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Lipid evaluation in HIV-1-positive patients treated with protease inhibitors

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There is accumulating evidence that human immunodeficiency virus type 1 (HIV-1) protease inhibitors (PIs) can induce hyperlipidaemia. To evaluate the frequency and type of hyperlipidaemia in PI-treated patients, 98 outpatients were prospectively analysed for their lipoprotein characteristics at the Medizinische Hochschule in Hannover, Germany. Fifty-seven percent of the patients studied presented with hyperlipidaemia. Both hypertrigylceridaemia (type IV and V hyperlipoproteinaemia, 33%) and hypercholesterolaemia (type IIa hyperlipoproteinaemia, 6%) were detectable. The remaining 18% had a type IIb hyperlipoproteinaemia. Increased lipid levels were highly statistically significant compared to a control group of PI-naive HIV-1-infected patients [low-density lipoprotein (LDL) 146 mg/dl (range, 53–274 mg/dl) versus 105 mg/dl (range, 22–188 mg/dl; *P*=0.0006); very-low-density lipoprotein (VLDL) 35.5 mg/dl (5–253 mg/dl) versus 18 mg/dl (range, 3–94 mg/dl; *P*=0.0002)]. All PIs used (saquinavir, indinavir, nelfinavir and ritonavir) were associated with this variable form of hyperlipidaemia according to the Fredrickson classification. There was no significant correlation of any determined lipid value with the duration of treatment. A higher frequency of the apolipoprotein E2 allele and E4 allele was observed in the hyperlipidaemic subjects. Patients with excessive hypertriglyceridaemia showed a reduced lipoprotein lipase activity. Lipodystrophy was observed especially in hyperlipidaemic patients and to a lesser extent in normolipidaemic subjects. The frequency of hyperlipidaemic risk factors was surprisingly high in the group studied, which in turn may explain the proposed increased risk of atherogenesis in HIV-1 PI-treated patients. Therefore, PI-treated subjects should also be evaluated for their lipoprotein pattern, which may require antihyperlipidaemic interventions.

Introduction

The early introduction of powerful protease inhibitors (PIs) in the treatment of human immunodeficiency virus type 1 (HIV-1)-infected patients has resulted in unexpected side effects over time [1–10]. The HIV-1 protease consists of two 99 amino acid subunits that form a homodimer with a typical retroviral aspartyl protease activity [11]. PIs bind with high affinity to the catalytic site of HIV-1 protease and prevent cleavage of HIV precursor proteins in infected cells. In the presence of PIs, the virion is unable to mature and the amount of infectious virus rapidly decreases. The four common PIs used (indinavir, nelfinavir, ritonavir and saquinavir) are structurally related substances that inhibit the cleavage of HIV precursor proteins competitively [11].

There is accumulating evidence that PIs can induce hyperlipidaemia in a subgroup of treated patients [5,12]. Patients reported to date have presented with hypertriglyceridaemia. In addition, there are also rare events of coronary artery and peripheral vascular disease published so far [13–15]. The accelerated atherogenesis described in these subjects may result from the observed dyslipidaemia. To further evaluate the lipid pattern and its potency as an atherogenic risk factor we analysed PI-treated subjects in detail.

Patients and Methods

Patients

HIV-1-infected patients who were admitted to our HIV outpatient clinic from March to July 1998 were enrolled in the study and analysed for lipid abnormalities. Specimen collection followed a 12 h overnight fasting period. Lipid parameters of patients receiving PI therapy were compared with a control group of PI-naive patients [13 patients treated with two nucleoside reverse transcriptase inhibitors (NRTI) and/or non-

Table 1. The clinical characteristics of the various hyperlipoproteinaemic phenotypes using the Fredrickson classification are depicted

Phenotype	*n*	Age (years)	BMI	CD4 cells (cells/mm^3)	Viral load (copies/ml)	Lipodystrophy (%)
Type IV/V	32	44.2±10.6	24.3±4.1	294.8±186.7	$52.1\times10^3\pm88.3\times10^3$	28
Type IIa	6	54.2±6.6	24.6±2.4	281.5±169.0	$2.3\times10^3\pm3.7\times10^3$	33
Type IIb	18	53.4±11.4	24.7±3.0	287.2±159.2	$56\times10^3\pm14\times10^3$	28
Normal	42	45.9±12.7	24.0±3.5	314.2±189.8	$35.8\times10^3\pm12.3\times10^3$	7

n, number of patients.
BMI, body mass index.

nucleoside reverse transcriptase inhibitor (NNRTI) and 10 therapy-naive patients]. None of the patients received parenteral nutrition, nutrition via percutaneous gastrostomy, growth hormones or anabolic steroids. Body fat changes in patients with HIV infection were defined as signs of abnormal fat accumulation (dorsocervical fat pad, benign symmetric lipomatosis, increased abdominal girth/visceral obesity, breast hypertrophy) and/or loss of the subcutaneous fat in the face, limbs, gluteal regions or buttocks. The symptoms were defined clinically by physical examination and patient's reports. The different forms of hyperlipoproteinaemia were defined as total cholesterol >200 mg/dl, triglycerides >200 mg/dl, low-density lipoprotein (LDL) >155 mg/dl, and very-low-density lipoprotein (VLDL) >35 mg/dl. Reference values for other lipid parameters were high-density lipoprotein (HDL) >35 mg/dl and lipoprotein(a) [Lp(a)] >30 mg/dl.

Determination of triglycerides and total cholesterol

Serum concentrations of triglycerides and total cholesterol were determined using semi-automatic enzymatic methods. For the triglyceride assay the Peridochrom Triglyceride GPO-PAP Test kit was used (Boehringer Mannheim, Germany). The reaction buffer contained 0.5 mM ATP, 0.35 mM 4-aminophenazone, 3 U/ml lipase, 2.5 U/ml glycerol phosphatoxidase, 0.2 U/ml glycerol kinase, 0.15 U/ml peroxidase and 3.5 mM 4-chlorophenol. For the total cholesterol assay the CHOD-PAP Test kit (Boehringer Mannheim, Germany) was used. The reaction buffer contained 50 mM $MgCl_2$, 1 mM 4-aminophenazone, 10 mM sodium cholate, 6 mM phenol, 4 mM 3,4-dichlorophenol, 0.3% polyglycol ether, 0.4 U/ml cholesterol esterase, 0.25 U/ml cholesterol oxidase, 0.2 U/ml peroxidase. Extinction was measured at 546 nm. The assays were performed according to manufacturer's instructions.

Lipid electrophoresis of VLDL, LDL and HDL

The concentrations of VLDL-, LDL- and HDL-cholesterol were determined using an electrophoretic and densitometric method (REP Lipoprotein-Kit, Helena Diagnostics, Germany). The plasma lipoproteins were separated on an agarose gel according to the manufacturer's instructions. The gel was stained and scanned in a specific densitometer. The lipoprotein concentrations were automatically calculated according to their densitometric value. Hyperlipoproteinaemia was classified according to the Fredrickson classification.

Nephelometric quantification of apolipoproteins [apoA-I, apoA-II, apoB, apoE and Lp(a)]

This method is based on particle agglutination technology. The assays were semi-automatically performed on the BN II (Behring Nephelometer, Behringwerke AG, Marburg, Germany) using rabbit polyclonal antisera against human apolipoproteins. For Lp(a), the antibody is conjugated to latex-particles to enhance the turbidity of the antigen–antibody complexes [16]. The turbidity of the formed complexes was proportional to the concentration of the used antigen. The reactions were measured at time 0 and 6 minutes at 840 nm. The differences of the measured values were calculated automatically. This approach excludes non-specific turbidity. From each of the serum samples, 30 µl was diluted by 1:100. A lyophilized serum control and standard serum were used for the quantification. The

Table 2. The lipoprotein parameters of the various hyperlipoproteinaemic phenotypes are illustrated

Phenotype	Serum cholesterol	Serum triglycerides	VLDL	LDL	HDL	Lipoprotein (a) (mg/dl)
Type IV/V	249.7±74.9	654.3±542.4	84.6±56.4	139.4±36.6	27±9.2	19.9±30.9
Type IIa	268.7±30.5	169±40.8	25±7.8	203.5±22.1	40.2±9.7	26.8±27.3
Type IIb	298.2±42.5	425.6±201.9	65.6±34.1	188.9±37.4	33.4±8.2	21.2±42.9
Normal	184.9±39.3	145±67.6	20.2±8.6	122.6±30.9	42.2±14.6	10.8±14.5

VLDL, very-low-density lipoprotein.
LDL, low-density lipoprotein.
HDL, high-density lipoprotein.

Table 3. Lipid parameters, CD4 cell count and viral load of PI-treated and PI-naive HIV-infected patients

	PI-treated patients	PI-naive patients	*P* value
Cholesterol (mg/dl)	227 (100–450)	159.5 (75–307)	<0.00001
Triglycerides (mg/dl)	252.5 (58–2192)	125.5 (22–538)	0.0003
LDL (mg/dl)	146 (53–274)	105 (22–188)	0.0006
VLDL (mg/dl)	35.5 (5–253)	18 (3–94)	0.0002
HDL (mg/dl)	31.5 (15–91)	36 (20–50)	0.3
ApoA-I (mg/dl)	135 (93–189)	140 (95–238)	0.8
ApoA-II (mg/dl)	30 (18–43)	29 (22–54)	0.2
ApoB (mg/dl)	125 (2–33)	84 (53–180)	0.0004
ApoE (mg/dl)	5 (2–33)	4 (2–10)	0.001
CD4 cell count (cells/mm^3)	275 (5–814)	336 (123–652)	0.02
HIV RNA copies/ml ($\log_{10}$)	3.20 (1.69–5.88)	3.097 (1.69–4.72)	0.4

Data are expressed as median (range) and *P* values are indicated for between-group comparison. VLDL, very-low-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol

reaction buffer consisted of polyethylene glycol, sodium chloride (11.6 g/l) in 0.05 M phosphate buffer pH 7.0 and sodium azide (<1 g/l). N-Diluens is a phosphate-buffered saline, pH 7.5, containing sodium azide (<1 g/l). Samples were measured in duplicate.

Lipoprotein lipase and hepatic lipase activity

Lipoprotein lipase (LPL) activity and hepatic lipase (HL) activity were measured according to the methods described in [17].

ApoE genotype

DNA was extracted from patients blood using a DNA purification kit (Invitek, Germany). Purification was performed according to the manufacturer's instructions. The relevant region within the *apoE* gene was amplified by PCR. PCR products were digested with the restriction enzyme *Hha*I for 4 h at 37°C. After digestion, samples were analysed on a 3% Nusieve, 1% Seakem agarose gel (FMC Bioproducts, USA) was used to detect the different *apoE* alleles.

CD4 cell count and viral load

CD4 lymphocyte cell count was determined by flow cytometry and HIV RNA copies by quantitative PCR (Amplicor HIV-1 Monitor Test Kit, Roche Molecular Systems).

Statistical analysis

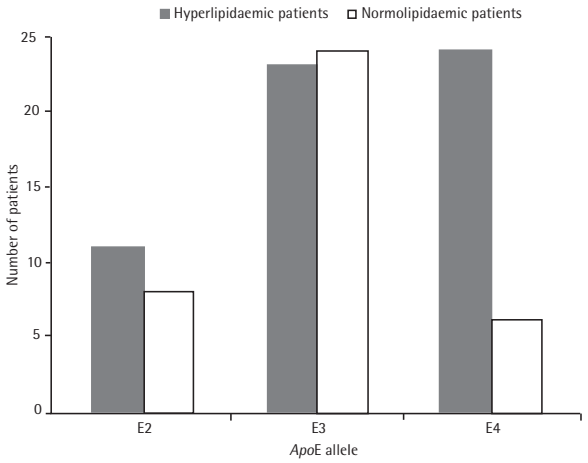
The analysis included the calculation of the mean and standard deviation (SD). Statistical significance for lipid profiles between PI-treated patients and control group was determined by analysis with non-parametric Mann–Whitney U test or Student's *t*-test where appropriate. Differences between the PI regimens and types of hyperlipoproteinaemia were determined by analysis of variance (ANOVA). *P* values of <0.05 were considered to indicate statistical significance.

Results

Ninety-eight HIV-1-positive patients treated with PIs (78 male, 20 female) were analysed for lipid abnormalities. None of the studied subjects had overt diabetes mellitus. The analysis of the lipid pattern revealed that 56 (57%) had detectable hyperlipidaemia showing that the prevalence of hyperlipidaemia in HIV-1 PI-treated subjects was very high. In addition, 10 patients (19%) presented with an elevated Lp(a) serum concentration (range, 33–158 mg/dl, mean 81±41 mg/dl), which represents an additional cardiovascular risk factor. Interestingly, seven out of these 10 patients with increased Lp(a) levels were treated with indinavir. In contrast, increased levels for Lp(a) were observed in 7% of the 42 normolipidaemic subjects (range, 44–71 mg/dl, mean 54±15 mg/dl) and in two patients (9%) of the control group (range, 44–100 mg/dl, mean 77 mg/dl).

We also determined the type of hyperlipoproteinaemia using the Fredrickson classification [18]. A hypertriglyceridaemic lipid pattern was detectable in 32 subjects, three of them presented with type V hyperlipidaemia (3%) and 29 of them with type IV hyperlipidaemia (30%). Eighteen subjects had an increase of both VLDL- and LDL-cholesterol, the so-called type IIb hyperlipidaemia (18%). In addition, six subjects had an isolated hypercholesterolaemia, type IIa hyperlipidaemia (6%). Thus, besides hypertriglyceridaemia, PI-treated HIV-1-positive patients can also develop isolated hypercholesterolaemia. The clinical characteristics of the various hyperlipoproteinaemic phenotypes are depicted in Table 1, whereas Table 2 reflects their lipoprotein parameters. Since type V hyperlipidaemia is commonly an exacerbation of an existing type IV hyperlipidaemia, we grouped these affected subjects for the subsequent statistical analysis. The average body mass index (BMI) was calculated in all studied groups within the non-obese range (<25

Figure 1. The *apoE* allele (ApoE2, E3 and E4) and corresponding hyperlipoproteinaemia



96 of 98 patients were evaluated.

kg/m^2). The CD4 cell count and the viral load in all groups were within the same range. Interestingly, lipodystrophy was physically detectable in both hyperlipidaemic and normolipidaemic subjects. However, lipodystrophy was more commonly observed among the hyperlipidaemic patients.

The statistical evaluation of patients receiving PI compared to the PI-naive group (20 male, three female) revealed highly statistically significant differences (Table 3). PI treatment was associated with elevated levels for fasting cholesterol (median, 227 mg/dl; range, 100–450 mg/dl; $P<0.00001$), triglycerides (median, 252.5 mg/dl; range, 58–2192 mg/dl; $P=0.0003$), LDL (median, 146 mg/dl; range, 53–274 mg/dl; $P=0.0006$), VLDL (median, 35.5 mg/dl; range, 5–253 mg/dl; $P=0.0002$). However, HDL values were within the same range in PI-treated (median, 31.5 mg/dl; range, 15–91 mg/dl) and PI-naive patients (median, 36 mg/dl; range, 20–50 mg/dl; $P=0.3$).

The additional quantification of apolipoprotein A-I (apoA-I), apolipoprotein B (apoB), apolipoprotein A-II (apoA-II), and apolipoprotein E (apoE) did reveal significant higher levels of apoB ($P=0.0004$) and apoE ($P=0.001$) in the PI-treated group (Table 3), which is characteristic for patients with increased levels of VLDL- and/or LDL-cholesterol. The determination of the LPL and HL activity in eight patients with increased serum trigylcerides above 800 mg/dl revealed that in seven out of eight patients the mean LPL decreased by 30% (range 20–70%) in comparison to control subjects, whereas the hepatic lipase activity was normal in the eight subjects studied. LPL from PI-treated patients was compared to normolipidaemic HIV-negative control subjects with an *apoE* 3/3 genotype.

To determine the impact of the *apoE* gene on lipid patterns in the studied subjects, we evaluated the genotype, illustrated in Figure 1. Since *apoE* 3/3 is the normal and most common genotype in individuals, the analysis revealed a relatively higher association of the *apoE* 2 and statistically significant higher rate of *apoE* 4 allele with patients presenting with hyperlipoproteinaemia. Moreover, carriers of the *apoE* 4 allele had higher total triglyceride values than patients without the *apoE* 4 allele (mean 534.0±606.3 mg/dl versus 321.9±278.0 mg/dl, $P=0.02$). No significant differences were observed for the other lipid parameters. The percentages of each *apoE* genotype compared to the frequency of *apoE* genotype of three different German population studies [19–21] revealed higher frequencies of apoE 2/3 (19.8% versus 10.7–12.0%), *apoE* 2/4 (7.3% versus 1.5-3.0%), and *apoE* 4/4 (8.3% versus 1.3–2.8%) in our study group.

The duration of treatment with PIs was comparable between the normolipidaemic and hyperlipidaemic groups: median 19 months (range, 2–30) in the normolipidaemic group, whereas median 21.5 months (range, 17–29 months), median 18.5 months (range, 5–29 months), median 20 months (range, 2–28 months), and median 16 months (range, 2–26 months) in type IIa, type IIb and type IV/V hyperlipidaemic subjects, respectively. Since the onset of hyperlipidaemia might already be within several months after initiation of PI treatment, the duration of therapy seems not to be critical for triggering hyperlipidaemia. The patients in the major groups of PI therapy were on drugs for an equivalent time [saquinavir: median 14 month (range, 7–30), indinavir: median 17 month (range, 1–29), nelfinavir: 21.5 month (range, 3–30), ritonavir plus saquinavir: 21 month (range, 2–26)]. There was no significant correlation of any determined lipid value with the duration of PI treatment. Other individual factors have to be responsible for the development of hyperlipidaemia. The individually applied PIs were also not significantly correlated with both hypertriglyceridaemia (VLDL-cholesterol) and hypercholesterolaemia (LDL-cholesterol) (Figures 2–4). More importantly, the combination of two PIs (especially ritonavir plus saquinavir) seems to have an synergistic effect on lipid metabolism as the prevalence of hyperlipidaemia is particulary high in these groups (Figure 2). These data reflect that all PIs used are associated with hyperlipidaemia, which in turn can occur in variable phenotypes. Figure 5 illustrates as an example, the lipoprotein profile in patients under the treatment with indinavir, reflecting the variable lipoprotein phenotype once hyperlipidaemia is detectable.

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