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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.

					Plasma drug concentrations (µg/l)					PR resistance mutations	Gag CS mutations				
Patient	Subtype	Sample	Viral load (copies/ml)	Treatment (Ritonavir boosted PI)	Ritonavir Therap. limit >210*	Saquinavir Therap. limit >100	Indinavir Therap. limit >250	Lopinavir Therap. Limit >4000	Atazanavir Therap. limit >200**	major (minor)	128-137 VSQNYPIVQN p24/p17	359-368 KARVLAEAMS p17/p2	373-382 SATIMMQRGN p2/p7	428-437 ERQANFLGKI p7/p1	444-453 RPGNFILQSRP p1/p6
1	B	baseline, week 0	7,150,000							NONE	128G		373P	431V, 437F	
		failure, week 127	10,500	saquinavir	0	0				NONE	**128S**		373P	431V, 437F	
2	C	baseline, week 0	15,300							NONE (A71AT)			373del, 374N, 376L, 381S		451N
		failure, week 94	870	indinavir	457		1,191			NONE (A71AT)			373del, 374N, **376M**, 381S		451N
3	CRF-01	baseline, week 0	284,000							NONE			373P, 375N		449P
		failure, week 205	17,600	saquinavir	4,758	1,021				NONE			373P, 375N		449P
4	B	baseline, week 0	253,000							NONE			374V, 375A		
		failure, week 141	118,000	saquinavir	113	0				NONE			374V, 375A		
5	B	baseline, week 0	68,976							NONE		362I	380K		
		failure, week 109	30,500	saquinavir	5,924	388				NONE		362I	380K		
6	B	baseline, week 0	21,100							NONE (L10I)			374N, 376V, 378M	431A	
		failure, week 147	9,180	saquinavir	211	0				L**90LM** (L10I)			374N, 376V, **376I/M**	**431A/V**	
7	B	baseline, week 0	1,002,000							NONE			373ins, 380R/L		451S
		failure, week 214	1,050	saquinavir	3,272	940				NONE (**L33I, A71T**)			373ins, 380R		**451del**
8	B	baseline, week 0	17,700							NONE			376V, 380K	437V	
		failure, week 308	442	indinavir			4,703			NONE			376V, 380K	437V	
9	B	baseline, week 0	not available							NONE			373P, 380K	428G	451ins
		failure, week 248	17,500	indinavir			0			NONE			373P, 380K	428G	451ins
10	B	baseline, week 0	205,510							NONE			375N		452R
		failure, week 154	4,770	saquinavir		584				**F53L, I54V, L90M (A71V, G73S, I85V)**			375N		**452S**
11	B	baseline, week 0	98,326							NONE			373S/A, 375A, 374N, 381G		
		failure, week 103	150,000	saquinavir		0				NONE			373A, 375A, 374N, **381Q/S**		
12	B	baseline, week 0	753,200							NONE (A71T)			374T, 375A, 376M		
		failure, week 157	85,600	saquinavir		0				NONE (A71ATV)			374T, 375A, 376M		
13	B	baseline, week 0	26,222							NONE			374S, 375A		
		failure, week 246	3,200	saquinavir	0					NONE (L10LV)			374S, 375A		
14	B	baseline, week 0	315,774							NONE	132Y	362I	73S/A, 375T/A, 380R/L		
		failure, week 150	16,400	saquinavir	1,046	147				NONE	**132F**	362I	373S, 375A, 380R		
15	A	baseline, week 0	17,735							NONE (L10I)	128V		373del, 375N		449P
		failure, week 150	321,000	indinavir	0					NONE (L10I)	**128VS**		373del, 375N		449P
16	B	baseline, week 0	29,359							NONE (A71V)	130K		373P, 375V, 380N	428G	
		failure, week 127	510	saquinavir		324				NONE (A71V)			n o s e q u e n c e a v a i l a b l e		
17	B	baseline, week 0	2,275,486							NONE (L10I)			380K		
		failure, week 529	1,450	lopinavir				13,243		**M46I, G48V, I54T, V82A, L90M** (L10I, **A71V**)			n o s e q u e n c e a v a i l a b l e		
18	G	baseline, week 0	140,000							NONE (L10IL)			381S/G, 378M/V		453L
		failure, week 322	751	atazanavir	0				0	NONE			381S, **376del**		453L
19	B	baseline, week 0	464,000							NONE		362I			
		failure, week 124	59,400	atazanavir	64				143	NONE		362I			

*Administered as the only protease inhibitor **A minimum effective concentration has not been established. The value is based on in vitro data and mean/median concentrations from clinical trials (Goldsmith & Perry 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, PR: Protease, CS: Cleavage site. Aminoacids are numbered according to the HXB2 reference sequence for the pol and gag genes seperately. 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 seperately.
297x210mm (600 x 600 DPI)

TITLE PAGE

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

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Short title: HIV-1 patients failing boosted PI

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ABSTRACT

The use of highly active antiretroviral treatment (HAART) regimens with unboosted protease 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 patients, 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 treatment between 1995 and 2008 were identified. Twenty-three (2.3%) experienced virological failure, of whom 19 (83%) started PI/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.

KEY WORDS

Ritonavir, resistance, PI plasma concentrations, gag CS

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INTRODUCTION

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 infectious 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 specific 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 primarily 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 [Cooper et al., 2003; Lima et al., 2008].

A previous study [Ananworanich et al., 2006] found a low prevalence (3.8%) of virological 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 failure on PI/r regimens in the absence of resistance have not been determined, though theories such as altered absorption and metabolism of the PIs in patients failing PI/r

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have been suggested as an explanation for the low plasma concentrations and subsequent virological failure seen in some patients [Zazzi, 2007].

Since *gag* is the natural substrate for PR, mutations in or around the *gag* cleavage sites have been linked to PI resistance development. Some studies assign a compensatory fitness role to *gag* CS mutations [Zhang et al., 1997; Doyon et al., 1996; Bally et al., 2000], whereas others found resistance development based exclusively on mutations in *gag* [Verheyen et al., 2006; Nijhuis et al., 2007; Prabu-Jeyabalan et al., 2004]. Some studies have looked in *gag* outside the CS and found non-CS mutations leading to improved viral replication [Gatanaga et al., 2002; Myint et al., 2004; Aoki et al., 2009]. Ghosn et al., 2009, suggested that the emergence of certain *gag* CS mutations could predict virological failure. Though the exact role of *gag* CS and non-CS mutations is yet to be determined, it is widely accepted that specific mutations such as A431V and I437V in p7/p1 and R452S in p1/p6 can contribute to continued viral replication in the presence of PIs [Zhang et al., 1997; Verheyen et al., 2006; Nijhuis et al., 2007; Myint et al., 2004; Malet et al., 2007].

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 patients was selected for molecular analysis to study the development of PR and *gag* mutations during PI/r treatment as well as the plasma concentration levels of the PIs at 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.

Study population

Patients were identified through The Danish HIV Cohort Study, described earlier [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 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 including Saquinavir, Indinavir, Atazanavir or Lopinavir and with no prior failure to unboosted PI were identified from the cohort. Patients with previous virological failure to a non-PI containing regimen were included in the study population.

The patients with virological failure while compliant with a boosted PI regimen were identified from the study population. Virological failure was defined as plasma HIV-1-RNA >400 copies/ml in two consecutive measurements minimum four weeks apart after at least three months of compliance to a continuous PI/r regimen or after HIV-1-RNA had been <20 copies/ml. Patient files of the failure patients were scrutinised to evaluate compliance. For almost all patients with virological failure or rebound the reasons for this had been discussed with the patient. Patients who had stopped treatment or who reported regularly missing treatment doses were excluded. Information on the

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degree of compliance (i.e. percentage of missed doses) had not been collected systematically.

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 received 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 patients. 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 sequenced 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 ºC, 10 min; 42 ºC, 5 min; 42 ºC, 1 h, 99 ºC, 3 min) and first round of PCR (94 ºC, 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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round of PCR (94 ºC, 30 sec; 65 ºC, 30 sec; 72 ºC, 1 min) 30 cycles; 72 ºC, 10 min) primers LF3 [Gatanaga et al., 2002] and TVM1_R 5'-CCTCCAATTCCCCCTATCATTTTTG-3' (in-house) were used, generating a 1723 bp PCR-fragment including all five PR cleavage sites.

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'-AAGACACCAAGGAAGC-3', TVM_seq2 5'-TTCCTAGGGGCCCTGCAATT-3', TVM_seq3 5'-ACTTTTACCCATGCATT-3' and TVM_seq4 5'-ACATTAGAAGAAATGATG-3' (all in-house).

The population based sequences were run on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA, USA).

Sequence analysis

Sequences (Genbank accession numbers: AM933271-AM933291; AM933522-AM933542) were analysed using SeqScape v2.5 (Applied Biosystems, CA, USA) and Bionumerics v5.0 (Applied Maths, Sint-Martens-Latem, Belgium) and aligned using Clustal W in Geneious v4.7 (Biomatters Ltd, Auckland, New Zealand). Phylogenetic neighbour-joining tree analyses for subtyping and quality control were carried out using Geneious v4.7 (Biomatters Ltd, Auckland, New Zealand). Sequences from the Danish HIV Sequence Database and subtype specific references from the Los Alamos Sequence Database (http://www.hiv.lanl.gov/) were included as reference sequences. Resistance associated mutations in *pol* according to the Stanford HIV Drug Resistance Database [Liu and Shafer; 2006; last updated June 10, 2008] were identified. Full *gag* sequences from pre-treatment and failure samples were aligned pairwise in order to detect mutations in the five CS regions that had developed during treatment. CS regions were defined by five amino acids (aa) on each side of the CS.

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Plasma concentrations

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

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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 virological 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 PI/r 3.4% (18 out of 528) developed virological failure compared to 1.0% (5 out of 479) of the patients starting 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 Saquinavir: 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 Atazanavir 4.5/1000PYR (95% confidence interval 1.1-17.8) (total 449 PYR).

Nineteen patients attending either of the two clinics in Copenhagen experienced virological 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 failure sample due to the restricted amount of stored material.

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The median time between the baseline and failure samples was 44 months (range 24-124 months). Fifteen patients were infected with subtype B virus, the remaining four with subtypes A, CRF-01, C and G, respectively (Table II).

No patients had major PI resistance mutations at baseline. Patients 6, 10 and 17 developed major PI resistance mutations during PI/r treatment, in all three cases including L90M. Two had received boosted Saquinavir, and one had received boosted Lopinavir. Patients 7 and 13 developed only minor PI mutations. For the remaining four patients with minor PI mutations at failure (2, 12, 15 and 16), these were already present at baseline. From ten patients no major or minor PI resistance mutations could be detected at time of virological failure.

Fifteen patients had mutations related to RT inhibitors (data not shown). In ten patients the mutations developed during the study period and in the remaining five the mutations were already present at baseline.

Gag sequences were analysed from 19 baseline samples and 17 failure samples. All sequences deviated from the HXB2 reference sequence in one or more positions at the five cleavage sites. Subtype non-B specimens exhibited more nucleotide differences from the subtype B reference than the subtype B sequences. These deviations were typically maintained from baseline to failure and are likely to be subtype specific polymorphisms.

Nine patients developed aa mutations in one or more CS between baseline and failure. Most mutations and polymorphisms were observed at the p2/p7 cleavage site. The most conserved site was p17/p2 with no development of mutations and only one polymorph position (position 362). Only patients with major or minor mutations in PR developed mutations at the p7/p1 and p1/p6 CS during failure.

Several insertions and deletions relative to the HXB2 reference were observed 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 (Patients 1, 4, 6, 9, 11, 12, 13, 15, 18 and 19, see Table II). Saquinavir 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 Saquinavir/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 patients 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.

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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.

The patients included in this study received Indinavir, Saquinavir, Lopinavir or Atazanavir as part of the PI/r regimen. Virological failure during Lopinavir/ritonavir treatment without the development of PI mutations is well described [Hicks et al., 2004]. In this study comparing previous PR regimens the failure incidence rates were higher in those treated with Saquinavir and Indinavir than in those treated with Lopinavir and Atazanavir (10.8/1000PYR and 15.8/1000PYR vs. 1.4/1000PYR and 4.5/1000PYR respectively). These rates were not corrected for calendar year, backbone treatment and reasons of choice of treatment regimen.

Out of the 19 patients chosen for molecular analyses only three (16%) developed major PI resistance mutations in PR explaining the viral drug escape. Three patients developed minor mutations in the PR gene, but no major mutations were detected, that could account for the virological failure observed. For the remaining patients sequencing of PR revealed no development of resistance mutations, and the virological failure must, thus, be explained by other factors. These findings are in concordance with findings from other studies, were 0-30% of virological failures to PI/r were explained by PR 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., 2007]. In accordance with these observations the present study found that drug concentrations were below target trough value in ten out of the 19 patients. Only one patient with measured plasma concentrations below target trough value developed major mutations. In the remaining nine patients with low PI concentrations the detected VL was caused by replicating wild type virus. Patients who are incompliant, but take their medication prior to sampling, will appear with measureable plasma drug concentra-

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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 possible that some of these patients may in fact have had too low trough levels. Drug concentrations were only measured for one time point for each patient. Sequential measurements 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 compliance 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* mutation development from baseline to failure at the p7/p1 and p1/p6 CS was only seen together 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 development.

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-

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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 observed.

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 patients 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 in Denmark.

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