

Characterisation of HIV-1 from patients with virological failure to a Boosted protease inhibitor regimen

Marie Rathcke Lillemark, Jan Gerstoft, Niels Obel, Gitte Kronborg, Court Pedersen, Louise Bruun Jørgensen, Tina Vasehus Madsen, Terese Lea Katzenstein

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Characterisation of HIV-1 from patients with virological failure to a Boosted protease inhibitor regimen

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Complete List of Authors:	Lillemark, Marie; State Serum Institute, Department of Virology Gerstoft, Jan; University Hospital of Copenhagen, Department of Infectious Diseases Obel, Niels; University Hospital of Copenhagen, Department of Infectious Diseases Kronborg, Gitte; University Hospital of Copenhagen, Hvidovre Hospital, Department of Infectious Diseases Pedersen, Court; Odense University Hospital, Department of Infectious Diseases Jørgensen, Louise; State Serum Institute, Department of Virology Madsen, Tina; State Serum Institute, Department of Virology Katzenstein, Terese; University Hospital of Copenhagen, Department of Infectious Diseases
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TABLE I.

Sex, origin, mode of transmission and treatment initiation information on the 1007 patients receiving Pl/r during the study period, and the 23 patients failing this treatment.

	Pl/r	Success	Failure	%
Sex				
Male	707	688	19	(2,7)
Female	300	296	4	(1,3)
Origin)
Denmark	719	703	16	(2,2)
Europe	46	44	2	(4,3)
Africa	148	144	4	(2,7)
Other/unknown	94	93	1	(1,1)
Mode of transmission)
Homosexual	427	419	8	(1,9)
Heterosexual	397	383	14	(3,5)
Other/unknown	183	182	1	(0,5)
Start Pl/r (year))
1996	33	31	2	(6,1)
1997	91	83	8	(8.8)
1998	74	71	3	(4,1)
1999	149	146	3	(2.0)
2000	139	136	3	(2.2)
2001	89	88	1	(1,1)
2002	48	47	1	(2.1)
2003	68	68	0	(0.0)
2004	87	86	1	(1.1)
2005	99	98	1	(1.0)
2006	70	70	0	(0.0)
2007	46	46	0	(0.0)
2008	14	14	0	(0.0)
Firstline treatment)
PI/r	528	510	18	(3.4)
not PI/r	479	474	5	(1.0)
Start of any treatment				
Before 1995	365	357	8	(2.2)
After 1995	642	627	15	(2.3)
Total	1007	984	23	(2.3)

Sex, origin, mode of transmission and treatment initiation information on the 1007 patients receiving PI/r during the study period, and the 23 patients failing this treatment. 210x297mm (600 x 600 DPI)

						Plasma de	ug concentral	tions (µgil)		PR resistance mutations			Gag CS mutations		
Patient	Subtype	Sample	Viral land (copies/mi)	Treatment (ritensvir boosted PI)	Ritonavir Therap. Emit >2100*	Sequinavir Therap limit >100	Indinavir Thecap. limit ⊁250	Lopinavir Therap. Limit >4000	Atazanavir Tharap limit ≻200 [™]	major (minor)	128-137 VSQNYJPIVQN p24/p17	359-358 KARVUJAEAM p17/p2	373-382 S SATIMMAQRON p2ip7	428-437 ERQANIFLGKI p7ip1	444-453 RPGNF/LQSF p1/p5
2	в	baseline, week 0 failure, week 127	7.150.000 10.900	seguinavir	ō	ō				NONE	1283 1288		373P 373P	431V. 437F 431V, 437F	
2	с	baseline, week 0 failure, week 94	15.300 870	indinavir	457		1.191			NONE (A71AT) NONE (A71AT)			373del 374N, 376L, 381S 373del, 374N, 376M, 381S		451N 451N
3	CRF-01	baseline, week 0 failure, week 205	254.000 17.600	saquinavir	4.758	1.021				NONE			373P, 375N 373P, 375N		449P 449P
4	в	baseline, week 0 failure, wask 141	253.000 118.000	saquinavir	113	0				NONE			374V, 375A 374V, 375A		
5	В	baseline, week 0 failure, week 109	68.976 30.500	sequinavir	5.924	368				NONE		3621 3621	380K 380K		
6	в	baseline, week 0 failure, week 147	21.100 9.180	siquitavir	211	0				NONE (L10) LBOLM (L10)			374N, 376V, 378M 374N, 376V, 378M	431A 431A/V	
7	в	baseline, week 0 failura, week 214	1.002.000	saquinavir	3.272	940				NONE NONE (L33L A71T)			373ins, 360R/L 373ins, 360R		4518 451del
8	в	baseline, week 0 failure, week 308	17.700 642	indinavir			4.793			NONE			376V, 360K 376V, 360K	437V 437V	
9	в	baseline, week 0 failure, week 245	not available 17.600	indinavir			D			NONE			373P, 350K 373P, 350K	4283 428G	451ins 451ins
10	в	baseline, week 0 failure, week 154	205.510 4.770	sequinavir		584			F5	NONE 3L, 154V, L90M (A71V, G73S, 185V)			375N 375N		462R 452\$
11	в	baseline, week 0 failure, week 103	\$8.326 150.000	saquinavir		0				NONE			3735/A, 375A, 374N, 3810 373A, 375A, 374N, 3810/5		
12	в	baseline, week 0 failure, week 187	763.200 85.600	saquinavir		0				NONE (A71T) NONE (A71AITV)			374T, 375A, 376M 374T, 375A, 376M		
13	В	baseline, week 0 failure, week 248	26.222 3.200	saquinavir	0					NONE NONE (L10LV)			3748, 375A 3745, 375A		
14	в	baseline, week 0 failure, week 150	315.774 16.400	sequinavir	1.045	147				NONE	132Y 132F	3621 3621	735/A. 3751/A. 380RAL 3735, 375A, 360R		
15	A.	baseline, week 0 failute, week 150	17.735 321.000	indinavir	0					NONE (L10) NONE (L10)	125V 128Wi		373del, 375N 373del, 375N		449P 449P
16	в	baseline, week 0 failure, week 127	29.309 510	saquinavir		324				NONE (471V) NONE (471V)	130K	no s	373P, 378V, 380N equence avai	428G 8 b 8	
17	В	baseline, week 0 failure, week 529	2.275.486 1.480	lopinavir				13.243	M46,	NONE (L.10) G48V, IS4T, V82A, L90M (L.10). A71	IV1	n o . s	380K equence avai	lable	
18	G	baseline, week 0 failure, week 322	140.000 751	atazanavir	0				0	NONE (L10L) NONE			3815/G, 378MU/V 3815, 376del		453L 453L
19	в	baseline, week 0 failure, week 124	484.000 59.400	atazanavir	64				143	NONE		362) 362)			

Subtype, viral load, plasma drug concentrations and mutations in the pol and gag genes from baseline and failure samples from the 19 patients from Copenhagen chosen for molecular analysis. Aminoacids are numbered according to the HXB2 reference sequence for the pol and gag genes seperately.

297x210mm (600 x 600 DPI)

2								
3 4	1	TITLE PAGE						
5 6	2	Characterisation of HIV-1	from patients with virological failure to a Boosted protease					
7 8	3	inhibitor regimen						
9 10	4	M.Sc. Marie Rathcke Lille	emark ¹ ; Prof. MD, DMSc. Jan Gerstoft ² ; MD, DMSc. Niels					
11 12	5	Obel ^{2,3} ; MD. DMSc. Gitte	Kronborg⁴; Prof. MD, DMSc. Court Pedersen⁵; M.Sc. PhD					
13 14 15	6	Louise Bruun Jørgensen	Louise Bruun Jørgensen ¹ ; M.Sc. PhD Tina Vasehus Madsen ¹ ; MD, DMSc. Terese Lea					
16 17	7	Katzenstein ²						
18 19	8							
20 21	9	¹ Department of Virology,	Statens Serum Institut, Copenhagen, Denmark					
22 23	10	² Department of Infectious	Diseases, University Hospital of Copenhagen, Copenhagen,					
24 25	11	Denmark						
26 27 28	12	³ The Danish HIV Cohort Study, University Hospital of Copenhagen, Copenhagen,						
28 29 30	13	Denmark						
31 32	14	⁴ Department of Infectious Diseases, University Hospital of Copenhagen, Hvidovre						
33 34	15	Hospital, Hvidovre, Denmark						
35 36	16	⁵ Department of Infectious Diseases, Odense University Hospital, Odense, Denmark						
37 38	17							
39 40 41	18	Short title: HIV-1 patients	s failing boosted PI					
41 42 43	19	Corresponding author:	Marie Rathcke Lillemark					
44 45	20		Statens Serum Institut					
46 47	21		Artillerivej 5					
48 49	22		Department of Virology					
50 51	23		Virus Biosafety (BL-3) Laboratory					
52 53	24		DK – 2300 Copenhagen S					
54 55 56	25		Phone: +45 3268 3407					
57 58	26		Fax: +45 3268 3082					
59 60	27		E-mail: <u>mml@ssi.dk</u>					

1 ABSTRACT

The use of highly active antiretroviral treatment (HAART) regimens with unboosted pro-tease inhibitors (PIs) has resulted in a high level of virological failure primarily due to the development of resistant virus. Current boosted PI regimens combine successfully low-dose ritonavir (r) with a second PI. The aim was to estimate the proportion of pa-tients, in a population based setting, who develop virological failure on a PI/r regimen. Through The Danish HIV Cohort Study 1007 patients who received PI/r based treat-ment between 1995 and 2008 were identified. Twenty-three (2.3%) experienced vi-rological failure, of whom 19 (83%) started Pl/r treatment before 2001. Patients from Copenhagen (n=19) were selected to study the development of protease (PR) and gag cleavage site (CS) mutations during PI/r treatment and PI plasma levels at the time of virological failure. Three patients (16%) developed major PI resistance mutations. Mutations in the p7/p1 and p1/p6 gag CS only developed in patients with major or minor mutations in PR. Drug concentrations were low or undetectable in 10 out of the 19 patients. In total PR resistance mutations and low drug levels could account for 12 (63%) of the failure cases. In conclusion, virological failure to PI/r is a low and decreasing problem primarily caused by low plasma drug levels and to a lesser extent major PR mutations. Gag CS mutations did not contribute significantly to resistance development and virological failure.

21 KEY WORDS

22 Ritonavir, resistance, PI plasma concentrations, gag CS

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During maturation of the Human immunodeficiency virus type 1 (HIV-1) viral particle the PR enzyme cleaves the gag polyprotein into functional proteins essential to the in-fectious virus [Loeb et al., 1989; Katz and Skalka 1994; Mammano et al., 1998]. The gag polyprotein contains five specific protease cleavage sites (CS), p24/p17, p17/p2, p2/p7, p7/p1 and p1/p6 with different affinity for the enzyme. These are cleaved at spe-cific rates and order to obtain the mature infectious viral particle. Protease inhibitors (PI) block the cleavage activity of PR by binding to the active site of the enzyme and thereby hinder the maturation of the virus [Ashorn et al., 1990; McQuade et al., 1990]. HAART regimens with unboosted PIs have shown a high level of virological failure pri-marily due to the development of resistant virus. Resistance towards PIs arises when specific major resistance mutations appear in the PR gene, causing a conformational change in the enzyme's active site. This lowers the affinity to the PIs and allows for cleavage to occur. Minor mutations develop secondarily to restore loss of viral fitness and replication capacity implied by the major mutations [Gulnik et al., 1995; Croteau et al., 1997; Borman et al., 1996; Nijhuis et al., 1999; Mammano et al., 2000]. Current boosted PI regimens combine low-dose ritonavir with a second high-dose PI (PI/r). This decreases the degradation rate by the cytochrome P450 enzyme in the liver and increases blood and intracellular levels of the PIs [Moyle and Back, 2001] which has proven to decrease the risk of virological failure and resistance development [Coo-per et al., 2003; Lima et al., 2008]. A previous study [Ananworanich et al., 2006] found a low prevalence (3.8%) of virologi-cal failure in patients on PI/r regimens. Von Wyl et al., [2007], found that only 30% of

PI/r failure patients harboured resistance mutations and Ananworanich et al., 2006,
found no resistance mutations in nine patients with PI/r failure. The major reasons for

26 failure on PI/r regimens in the absence of resistance have not been determined, though

27 theories such as altered absorption and metabolism of the PIs in patients failing PI/r

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1 have been suggested as an explanation for the low plasma concentrations and subse-2 quent virological failure seen in some patients [Zazzi, 2007]. 3 Since gag is the natural substrate for PR, mutations in or around the gag cleavage 4 sites have been linked to PI resistance development. Some studies assign a compen-5 satory fitness role to gag CS mutations [Zhang et al., 1997; Doyon et al., 1996; Bally et 6 al., 2000], whereas others found resistance development based exclusively on muta-7 tions in gag [Verheyen et al., 2006; Nijhuis et al., 2007; Prabu-Jeyabalan et al., 2004]. 8 Some studies have looked in gag outside the CS and found non-CS mutations leading 9 to improved viral replication [Gatanaga et al., 2002; Myint et al., 2004; Aoki et al., 10 2009]. Ghosn et al., 2009, suggested that the emergence of certain gag CS mutations 11 could predict virological failure. Though the exact role of gag CS and non-CS mutations 12 is yet to be determined, it is widely accepted that specific mutations such as A431V 13 and I437V in p7/p1 and R452S in p1/p6 can contribute to continued viral replication in 14 the presence of PIs [Zhang et al., 1997; Verheyen et al., 2006; Nijhuis et al., 2007; My-15 int et al., 2004; Malet et al., 2007]. 16 The purpose of the present study was to estimate the proportion of patients, in a population based setting, who develop virological failure on a PI/r based regimen. A group of 17

19 mutations during PI/r treatment as well as the plasma concentration levels of the PIs at

patients was selected for molecular analysis to study the development of PR and gag

20 the time of virological failure.

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PATIENTS AND METHODS
 Setting

The estimated HIV-1 prevalence among adults in Denmark is 0.07% [Lohse et al.,
2005]. About 60% of the patients are infected with subtype B virus [Jørgensen et al;
2003]. Medical care, including antiretroviral treatment (ARV), is tax-paid and provided
free-of-charge to all residents of Denmark infected with HIV-1. During the study's follow-up period, national criteria for initiating HAART were HIV-1-related disease, acute
HIV-1 infection, pregnancy, CD4 cell count < 300 cells/µl, and, until 2001, plasma HIV-
1-RNA >100,000 copies/ml.

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11 Study population

12 Patients were identified through The Danish HIV Cohort Study, described earlier 13 [Lohse et al., 2005]. In brief, this cohort encompasses all patients infected with HIV-1 seen at Danish HIV treatment clinics since 1st of January 1995. The Danish HIV Cohort 14 15 Study is approved by the Danish Data Protection Agency (J-no: 2001-41-0624). All patients who, as of December 31st 2008, had received treatment with a PI/r regimen 16 17 including Saguinavir, Indinavir, Atazanavir or Lopinavir and with no prior failure to un-18 boosted PI were identified from the cohort. Patients with previous virological failure to a 19 non-PI containing regimen were included in the study population. 20 The patients with virological failure while compliant with a boosted PI regimen were 21 identified from the study population. Virological failure was defined as plasma HIV-1-22 RNA >400 copies/ml in two consecutive measurements minimum four weeks apart af-23 ter at least three months of compliance to a continuous PI/r regimen or after HIV-1-24 RNA had been <20 copies/ml. Patient files of the failure patients were scrutinised to 25 evaluate compliance. For almost all patients with virological failure or rebound the rea-

sons for this had been discussed with the patient. Patients who had stopped treatment

27 or who reported regularly missing treatment doses were excluded. Information on the

degree of compliance (i.e. percentage of missed doses) had not been collected sys-tematically. For the molecular analysis of the *pol* and *gag* genes, all failure patients seen at the two HIV treating centres in Copenhagen, managing 2/3 of the Danish HIV patients, were selected. Plasma samples obtained at or after the time of failure, and baseline samples obtained prior to any PI treatment were analysed. Failure samples taken more than two months after the failure criteria had been fulfilled were only included if the patient re-ceived the same PI/r treatment at failure and sample date. The study was approved by The Danish National Committee on Biomedical Research Ethics (H-D-2009-017). Informed consent was obtained from each patient. PCR and sequencing Sequences of the pol region were extracted from the Danish HIV Sequence Database housed at the Department of Virology, State Serum Institute, which includes sequences from resistance tests performed on samples from Danish HIV patients from 2000 to 2008. The database currently includes approximately 5000 sequences from 1200 pa-tients. The Danish HIV Sequence Database is approved by the Danish Data Protection Agency (J-no: 2006-41-6709). If the sequence was not available from the database. RNA from stored plasma samples was extracted using QiaAmp viral RNA mini kit (Qiagen, Hilden, Germany). The pol gene including the PR and reverse transcriptase (RT) regions was amplified and se-quenced using a previously described in house nested RT-PCR method amplifying 1302 bp [Madsen et al., 2007]. Amplification of the complete gag gene was done using primers JA152 [Leitner et al., 1995] and XM1 mod (modified from Gatanaga et al., 2002) for both cDNA (65 °C, 10 min; 42 °C, 5 min; 42 °C, 1 h, 99 °C, 3 min) and first round of PCR (94 °C, 10 min; (94 ^oC, 30 sec; 65 ^oC, 30 sec; 72 ^oC, 1 min) 40 cycles; 72 ^oC, 10 min). For the second

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50 51	23
52 53	24
54 55 56	25
57 58	26
59 60	27

round of PCR (94 °C, 30 sec; 65 °C, 30 sec; 72 °C, 1 min) 30 cycles; 72 °C, 10 min) 1

- 2 primers LF3 [Gatanaga et al., 2002] and TVM1_R 5'-
- 3 CCTCCAATTCCCCCTATCATTTTTG-3' (in-house) were used, generating a 1723 bp
- 4 PCR-fragment including all five PR cleavage sites.
- 5 The gag sequences were obtained using BigDye® Terminator v1.1 Cycle Sequencing
- Kit (Applied Biosystems, CA, USA) with primers LF3, TVM1_R, TVM_seq1 5'-6
- AAGACACCAAGGAAGC-3', TVM_seq2 5'-TTCCTAGGGGCCCTGCAATT-3', 7
- 8 TVM seq3 5'-ACTTTTACCCATGCATT-3' and TVM seq4 5'-
- 9 ACATTAGAAGAAATGATG-3' (all in-house).

10 The population based sequences were run on an ABI PRISM® 3100 Genetic Analyzer

- 11 (Applied Biosystems, CA, USA).
- 12

13 Sequence analysis

14 Sequences (Genbank accession numbers: AM933271-AM933291; AM933522-

15 AM933542) were analysed using SegScape v2.5 (Applied Biosystems, CA, USA) and

16 Bionumerics v5.0 (Applied Maths, Sint-Martens-Latern, Belgium) and aligned using

17 Clustal W in Geneious v4.7 (Biomatters Ltd, Auckland, New Zealand). Phylogenetic

18 neighbour-joining tree analyses for subtyping and quality control were carried out using

- 19 Geneious v4.7 (Biomatters Ltd, Auckland, New Zealand). Sequences from the Danish
- 20 HIV Sequence Database and subtype specific references from the Los Alamos Se-
- 21 quence Database (http://www.hiv.lanl.gov/) were included as reference sequences.

22 Resistance associated mutations in *pol* according to the Stanford HIV Drug Resistance

23 Database [Liu and Shafer; 2006; last updated June 10, 2008] were identified. Full gag

- 24 sequences from pre-treatment and failure samples were aligned pairwise in order to
- 25 detect mutations in the five CS regions that had developed during treatment. CS re-

26 gions were defined by five amino acids (aa) on each side of the CS.

- 1 Plasma concentrations
- 2 PI plasma concentrations from the time of virological failure were measured using a
- 3 validated method based on high performance liquid chromatography [Justesen et al.,
- 4 2003].

To per period

1 RESULTS

1007 patients in The Danish HIV Cohort Study database had received ritonavir boosted
regimens with Saquinavir, Indinavir, Lopinavir or Atazanavir and had no prior failure to
unboosted PI. Among these 1007 patients 36 had experienced virological failure. For
13 of these incompliance was reported in the patient files. The remaining 23 patients
(2.3%) had thus experienced virological failure according to the definition described in
the 'Methods' section.

Characteristics of the study population as well as the 23 patients experiencing virologi-cal failure are given in Table I. Virological failure experienced by 2.7% of males (19 out of 707) versus 1.3% of females (4 out of 300). In total 521 patients started boosted PI after year 2000 of whom 4 (0.8%) experienced virological failure. This incidence was higher for the years 1995 to 2000 where 3.9% (19 out of 486) starting PI/r developed virological failure. For patients with a first-line regimen including Pl/r 3.4% (18 out of 528) developed virological failure compared to 1.0% (5 out of 479) of the patients start-ing a non-PI/r regimen. The failure rates were similar in the groups of patients starting any treatment before and after 1995 (2.3% vs. 2.2%). The incidence rates of failure for each specific PI/r regimen were: boosted Saguinavir: 10.8/1000 person years of follow up (PYR) (95% confidence interval 6.4-18.2) (total 1297 PYR), boosted Indinavir 15.8/1000PYR (95% confidence interval 7.1-35.2) (total 380 PYR), boosted Lopinavir 1.4/1000PYR (95% confidence interval 0.2-9.7) (total 731 PYR), and boosted Ata-zanavir 4.5/1000PYR (95% confidence interval 1.1-17.8) (total 449 PYR). Nineteen patients attending either of the two clinics in Copenhagen experienced vi-rological failure on PI/r based treatment. These were included for sequence analyses of the pol and gag regions at baseline and at time of virological failure (Table II). For two patients (patients 16 and 17), it was not possible to obtain gag sequences from the fail-ure sample due to the restricted amount of stored material.

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1 The median time between the baseline and failure samples was 44 months (range 24-2 124 months). Fifteen patients were infected with subtype B virus, the remaining four 3 with subtypes A, CRF-01, C and G, respectively (Table II). 4 No patients had major PI resistance mutations at baseline. Patients 6, 10 and 17 de-5 veloped major PI resistance mutations during PI/r treatment, in all three cases including 6 L90M. Two had received boosted Saguinavir, and one had received boosted Lopinavir. 7 Patients 7 and 13 developed only minor PI mutations. For the remaining four patients 8 with minor PI mutations at failure (2, 12, 15 and 16), these were already present at 9 baseline. From ten patients no major or minor PI resistance mutations could be de-10 tected at time of virological failure. 11 Fifteen patients had mutations related to RT inhibitors (data not shown). In ten patients 12 the mutations developed during the study period and in the remaining five the muta-13 tions were already present at baseline. 14 Gag sequences were analysed from 19 baseline samples and 17 failure samples. All 15 sequences deviated from the HXB2 reference sequence in one or more positions at the 16 five cleavage sites. Subtype non-B specimens exhibited more nucleotide differences 17 from the subtype B reference than the subtype B sequences. These deviations were 18 typically maintained from baseline to failure and are likely to be subtype specific poly-19 morphisms. 20 Nine patients developed aa mutations in one or more CS between baseline and failure. 21 Most mutations and polymorphisms were observed at the p2/p7 cleavage site. The 22 most conserved site was p17/p2 with no development of mutations and only one poly-23 morph position (position 362). Only patients with major or minor mutations in PR devel-24 oped mutations at the p7/p1 and p1/p6 CS during failure. 25 Several insertions and deletions relative to the HXB2 reference were observed 26 throughout the gene especially in the p1 and p6 regions (data not shown). All except

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one were identical at baseline and failure. This was a four aa insertion in p24 in the
 failure sample of patient 12 (data not shown).

Drug concentrations were below target trough value in ten out of the 19 patients (Pa-tients 1, 4, 6, 9, 11, 12, 13, 15, 18 and 19, see Table II). Saguinavir or Indinavir were not detectable in six out of the 14 patients in whom it was measured. In two of these patients ritonavir was detectable. The remaining eight patients had Saguinavir/Indinavir drug concentrations in a range above the therapeutic limit. For two patients ritonavir was the only drug measured, and in both cases it was undetectable, suggesting the additional PI to be present at low concentrations as well. Lopinavir was detected in one patient receiving this PI, but Atazanavir was below the therapeutic limit in the two pa-tients receiving it. For nine patients, drug concentrations were above target trough value. In these patients the median viral load (VL) was 1,480 copies/ml (442-30,500) at the time of virological failure. For the three patients with major resistance mutations, one had plasma concentrations below target trough value and two had values above.

O C C

1 DISCUSSION

In the present study, 23 (2.3%) of Danish HIV-1 patients on PI/r treatment in the period
from 1995 to 2008 developed virological failure.

4 The patients included in this study received Indinavir, Saquinavir, Lopinavir or Ata-

5 zanavir as part of the PI/r regimen. Virological failure during Lopinavir/ritonavir treat-

6 ment without the development of PI mutations is well described [Hicks et al., 2004]. In

7 this study comparing previous PR regimens the failure incidence rates were higher in

8 those treated with Saquinavir and Indinavir than in those treated with Lopinavir and

9 Atazanavir (10.8/1000PYR and 15.8/1000PYR vs. 1.4/1000PYR and 4.5/1000PYR re-

spectively). These rates were not corrected for calendar year, backbone treatment and
reasons of choice of treatment regimen.

12 Out of the 19 patients chosen for molecular analyses only three (16%) developed major

13 PI resistance mutations in PR explaining the viral drug escape. Three patients devel-

14 oped minor mutations in the PR gene, but no major mutations were detected, that

15 could account for the virological failure observed. For the remaining patients sequenc-

16 ing of PR revealed no development of resistance mutations, and the virological failure

17 must, thus, be explained by other factors. These findings are in concordance with find-

18 ings from other studies, were 0-30% of virological failures to PI/r were explained by PR

19 mutations [Ananworanich et al., 2006; von Wyl et al., 2007].

Previous studies have shown low PI concentrations in patients failing PI/r regimens and
high intra- and inter-patient variability [Zazzi, 2007; Masquelier et al., 2005; Best et al.,

22 2007]. In accordance with these observations the present study found that drug con-

23 centrations were below target trough value in ten out of the 19 patients. Only one pa-

24 tient with measured plasma concentrations below target trough value developed major

25 mutations. In the remaining nine patients with low PI concentrations the detected VL

26 was caused by replicating wild type virus. Patients who are incompliant, but take their

27 medication prior to sampling, will appear with measureable plasma drug concentra-

tions, but high VL and wild type virus due to the delayed viral response. This could explain the observed pattern in these patients.

Samples were unlikely to have been collected immediately before a dose, so it is pos-sible that some of these patients may in fact have had too low trough levels. Drug con-centrations were only measured for one time point for each patient. Sequential meas-urements might have given a more complete picture of the actual situation. The drug concentrations below target trough level are likely to be explained by poor compliance even though this was considered when selecting the study population. Information on compliance was not collected prospectively but evaluated looking through patient files and no data for successful compliance were collected. Even for patients with no com-pliance issues registered these might have been contributing to the virological failure observed. Drug levels equal to zero makes altered absorption or metabolism unlikely explanations.

Baseline and failure gag sequences were analysed for seventeen patients. Gag muta-tion development from baseline to failure at the p7/p1 and p1/p6 CS was only seen to-gether with the development of major or minor PR mutations. In none of the 19 failure cases analysed in this study it could be confirmed that these gag mutations had given rise to resistance development and virological failure on their own. Mutations in p24/p17 and p2/p7 were observed independently of PR mutations at positions 128. 132, 376, 381. These positions have not been linked previously to PR resistance de-velopment.

Variations from the reference sequence in p7/p1 and p1/p6 were observed at positions
428, 431, 437, 449, 451 and 452 including the 431V, 437V and 452S variants described in previous studies in relation to PI resistance development [Zhang et al., 1997;
Verheyen et al., 2006; Nijhuis et al., 2007; Myint et al., 2004; Malet et al., 2007]. These
variants were present at baseline as well as at the time of virological failure. They were
therefore most likely present during the period of viral suppression observed in all pa-

tients according to the criteria and did thus not prevent viral suppression. Previous studies have shown that certain CS mutations might contribute directly to PI resistance by improving cleavage efficiency by wild type PR [Verheyen et al., 2006; Nijhuis et al., 2007; Prabu-Jeyabalan et al., 2004]. In the present study it could not be confirmed that this was of any significance for the limited amounts of virological failure cases ob-served. In conclusion, virological failure is infrequent in patients on a PI/r based regimen. The proportion of patients failing PI/r treatment has decreased during the years 1995-2008. PR resistance mutations and low drug levels could account for 12 (63%) of the failure cases observed. The analyses of gag sequences did not confirm a significant role of gag CS mutations in resistance development among the limited amount of failure pa-tients with virological failure to PI/r. The use of regimens evaluated in the present study has decreased over the years, and are only used for a minority of present day patients

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