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

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TABLE I.

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.

	PI/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 PI/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)

TABLE II

Patient	Subtype	Sample	Viral load (copies/mL)	Treatment (Protease Inhibitor)	Plasma drug concentrations (µg/L)					PI resistance mutations	Gag CS mutations				
					Ritonavir Therap. limit >100*	Saquinavir Therap. limit >100	Indinavir Therap. limit >250	Lopinavir Therap. Limit >600	Atazanavir Therap. limit >200*		128-137 VIGVIVIVVGN Q220R†	359-368 KAVVLAIVMS p170‡	373-382 SATVWVGRGL 226‡	428-437 ERQANPLGKI 375‡	444-453 RFDVFLGSDP 87‡
1	B	baseline, week 0	7,150,000	saquinavir	0	0				NONE	128G		373P	431V, 437P	
		failure, week 127	10,500						NONE	128S		373P	431V, 437P		
2	C	baseline, week 0	19,300	indinavir	457		1,191		NONE (A71A†)			373AA, 374K, 375L, 381S		451N	
		failure, week 34	870						NONE (A71A†)			373AA, 374K, 376M, 381S		451N	
3	CRF-01	baseline, week 0	254,000	saquinavir	4,758	1,021			NONE			373P, 375M		449P	
		failure, week 205	17,600						NONE			373P, 375M		449P	
4	B	baseline, week 0	250,000	saquinavir	113	0			NONE			374V, 375A			
		failure, week 141	118,000						NONE			374V, 375A			
5	B	baseline, week 0	68,878	saquinavir	5,024	388			NONE		362	383K			
		failure, week 109	35,500						NONE		362	383P			
6	B	baseline, week 0	31,100	saquinavir	211	0			NONE (L10I)			374K, 375V, 375M	431A		
		failure, week 147	9,190						NONE (L10I)			374K, 375V, 376M	431AV		
7	B	baseline, week 0	1,023,000	saquinavir	3,272	940			NONE			373K, 380R/L		451S	
		failure, week 274	1,190						NONE (E33L, A71T)			373K, 380R		451M	
8	B	baseline, week 0	17,700	indinavir			4,700		NONE			376V, 380K	437V		
		failure, week 308	642						NONE			376V, 380K	437V		
9	B	baseline, week 0	not available	indinavir			0		NONE			373P, 380K	428G	451H	
		failure, week 245	17,600						NONE			373P, 380K	428G	451H	
10	B	baseline, week 0	286,510	saquinavir	584				NONE			375N		452H	
		failure, week 154	4,770						F63L, I54V, L10M (A71V, G73S, 86V)			375N		452S	
11	B	baseline, week 0	99,328	saquinavir	0				NONE			373SA, 375A, 376L, 381G			
		failure, week 152	150,000						NONE			375A, 375A, 376L, 381S			
12	B	baseline, week 0	753,200	saquinavir	0				NONE (A71T)			374T, 375A, 375M			
		failure, week 187	85,000						NONE (A71T/T)			374T, 375A, 375M			
13	B	baseline, week 0	26,222	saquinavir	0				NONE			374S, 375A			
		failure, week 246	3,300						NONE (L10L,V)			374S, 375A			
14	B	baseline, week 0	315,774	saquinavir	1,048	147			NONE	132Y	362	375A, 375T, 380R/L			
		failure, week 150	16,400						NONE	132P	362	375S, 376L, 380R			
15	A	baseline, week 0	17,739	indinavir	0				NONE (L10I)	128V		373AA, 375N		449P	
		failure, week 152	321,000						NONE (L10I)	128VH		373AA, 375N		449P	
16	B	baseline, week 0	29,309	saquinavir		324			NONE (A71V)	130K		373P, 375V, 380N	428G		
		failure, week 127	510						NONE (A71V)			n o n e a v a i l a b l e			
17	B	baseline, week 0	2,275,495	lopinavir				13,243	NONE (L10I)			383K			
		failure, week 529	1,450						M46, G49V, S47, V83A, L10M (L10I, A71V)			n o n e a v a i l a b l e			
18	G	baseline, week 0	142,000	atazanavir	0				NONE (L10L)			381S/L, 376M/L		451L	
		failure, week 322	751						NONE			381S, 376M		451L	
19	B	baseline, week 0	454,000	atazanavir	64			143	NONE			362			
		failure, week 124	59,400						NONE			362			

*Parameter as the only protease inhibitor. †Minimum effective concentration has not been established. ‡The value is based on in vitro data and the mean molecular concentrations from clinical trials (Costenbiller & Penn 2003). §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. PI: Protease inhibitor, PSI: Protease, CS: Cleavage site. Aminoacids are numbered according to the HXB2 reference sequence for the pol and gag genes separately. Mutations developed during treatment are in bold.

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 separately.

297x210mm (600 x 600 DPI)

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3 1 TITLE PAGE
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5 2 Characterisation of HIV-1 from patients with virological failure to a Boosted protease
6
7 3 inhibitor regimen
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9
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2
3 1 ABSTRACT
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5 2 The use of highly active antiretroviral treatment (HAART) regimens with unboosted pro-
6 3 tease inhibitors (PIs) has resulted in a high level of virological failure primarily due to
7 4 the development of resistant virus. Current boosted PI regimens combine successfully
8 5 low-dose ritonavir (r) with a second PI. The aim was to estimate the proportion of pa-
9 6 tients, in a population based setting, who develop virological failure on a PI/r regimen.
10 7 Through The Danish HIV Cohort Study 1007 patients who received PI/r based treat-
11 8 ment between 1995 and 2008 were identified. Twenty-three (2.3%) experienced vi-
12 9 rological failure, of whom 19 (83%) started PI/r treatment before 2001. Patients from
13 10 Copenhagen (n=19) were selected to study the development of protease (PR) and *gag*
14 11 cleavage site (CS) mutations during PI/r treatment and PI plasma levels at the time of
15 12 virological failure. Three patients (16%) developed major PI resistance mutations.
16 13 Mutations in the p7/p1 and p1/p6 *gag* CS only developed in patients with major or
17 14 minor mutations in PR. Drug concentrations were low or undetectable in 10 out of the
18 15 19 patients. In total PR resistance mutations and low drug levels could account for 12
19 16 (63%) of the failure cases. In conclusion, virological failure to PI/r is a low and
20 17 decreasing problem primarily caused by low plasma drug levels and to a lesser extent
21 18 major PR mutations. Gag CS mutations did not contribute significantly to resistance
22 19 development and virological failure.

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21 KEY WORDS

22 Ritonavir, resistance, PI plasma concentrations, gag CS
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1 INTRODUCTION

2 During maturation of the *Human immunodeficiency virus type 1* (HIV-1) viral particle
3 the PR enzyme cleaves the *gag* polyprotein into functional proteins essential to the in-
4 fectious virus [Loeb et al., 1989; Katz and Skalka 1994; Mammano et al., 1998]. The
5 *gag* polyprotein contains five specific protease cleavage sites (CS), p24/p17, p17/p2,
6 p2/p7, p7/p1 and p1/p6 with different affinity for the enzyme. These are cleaved at spe-
7 cific rates and order to obtain the mature infectious viral particle. Protease inhibitors
8 (PI) block the cleavage activity of PR by binding to the active site of the enzyme and
9 thereby hinder the maturation of the virus [Ashorn et al., 1990; McQuade et al., 1990].
10 HAART regimens with unboosted PIs have shown a high level of virological failure pri-
11 marily due to the development of resistant virus. Resistance towards PIs arises when
12 specific major resistance mutations appear in the PR gene, causing a conformational
13 change in the enzyme's active site. This lowers the affinity to the PIs and allows for
14 cleavage to occur. Minor mutations develop secondarily to restore loss of viral fitness
15 and replication capacity implied by the major mutations [Gulnik et al., 1995; Croteau et
16 al., 1997; Borman et al., 1996; Nijhuis et al., 1999; Mammano et al., 2000].
17 Current boosted PI regimens combine low-dose ritonavir with a second high-dose PI
18 (PI/r). This decreases the degradation rate by the cytochrome P450 enzyme in the liver
19 and increases blood and intracellular levels of the PIs [Moyle and Back, 2001] which
20 has proven to decrease the risk of virological failure and resistance development [Coo-
21 per et al., 2003; Lima et al., 2008].
22 A previous study [Ananworanich et al., 2006] found a low prevalence (3.8%) of virologi-
23 cal failure in patients on PI/r regimens. Von Wyl et al., [2007], found that only 30% of
24 PI/r failure patients harboured resistance mutations and Ananworanich et al., 2006,
25 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

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 popu-
17 lation based setting, who develop virological failure on a PI/r based regimen. A group of
18 patients was selected for molecular analysis to study the development of PR and *gag*
19 mutations during PI/r treatment as well as the plasma concentration levels of the PIs at
20 the time of virological failure.

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3 1 PATIENTS AND METHODS
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5 2 Setting
6

7 3 The estimated HIV-1 prevalence among adults in Denmark is 0.07% [Lohse et al.,
8 4 2005]. About 60% of the patients are infected with subtype B virus [Jørgensen et al;
9 5 2003]. Medical care, including antiretroviral treatment (ARV), is tax-paid and provided
10 6 free-of-charge to all residents of Denmark infected with HIV-1. During the study's fol-
11 7 low-up period, national criteria for initiating HAART were HIV-1-related disease, acute
12 8 HIV-1 infection, pregnancy, CD4 cell count < 300 cells/ μ l, and, until 2001, plasma HIV-
13 9 1-RNA >100,000 copies/ml.
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25 11 Study population
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27 12 Patients were identified through The Danish HIV Cohort Study, described earlier
28 13 [Lohse et al., 2005]. In brief, this cohort encompasses all patients infected with HIV-1
29 14 seen at Danish HIV treatment clinics since 1st of January 1995. The Danish HIV Cohort
30 15 Study is approved by the Danish Data Protection Agency (J-no: 2001-41-0624).
31 16 All patients who, as of December 31st 2008, had received treatment with a PI/r regimen
32 17 including Saquinavir, Indinavir, Atazanavir or Lopinavir and with no prior failure to un-
33 18 boosted PI were identified from the cohort. Patients with previous virological failure to a
34 19 non-PI containing regimen were included in the study population.
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44 20 The patients with virological failure while compliant with a boosted PI regimen were
45 21 identified from the study population. Virological failure was defined as plasma HIV-1-
46 22 RNA >400 copies/ml in two consecutive measurements minimum four weeks apart af-
47 23 ter at least three months of compliance to a continuous PI/r regimen or after HIV-1-
48 24 RNA had been <20 copies/ml. Patient files of the failure patients were scrutinised to
49 25 evaluate compliance. For almost all patients with virological failure or rebound the rea-
50 26 sons for this had been discussed with the patient. Patients who had stopped treatment
51 27 or who reported regularly missing treatment doses were excluded. Information on the
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1 degree of compliance (i.e. percentage of missed doses) had not been collected sys-
2 tematically.

3 For the molecular analysis of the *pol* and *gag* genes, all failure patients seen at the two
4 HIV treating centres in Copenhagen, managing 2/3 of the Danish HIV patients, were
5 selected. Plasma samples obtained at or after the time of failure, and baseline samples
6 obtained prior to any PI treatment were analysed. Failure samples taken more than two
7 months after the failure criteria had been fulfilled were only included if the patient re-
8 ceived the same PI/r treatment at failure and sample date.

9 The study was approved by The Danish National Committee on Biomedical
10 Research Ethics (H-D-2009-017). Informed consent was obtained from each patient.

11 12 PCR and sequencing

13 Sequences of the *pol* region were extracted from the Danish HIV Sequence Database
14 housed at the Department of Virology, State Serum Institute, which includes sequences
15 from resistance tests performed on samples from Danish HIV patients from 2000 to
16 2008. The database currently includes approximately 5000 sequences from 1200 pa-
17 tients. The Danish HIV Sequence Database is approved by the Danish Data Protection
18 Agency (J-no: 2006-41-6709).

19 If the sequence was not available from the database, RNA from stored plasma samples
20 was extracted using QiaAmp viral RNA mini kit (Qiagen, Hilden, Germany). The *pol*
21 gene including the PR and reverse transcriptase (RT) regions was amplified and se-
22 quenced using a previously described in house nested RT-PCR method amplifying
23 1302 bp [Madsen et al., 2007].

24 Amplification of the complete *gag* gene was done using primers JA152 [Leitner et al.,
25 1995] and XM1_mod (modified from Gatanaga et al., 2002) for both cDNA (65 °C, 10
26 min; 42 °C, 5 min; 42 °C, 1 h, 99 °C, 3 min) and first round of PCR (94 °C, 10 min; (94
27 °C, 30 sec; 65 °C, 30 sec; 72 °C, 1 min) 40 cycles; 72 °C, 10 min). For the second

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1 round of PCR (94 °C, 30 sec; 65 °C, 30 sec; 72 °C, 1 min) 30 cycles; 72 °C, 10 min)
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
6 Kit (Applied Biosystems, CA, USA) with primers LF3, TVM1_R, TVM_seq1 5'-
7 AAGACACCAAGGAAGC-3', TVM_seq2 5'-TTCCTAGGGGCCCTGCAATT-3',
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 SeqScape v2.5 (Applied Biosystems, CA, USA) and
16 Bionumerics v5.0 (Applied Maths, Sint-Martens-Latem, 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.

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3 1 Plasma concentrations
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5 2 PI plasma concentrations from the time of virological failure were measured using a
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7 3 validated method based on high performance liquid chromatography [Justesen et al.,
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9 4 2003].
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For Peer Review

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3 1 RESULTS

4 1007 patients in The Danish HIV Cohort Study database had received ritonavir boosted
5 regimens with Saquinavir, Indinavir, Lopinavir or Atazanavir and had no prior failure to
6 unboosted PI. Among these 1007 patients 36 had experienced virological failure. For
7 13 of these incompliance was reported in the patient files. The remaining 23 patients
8 (2.3%) had thus experienced virological failure according to the definition described in
9 the 'Methods' section.

10 Characteristics of the study population as well as the 23 patients experiencing virologi-
11 cal failure are given in Table I. Virological failure experienced by 2.7% of males (19 out
12 of 707) versus 1.3% of females (4 out of 300). In total 521 patients started boosted PI
13 after year 2000 of whom 4 (0.8%) experienced virological failure. This incidence was
14 higher for the years 1995 to 2000 where 3.9% (19 out of 486) starting PI/r developed
15 virological failure. For patients with a first-line regimen including PI/r 3.4% (18 out of
16 528) developed virological failure compared to 1.0% (5 out of 479) of the patients start-
17 ing a non-PI/r regimen. The failure rates were similar in the groups of patients starting
18 any treatment before and after 1995 (2.3% vs. 2.2%). The incidence rates of failure for
19 each specific PI/r regimen were: boosted Saquinavir: 10.8/1000 person years of follow
20 up (PYR) (95% confidence interval 6.4-18.2) (total 1297 PYR), boosted Indinavir
21 15.8/1000PYR (95% confidence interval 7.1-35.2) (total 380 PYR), boosted Lopinavir
22 1.4/1000PYR (95% confidence interval 0.2-9.7) (total 731 PYR), and boosted Ata-
23 zanavir 4.5/1000PYR (95% confidence interval 1.1-17.8) (total 449 PYR).

24 Nineteen patients attending either of the two clinics in Copenhagen experienced vi-
25 rological failure on PI/r based treatment. These were included for sequence analyses of
26 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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3 1 The median time between the baseline and failure samples was 44 months (range 24-
4 124 months). Fifteen patients were infected with subtype B virus, the remaining four
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7 with subtypes A, CRF-01, C and G, respectively (Table II).
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10 4 No patients had major PI resistance mutations at baseline. Patients 6, 10 and 17 de-
11 5
12 veloped major PI resistance mutations during PI/r treatment, in all three cases including
13 6
14 L90M. Two had received boosted Saquinavir, and one had received boosted Lopinavir.
15 7
16 Patients 7 and 13 developed only minor PI mutations. For the remaining four patients
17 8
18 with minor PI mutations at failure (2, 12, 15 and 16), these were already present at
19 9
20 baseline. From ten patients no major or minor PI resistance mutations could be de-
21 10
22 tected at time of virological failure.
23 11
24 Fifteen patients had mutations related to RT inhibitors (data not shown). In ten patients
25 12
26 the mutations developed during the study period and in the remaining five the muta-
27 13
28 tions were already present at baseline.
29 14
30 *Gag* sequences were analysed from 19 baseline samples and 17 failure samples. All
31 15
32 sequences deviated from the HXB2 reference sequence in one or more positions at the
33 16
34 five cleavage sites. Subtype non-B specimens exhibited more nucleotide differences
35 17
36 from the subtype B reference than the subtype B sequences. These deviations were
37 18
38 typically maintained from baseline to failure and are likely to be subtype specific poly-
39 19
40 morphisms.
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42 Nine patients developed aa mutations in one or more CS between baseline and failure.
43 21
44 Most mutations and polymorphisms were observed at the p2/p7 cleavage site. The
45 22
46 most conserved site was p17/p2 with no development of mutations and only one poly-
47 23
48 morph position (position 362). Only patients with major or minor mutations in PR devel-
49 24
50 oped mutations at the p7/p1 and p1/p6 CS during failure.
51 25
52 Several insertions and deletions relative to the HXB2 reference were observed
53 26
54 throughout the gene especially in the p1 and p6 regions (data not shown). All except
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1 one were identical at baseline and failure. This was a four aa insertion in p24 in the
2 failure sample of patient 12 (data not shown).
3 Drug concentrations were below target trough value in ten out of the 19 patients (Pa-
4 tients 1, 4, 6, 9, 11, 12, 13, 15, 18 and 19, see Table II). Saquinavir or Indinavir were
5 not detectable in six out of the 14 patients in whom it was measured. In two of these
6 patients ritonavir was detectable. The remaining eight patients had Saquinavir/Indinavir
7 drug concentrations in a range above the therapeutic limit. For two patients ritonavir
8 was the only drug measured, and in both cases it was undetectable, suggesting the
9 additional PI to be present at low concentrations as well. Lopinavir was detected in one
10 patient receiving this PI, but Atazanavir was below the therapeutic limit in the two pa-
11 tients receiving it. For nine patients, drug concentrations were above target trough
12 value. In these patients the median viral load (VL) was 1,480 copies/ml (442-30,500) at
13 the time of virological failure. For the three patients with major resistance mutations,
14 one had plasma concentrations below target trough value and two had values above.

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3 1 DISCUSSION
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5 2 In the present study, 23 (2.3%) of Danish HIV-1 patients on PI/r treatment in the period
6
7 3 from 1995 to 2008 developed virological failure.

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9 4 The patients included in this study received Indinavir, Saquinavir, Lopinavir or Ata-
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11 5 zanavir as part of the PI/r regimen. Virological failure during Lopinavir/ritonavir treat-
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13 6 ment without the development of PI mutations is well described [Hicks et al., 2004]. In
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15 7 this study comparing previous PR regimens the failure incidence rates were higher in
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17 8 those treated with Saquinavir and Indinavir than in those treated with Lopinavir and
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19 9 Atazanavir (10.8/1000PYR and 15.8/1000PYR vs. 1.4/1000PYR and 4.5/1000PYR re-
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21 10 spectively). These rates were not corrected for calendar year, backbone treatment and
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23 11 reasons of choice of treatment regimen.

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27 12 Out of the 19 patients chosen for molecular analyses only three (16%) developed major
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29 13 PI resistance mutations in PR explaining the viral drug escape. Three patients devel-
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31 14 oped minor mutations in the PR gene, but no major mutations were detected, that
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33 15 could account for the virological failure observed. For the remaining patients sequenc-
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35 16 ing of PR revealed no development of resistance mutations, and the virological failure
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37 17 must, thus, be explained by other factors. These findings are in concordance with find-
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39 18 ings from other studies, were 0-30% of virological failures to PI/r were explained by PR
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41 19 mutations [Ananworanich et al., 2006; von Wyl et al., 2007].

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44 20 Previous studies have shown low PI concentrations in patients failing PI/r regimens and
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46 21 high intra- and inter-patient variability [Zazzi, 2007; Masquelier et al., 2005; Best et al.,
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48 22 2007]. In accordance with these observations the present study found that drug con-
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50 23 centrations were below target trough value in ten out of the 19 patients. Only one pa-
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52 24 tient with measured plasma concentrations below target trough value developed major
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54 25 mutations. In the remaining nine patients with low PI concentrations the detected VL
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56 26 was caused by replicating wild type virus. Patients who are incompliant, but take their
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58 27 medication prior to sampling, will appear with measureable plasma drug concentra-
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1 tions, but high VL and wild type virus due to the delayed viral response. This could ex-
2 plain the observed pattern in these patients.

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

14 Baseline and failure *gag* sequences were analysed for seventeen patients. *Gag* muta-
15 tion development from baseline to failure at the p7/p1 and p1/p6 CS was only seen to-
16 gether with the development of major or minor PR mutations. In none of the 19 failure
17 cases analysed in this study it could be confirmed that these gag mutations had given
18 rise to resistance development and virological failure on their own. Mutations in
19 p24/p17 and p2/p7 were observed independently of PR mutations at positions 128,
20 132, 376, 381. These positions have not been linked previously to PR resistance de-
21 velopment.

22 Variations from the reference sequence in p7/p1 and p1/p6 were observed at positions
23 428, 431, 437, 449, 451 and 452 including the 431V, 437V and 452S variants de-
24 scribed in previous studies in relation to PI resistance development [Zhang et al., 1997;
25 Verheyen et al., 2006; Nijhuis et al., 2007; Myint et al., 2004; Malet et al., 2007]. These
26 variants were present at baseline as well as at the time of virological failure. They were
27 therefore most likely present during the period of viral suppression observed in all pa-

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3 1 tients according to the criteria and did thus not prevent viral suppression. Previous
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5 2 studies have shown that certain CS mutations might contribute directly to PI resistance
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7 3 by improving cleavage efficiency by wild type PR [Verheyen et al., 2006; Nijhuis et al.,
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9 4 2007; Prabu-Jeyabalan et al., 2004]. In the present study it could not be confirmed that
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11 5 this was of any significance for the limited amounts of virological failure cases ob-
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13 6 served.

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16 7 In conclusion, virological failure is infrequent in patients on a PI/r based regimen. The
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18 8 proportion of patients failing PI/r treatment has decreased during the years 1995-2008.
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20 9 PR resistance mutations and low drug levels could account for 12 (63%) of the failure
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22 10 cases observed. The analyses of *gag* sequences did not confirm a significant role of
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24 11 *gag* CS mutations in resistance development among the limited amount of failure pa-
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26 12 tients with virological failure to PI/r. The use of regimens evaluated in the present study
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28 13 has decreased over the years, and are only used for a minority of present day patients
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30 14 in Denmark.
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