., van Baelen, B., Lefebvre, E., for the POWER 2 Study Group. (2007) Week 24 efficacy and safety of TMC114/ritonavir in treatment-experienced HIV patients. *AIDS*, 21(6), F11-18.

Hemelaar, J., Gouws, E., Ghys, P.D., Osmanov, S. and WHO-UNAIDS Network for HIV Isolation and Characterisation. (2011) Global trends in molecular epidemiology of HIV-1 during 2000-2007. *AIDS*, 25(5), p. 679-689.

Henrard, D.R., Phillips, J.F., Muenz, L.R., Blattner, W.A., Wiesner, D., Eyster, M.E. and Goedert, J.J. (1995) Natural history of HIV-1 cell-free viremia. *The Journal of the American Medical Association*, 274(7), p. 554-558.

Hirsch, M.S., Brun-Vézinet, F., D'Aquila, R.T., Hammer, S.M., Johnson, V.A., Kuritzkes, D.R., Loveday, C., Mellors, J.W., Clotet, B., Conway, B., Demeter, L.M., Vella, S., Jacobsen, D.M. and Richman, D.D. (2000) Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel. *The Journal of the American Medical Association*, 283(18), p. 2417-2426.

References

Hirsch, M.S., Conway, B., D'Aquila, R.T., Johnson, V.A., Brun-Vézinet, F., Clotet, B., Demeter, L.M., Hammer, S.M., Jacobsen, D.M., Kuritzkes, D.R., Loveday, C., Mellors, J.W., Vella, S., Richman, D.D., for the International AIDS Society--USA Panel. (1998) Antiretroviral drug resistance testing in adults with HIV infection: implications for clinical management. *The Journal of the American Medical Association*, 279(24), p. 1984-1991.

Hirsch, M.S., Günthard, H.F., Schapiro, J.M., Brun-Vézinet, F., Clotet, B., Hammer, S.M., Johnson, V.A., Kuritzkes, D.R., Mellors, J.W., Pillay, D., Yeni, P.G., Jacobsen, D.M. and Richman, D.D. (2008) Antiretroviral drug resistance testing in adult HIV-1 infection: 2008 recommendations of an International AIDS Society-USA panel. *Clinical Infectious Diseases*, 47(2), p. 266-285.

Hoffmann, C. (2003) HIV Therapy 2003. In: Hoffmann, C. and Kamps, B.S. (eds) *HIV Medicine 2003*. Paris: Flying Publisher, p. 15-44.

Jaffe, H.W., Choi, K., Thomas, P.A., Haverkos, H.W., Auerbach, D.M., Guinan, M.E., Rogers, M.F., Spira, T.J., Darrow, W.W., Kramer, M.A., Friedman, S.M., Monroe, J.M., Friedman-Kien, A.E., Laubenstein, L.J., Marmor, M., Safai, B., Dritz, S.K., Crispi, S.J., Fannin, S.L., Orkwis, J.P., Kelter, A., Rushing, W.R., Thacker, S.B. and Curran, J.W. (1983) National case-control study of Kaposi's sarcoma and *Pneumocystis carinii* pneumonia in homosexual men: Part 1. Epidemiologic results. *Annals of Internal Medicine*, 99(2), p. 145-151.

Jayaweera, D.T., Espinoza, L. and Castro, J. (2008) Etravirine: the renaissance of non-nucleoside reverse transcriptase inhibitors. *Expert Opinion on Pharmacotherapy*, 9(17), p. 3083-3094.

Johnson, V.A., Brun-Vezinet, F., Clotet, B., Gunthard, H.F., Kuritzkes, D.R., Pillay, D., Schapiro, J.M. and Richman, D.D. (2009) Update of the Drug Resistance Mutations in HIV-1: December 2009. *Topics in HIV Medicine*, 17(5), p. 138-145.

Joint United Nations Programme on HIV/AIDS (UNAIDS). (2013) *UNAIDS Report on the Global AIDS Epidemic 2013* [Online] Available at: http://www.unaids.org/sites/default/files/media_asset/UNAIDS_Global_Report_2013_en_1.pdf [Accessed: 01/12/2015].

Kadiu, I. and Gendelman, H.E. (2011) Macrophage bridging conduit trafficking of HIV-1 through the endoplasmic reticulum and golgi network. *Journal of Proteome Research*, 10, p. 3225-3238.

Katlama, C., Campbell, T., Clotet, B., for the DUET-2 study group. (2007a) DUET-2: 24 week results of a phase III randomized double-blind trial to evaluate the efficacy and safety of TMC125 versus placebo in 591 treatment-experienced HIV-1 infected patients [Abstract]. In 4th IAS Conference on HIV Pathogenesis, Treatment and Prevention; July 22-25, 2007; Sydney, Australia. Abstract WESS204:2.

Katlama, C., Esposito, R., Gatell, J.M., Goffard, J.C., Grinsztejn, B., Pozniak, A., Rockstroh, J., Stoehr, A., Vetter, N., Yeni, P., Parys, W., Vangeneugden, T., for

the POWER 1 study group. (2007b) Efficacy and safety of TMC114/ritonavir in treatment-experienced HIV patients: 24-week results of POWER 1. *AIDS*, 21(4), p. 395-402.
Katlama, C., Haubrich, R., Lalezari, J., Lazzarin, A., Madruga, J.V., Molina, J.M., Schechter, M., Peeters, M., Picchio, G., Vingerhoets, J., Woodfall, B., De Smedt, G., for the DUET-1, DUET-2 study groups. (2009) Efficacy and safety of etravirine in treatment-experienced, HIV-1 patients: pooled 48 week analysis of two randomized, controlled trials. *AIDS*, 23(17), p. 2289-2300.

Kaye, S., Loveday, C. and Tedder, R.S. (1992) A microtitre format point mutation assay: Application to the detection of drug resistance in human immunodeficiency virus type-1 infected patients treated with zidovudine. *Journal of Medical Virology*, 37(4), p. 241-246.

Kinloch, S., MacRae, E., Johnson, M.A. and Loveday, C. (2003) Horizontal transmission of NNRTI resistance between two patients with recent HIV infection [Abstract]. In 9th European AIDS Conference (EACS); October 25-29 2003; Warsaw, Poland. Abstract number 3.1/7.

Konou, A.A., Dagnra, A.Y., Vidal, N., Salou, M., Adam, Z., Singo-tokofai, A., Delaporte, E., Prince-david, M. and Peeters, M. (2015) Alarming rates of virological failure and drug resistance in patients on long-term antiretroviral treatment in routine HIV clinics in Togo. *AIDS*, 29(18), p. 2527-2530.

Kouyos, R.D. and Günthard, H.F. (2015) Editorial Commentary: The Irreversibility of HIV Drug Resistance. *Clinical Infectious Diseases*, 61(5), p. 837-839.

Lambotte, O., Chaix, M-L., Gubler, B., Nasreddine, N., Wallon, C., Goujard, C., Rouzioux, C., Taoufik, Y. and Delfraissy, J-F. (2004) The lymphocyte HIV reservoir in patients on long-term HAART is a memory of virus evolution. *AIDS*, 18(8), p. 1147-1158.

Larder, B.A. and Kemp, S.D. (1989) Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to Zidovudine (AZT). *Science*, 246(4934), p. 1155-1158.

Larder, B.A., Kemp, S.D. and Harrigan, R. (1995) Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. *Science*, 269(5224), p. 696-699.

Lathouwers, E., Gupta, S., Haddad, M., Paquet, A., de Meyer, S. and Baugh, B. (2015) Trends in darunavir resistance-associated mutations and phenotypic resistance in commercially tested United States clinical samples between 2006 and 2012. *AIDS Research and Human Retroviruses*, 31(6), p. 628-635.

Lau, B., Gange, S.J. and Moore, R.D. (2007) Interval and Clinical Cohort Studies: Epidemiological Issues. *AIDS Research and Human Retroviruses*, 23(6), p. 769-776.

References

Lazzarin, A., Campbell, T., Clotet, B., Johnson, M., Katlama, C., Moll, A., Towner, W., Trottier, B., Peeters, M., Vingerhoets, J., de Smedt, G., Baeten, B., Beets, G., Sinha, R., Woodfall, B., for the DUET-2 study group. (2007) Efficacy and safety of TMC125 (etravirine) in treatment-experienced HIV-1-infected patients in DUET-2: 24-week results from a randomised, double-blind, placebo-controlled trial. *Lancet*, 370(9581), p. 39-48.

Levy, J.A., Hoffmann, A.D., Kramer, S.M., Landis, J.A., Shimabukuro, J.M. and Oshiro, L.S. (1984) Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science*, 225(4664), p. 840-842.

Llibre, J.M, Santos, J.R., Puig, T., Moltó, J., Ruiz, L., Paredes, R. and Clotet, B. (2008) Prevalence of etravirine-associated mutations in clinical samples with resistance to nevirapine and efavirenz. *Journal of Antimicrobial Chemotherapy*, 62(5), p. 909-913.

Los Alamos National Laboratory. (2015) *HIV Circulating Recombinant Forms (CRFs)* [Online] Available at: http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html [Accessed 10/04/2015].

Loveday, C., Devereux, H., Huckett, L., Gundlach, T. and Johnson, M. (1999) High prevalence of genotypic changes in antiretroviral drug-naïve patients in UK [Abstract]. *Antiretroviral Therapy*, 4(Suppl 1), A99.

Loveday, C., MacRae, E.D. and Johnson, M. (2003) A marked increase in prevalence of ART mutations in naïve patients with HIV / AIDS in the UK in 2002 – time to recommend resistance testing prior to therapy. [Abstract] In 10th Conference on Retroviruses and Opportunistic Infections; February 10-14 2003; Boston, Massachusetts, USA. Abstract number 632.

MacRae, E., Loveday, C., Johnson, M. and on behalf of the ICVC Collaborative Research Group. (2003) The rising prevalence of ART resistance mutations in naïve patients with HIV/AIDS in a UK cohort [Abstract]. In 9th European AIDS Conference (EACS); October 25-29 2003; Warsaw, Poland. Abstract number 3.1/6

Madruga, J.V., Cahn, P., Grinsztejn, B., Haubrich, R., Lalezari, J., Mills, A., Pialoux, G., Wilkin, T., Peeters, M., Vingerhoets, J., de Smedt, G., Leopold, L., Trefiglio, R., Woodfall, B., for the DUET-1 study group. (2007) Efficacy and safety of TMC125 (etravirine) in treatment-experienced HIV-1-infected patients in DUET-1: 24-week results from a randomised, double-blind, placebo-controlled trial. *Lancet*, 370(9581), p. 29-38.

Margot, N.A., Isaacson, E., McGowan, I., Cheng, A.K., Schooley, R.T. and Miller, M.D. (2002) Genotypic and phenotypic analyses of HIV-1 in antiretroviral-experienced patients treated with tenofovir DF. *AIDS*, 16(9), p. 1227-1235.

Masur, H., Michelis, M.A., Greene, J.B., Onorato, I., Stouwe, R.A., Holzman, R.S., Wormser, G., Brettman, L., Lange, M., Murray, H.W. and Cunnigham-Rundles, S. (1981) An outbreak of community acquired *Pneumocystis carinii* pneumonia: initial

manifestation of cellular immune dysfunction. *The New England Journal of Medicine*, 305(24), p. 1431-1438.

Mellors, J.W., Rinaldo, C.R. Jr, Gupta, P., White, R.M., Todd, J.A. and Kingsley, L.A. (1996) Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science*, 272(5265), p. 1167-1170.

Miller, V., Ait-Khaled, M., Stone, C., Griffin, P., Mesogiti, D., Cutrell, A., Harrigan, R., Staszewski, S., Katlama, C., Pearce, G. and Tisdale, M. (2000) HIV-1 reverse transcriptase (RT) genotype and susceptibility to RT inhibitors during abacavir monotherapy and combination therapy. *AIDS*, 14(2), p. 163-171.

Mitsuya, Y., Liu, T.F., Rhee, S-Y., Fessel, W.J. and Shafer, R.W. (2007) Prevalence of darunavir resistance-associated mutations: patterns of occurrence and association with past treatment. *The Journal of Infectious Diseases*, 196(8), p. 1177-1179.

Mitsuya, Y., Winters, M.A., Fessel, J., Rhee, S-Y., Hurley, L., Horberg, M., Schiffer, C.A., Zolopa, A.R. and Shafer, R.W. (2006) N88D facilitates the co-occurrence of D30N and L90M and the development of multidrug resistance in HIV Type 1 protease following nelfinavir treatment failure. *AIDS Research and Human Retroviruses*, 22(12), p. 1300-1305.

Mortimer, P.P. and Loveday, C. (2001) The virus and the tests. In: Adler, M.W. (ed) *ABC of AIDS (5th edition)*. London: BMJ Publishing Group, p. 6-11.

Myers, R.E., Gale, C.V., Harrison, A., Takeuchi, Y. and Kellam, P. (2005) A statistical model for HIV-1 sequence classification using the subtype analyser (STAR). *Bioinformatics*, 21(17), p. 3535-3540.

National AIDS Manual. (2014) *Antiretroviral drug chart* [Online] Available at: http://www.aidsmap.com/v635494203890000000/file/1187469/drug_chart_october_2014_web.pdf [Accessed: 01/12/2015].

Ortiz, R., Dejesus, E., Khanlou, H., Voronin, E., van Lunzen, J., Andrade-Villanueva, J., Fourie, J., De Meyer, S., De Pauw, M., Lefebvre, E., Vangeneugden, T. and Spinosa-Guzman, S. (2008) Efficacy and safety of once-daily darunavir/ritonavir versus lopinavir/ritonavir in treatment-naive HIV-1-infected patients at week 48. *AIDS*, 22(12), p. 1389-1397.

Palca, J. (1987) Settlement on AIDS finally reached between US and Pasteur. *Nature*, 326(6112), p. 533.

Paredes, R. and Clotet, B. (2009) Clinical management of HIV-1 resistance. *Antiviral Research*, 85(1), p. 245-265.

Paterson, D.L., Swindells, S., Mohr, J., Brester, M., Vergis, E.N., Squier, C. and Singh, N. (2000) Adherence to protease inhibitor therapy and outcomes in patients with HIV infection. *Annals of Internal Medicine*, 133(1), p. 21-30.

References

Perelson, A.S., Neumann, A.U., Markowitz, M., Leonard, J.M. and Ho, D.D. (1996) HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. *Science*, 271(5255), p. 1582-1586.

Picchio, G., Vingerhoets, J., Staes, M., Tambuyzer, L., Bacheler, L., Pattery, T. and de Bethune, M-P. (2008) Prevalence of etravirine (ETR, TMC125) resistance-associated mutations in a large panel of clinical isolates. [Abstract] In 15th Conference on Retroviruses and Opportunistic Infections; February 3-6 2008; Boston, Massachusetts, USA. Abstract number 866.

Pillay, D., Green, H., Matthias, R., Dunn, D., Phillips, A., Sabin, C., Evans, B.; UK Collaborative Group on HIV Drug Resistance. (2005) Estimating HIV-1 drug resistance in antiretroviral-treated individuals in the United Kingdom. *Journal of Infectious Diseases,* 192(6), p. 967-73.

Pineda-Peña, A.C., Faria, N.R., Imbrechts, S., Libin, P., Abecasis, A.B., Deforche, K., Gómez-López, A., Camacho, R.J., de Oliveira, T. and Vandamme, A.M. (2013) Automated subtyping of HIV-1 genetic sequences for clinical and surveillance purposes: performance evaluation of the new REGA version 3 and seven other tools*. Infection, Genetics and Evolution*, 19, p. 337-348.

Plantier, J-C., Leoz, M., Dickerson, J.E., De Oliveira, F., Cordonnier, F., Lemée, V., Damond, F., Robertson, D.L, and Simon, F. (2009) A new human immunodeficiency virus derived from gorillas. *Nature Medicine*, 15(8), p. 871-872.

Pratt, R. J. (2003) *HIV & AIDS: A foundation for nursing and healthcare practice* (5th edition). London: Arnold, a member of the Hodder Headline Group.

Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., Premsri, N., Namwat, C., de Souza, M., Adams, E., Benenson, M., Gurunathan, S., Tartaglia, J., McNeil, J.G., Francis, D.P., Stablein, D., Birx, D.L., Chunsuttiwat, S., Khamboonruang, C., Thongcharoen, P., Robb, M.L., Michael, N.L., Kunasol, P., Kim, J.H., for the MOPH-TAVEG Investigators. (2009) Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *The New England Journal of Medicine*, 361(23), p. 2209-2220.

Rhee, S.Y., Gonzales, M.J., Kantor, R., Betts, B.J., Ravela, J. and Shafer, R.W. (2003) Human immunodeficiency virus reverse transcriptase and protease sequence database. *Nucleic Acids Research*, 31(1), p. 298-303.

Robertson, D.L., Anderson, J.P., Bradac, J.A., Carr, J.K., Foley, B., Funkhouser, R.K., Gao, F., Hahn, B.H., Kuiken, C., Learn, G.H., Leitner, T., McCutchan, F., Osmanov, S., Peeters, M., Pieniazek, D., Kalish, M.L., Salminen, M., Sharp, P.M., Wolinsky, S. and Korber, B. (1999) HIV-1 Nomenclature Proposal. In: Kuiken, C.L., Foley, B., Hahn, B., Korber, B., McCutchan, F., Marx, P.A., Mellors, J.W., Mullins, J.I., Sodroski, J. and Wolinsky, S. (eds) *Human Retroviruses and AIDS 1999.* Los Alamos National Laboratory: Theoretical Biology and Biophysics Group, p. 492-505.

Robertson, D.L., Sharp, P.M., McCutchan, F.E. and Hahn, B.H. (1995) Recombination in HIV-1. *Nature*, 374(6518), p. 124-126.

References

Rogers, M.F., Morens, D.M., Stewart, J.A., Kaminski, R.M., Spira, T.J., Feorino, P.M., Larsen, S.A., Francis, D.P., Wilson, M. and Kaufman, L. (1983) National Case-Control Study of Kaposi's Sarcoma and *Pneumocystis carinii* pneumonia in Homosexual Men: Part 2, Laboratory Results. *Annals of Internal Medicine*, 99(2), p. 151-158.

Rozanov, M., Plikat, U., Chappey, C., Kochergin, A. and Tatusova, T. (2004) A web-based genotyping resource for viral sequences. *Nucleic Acids Research*, 32, 654-659.

Rubbert, A. and Ostrowski, M. (2003) Pathogenesis of HIV-1 Infection. In: Hoffmann, C. and Kamps, B.S. (eds) *HIV Medicine 2003*. Paris: Flying Publisher, p. 15-44.

Rusconi, S., Gianotti, N., Adorni, F., Boeri, E., Menzo, S., Gonnelli, A., Micheli, V., Meraviglia, P., Trezzi, M., Paolini, E., Giacometti, A., Corsi, P., Di Pietro, M., Monno, L., Punzi, G., Zazzi, M., for the ARCA Collaborative Group. (2007) Determinants of HIV-1 genotypic resistance to darunavir (TMC114) in a large Italian resistance database (Antiretroviral Resistance Cohort Analysis). *Journal of Acquired Immune Deficiency Syndrome*, 46(3), p. 373-375.

Salzwedel, K., Martin, D.E. and Sakalian, M. (2007) Maturation inhibitors: a new therapeutic class targets the virus structure. *AIDS Reviews*, 9(3), p. 162-172.

Scott, C., Grover, D. and Nelson, M. (2008) Is there a role for etravirine in patients with Nonnucleoside reverse transcriptase inhibitor resistance? *AIDS*, 22(8), p. 989–992.

Shafer, R.W. and Schapiro, J.M. (2008) HIV-1 drug resistance mutations: an updated framework for the second decade of HAART. *AIDS Reviews*, 10(2), p. 67-84.

Semple, M., Kaye, S., Loveday, C. and Tedder, R. (1993) HIV-1 plasma viraemia quantification: a non-culture measurement needed for clinical trials. *Journal of Virological Methods*, 41(2), p. 167-179.

Semple, M., Loveday, C., Weller, I. and Tedder, R. (1991) Direct measurement of viraemia in patients infected with HIV-1 and its relationship to disease progression and zidovudine therapy. *Journal of Medical Virology*, 35(1), p. 38-45.

Simon, F., Mauclère, P., Roques, P., Loussert-Ajaka, I., Müller-Trutwin, M.C., Saragosti, S., Georges-Courbot, M.C., Barré-Sinoussi, F. and Brun-Vézinet, F. (1998) Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nature Medicine*, 4(9), p. 1032-1037.

Spence, R.A., Kati, W.M., Anderson, K.S. and Johnson, K.A. (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science*, 267(5200), p. 988-993.

References

Tamalet, C., Fantini, J., Tourres, C. and Nouara, Y. (2003) Resistance of HIV-1 to multiple antiretroviral drugs in France: a 6-year survey (1997–2002) based on an analysis of over 7000 genotypes. *AIDS*, 17(16), p. 2383-2388.

The Antiretroviral Therapy Cohort Collaboration. (2008) Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *The Lancet*, 372(9635), p. 293-299.

The rgp120 HIV Vaccine Study Group. (2005) Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *The Journal of Infectious Diseases*, 191(5), p. 654-665.

The UK Collaborative Group for HIV and STI Surveillance. (2007) *Testing Times. HIV and other Sexually Transmitted Infections in the United Kingdom: 2007.* London: Health Protection Agency, Centre for Infections.

Torre, D. and Tambini, R. (2002) Antiretroviral drug resistance testing in patients with HIV-1 infection: a meta-analysis study. *HIV Clinical Trials*, 3(1), p. 1-8.

Townsend, C.L., Cliffe, S. and Tookey, P.A. (2006) Uptake of antenatal HIV testing in the United Kingdom: 2000–2003. *Journal of Public Health*, 28(3), p. 248-252.

Vandamme, A.M., Sönnerborg, A., Ait-Khaled, M., Albert, J., Asjo, B., Bacheler, L., Banhegyi, D., Boucher, C., Brun-Vézinet, F., Camacho, R., Clevenbergh, P., Clumeck, N., Dedes, N., De Luca, A., Doerr, H.W., Faudon, J-L., Gatti, G., Gerstoft, J., Hall, W.W., Hatzakis, A., Hellmann, N., Horban, A., Lundgren, J.D., Kempf, D., Miller, M., Miller, V., Myers, T.W., Nielsen, C., Opravil, M., Palmisano, L., Perno, C.F., Phillips, A., Pillay, D., Pumarola, T., Ruiz, L., Salminen, M., Schapiro, J., Schmidt, B., Schmit, J-C., Schuurman, R., Shulse, E., Soriano, V., Staszewski, S., Vella, S., Youle, M., Ziermann, R. and Perrin, L. (2004) Updated European recommendations for the clinical use of HIV drug resistance testing. *Antiviral Therapy* 9, p. 829–848.

Vingerhoets, J., Buelens, A., Peeters, M., Picchio, G., Tambuyzer, L., Van Marck, H., de Smedt, G., Woodfall, B. and de Béthune, M.P. (2007) Impact of baseline NNRTI mutations on the virological response to TMC125 in the Phase III clinical trials DUET-1 and DUET-2 [Abstract #32]. *Antiviral Therapy*, 12(Suppl 1), S34.

Vingerhoets, J., Peeters, M., Azijn, H., Tambuyzer, L., Hoogstoel, A., Nijs, S., de Béthune, M-P. and Picchio, G. (2008) An update of the list of NNRTI mutations associated with decreased virologic response to etravirine (ETR): multivariate analyses on the pooled DUET-1 and DUET-2 clinical trial data [Abstract 24]. *Antiviral Therapy*, 13(Suppl 3), A26.

Vingerhoets, J, Tambuyzer, L., Azijn, H., Hoogstoel, A., Nijs, S., Peeters, M., de Béthune, M.P., De Smedt, G., Woodfall, B. and Picchio, G. (2010) Resistance profile of etravirine: combined analysis of baseline genotypic and phenotypic data from the randomized, controlled Phase III clinical studies. *AIDS*, 24(4), p. 503-514.

References

Visible Genetics Inc. (2001) *Introducing the TRUGENE™ HIV-1 genotyping test. A total genotyping system designed to give you the complete picture in HIV resistance testing. Solve the puzzle of HIV resistance testing.* Toronto: Visible Genetics Inc.

Vitoria, M., Ford, N., Doherty, M. and Flexner, C. (2014) Simplification of antiretroviral therapy: a necessary step in the public health response to HIV/AIDS in resource-limited settings. *Antiviral Therapy*, 19(Suppl 3), p. 31-37.

Wensing, A.M.J., van de Vijver, D.A.M.C., Angarano, G., Asjo, B., Balotta, C., Boeri, E., Camacho, R., Chaix, M.L., Costagliola, D., de Luca, A., Derdelinck, I., Grossman, Z., Hamouda, O., Hatzakis, A., Hemmer, R., Hoepelman, A., Horban, A., Korn, K., Kucherer, C., Leitner, T., Loveday, C., MacRae, E., Maljkovic, I., de Mendoza, C., Meyer, L., Nielsen, C., Op de Coul, E.L., Ormaasen, V., Paraskevis, D., Perrin, L., Puchhammer-Stockl, E., Ruiz, L., Salminen, M., Schmit, J.C., Schneider, F., Schuurman, R., Soriano, V., Stanczak, G., Stanojevic, M., Vandamme, A.M., Van Laethem, K., Violin, M., Wilbe, K., Yerly, S., Zazzi, M. and Boucher, C.A.B. for the SPREAD Programme. (2005) Prevalence of Drug-Resistant HIV-1 Variants in Untreated Individuals in Europe: Implications for Clinical Management. *Journal of Infectious Diseases*, 192 (6), p. 958-966.

World Health Organisation (WHO) (2013a) *Global update on HIV treatment 2013: Results, Impact and Opportunities.* [Online]. Available at: http://www.who.int/hiv/pub/progressreports/update2013/en/ [Accessed: 01/12/2015].

World Health Organisation (WHO) (2013b) *Consolidated guideline on the use of antiretroviral drugs for treating and preventing HIV infection. Recommendations for a public health approach.* [Online]. Available at: http://www.who.int/hiv/pub/guidelines/arv2013/download/en/ [Accessed: 01/12/2015].

Wiley, C.A., Schrier, R.D., Nelson, J.A., Lampert, P.W. and Oldstone, M.B. (1986) Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. *Proceedings of the National Academy of Sciences of the United States of America*, 83(18), p. 7089-7093.

Williams, I., Churchill, D., Anderson, J., Boffito, M., Bower, M., Cairns, G., Cwynarski, K., Edwards, S., Fidler, S., Fisher, M., Freedman, A., Geretti, A.M., Gilleece, Y., Horne, R., Johnson, M., Khoo, S., Leen, C., Marshall, N., Nelson, M., Orkin, C., Paton, N., Phillips, A., Post, F., Pozniak, A., Sabin, C., Trevelion, R., Ustianowski, A., Walsh, J., Waters, L., Wilkins, E., Winston, A. and Youle, M. (2014) British HIV Association guidelines for the treatment of HIV-1 positive adults with antiretroviral therapy 2012 (updated June 2014). *HIV Medicine*, 15 (Suppl. 1), p. 1–85.

Yin, Z., Brown, A.E., Hughes, G., Nardone, A., Gill, O.N., Delpech, V.C. and contributors. (2014) *HIV in the United Kingdom 2014 Report: data to end 2013.* London: Public Health England.

References

Youree, B.E. and D'Aquila, R.T. (2002) Antiretroviral resistance testing for clinical management. *AIDS Review*, 4(1), p. 3-12.

Zaccarelli, M., Perno, C.F., Forbici, F., Cingolani, A., Liuzzi, G., Bertoli, A., Trotta, M.P., Bellocchi, M.C., Di Giambenedetto, S., Tozzi, V., Gori, C., D'Arrigo, R., De Longis, P., Noto, P., Girardi, E., De Luca, A. and Antinori, A. (2003) Using a database of HIV patients undergoing genotypic resistance test after HAART failure to understand the dynamics of M184V mutation. *Antiviral Therapy*, 8(1), p. 51-56.

Zhang, D., Caliendo, A.M., Eron, J.J., DeVore, K.M., Kaplan, J.C., Hirsch, M.S. and D'Aquila, R.T. (1994). Resistance to 2',3'-dideoxycytidine conferred by a mutation in codon 65 of the human immunodeficiency virus type 1 reverse transcriptase. *Antimicrobial Agents and Chemotherapy*, 38(2), p. 282-287.