Role of 3 Lipoprotein Lipase Variants in Triglycerides in Children Receiving Highly Active Antiretroviral Therapy

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Background: Lipoprotein lipase is a key enzyme in lipid metabolism, especially for plasma triglycerides (TGs). Genetic variants have been associated with lipid levels in healthy individuals, cardiovascular disease, obesity and diabetes. Our aim was to evaluate the influence of 3 polymorphisms: *Hind III*, *Pvu II* and S447X in plasma TG levels in human immunodeficiency virus-1-infected children under highly active antiretroviral therapy (HAART).

Methods: Fifty-two children diagnosed with human immunodeficiency virus-1 between 2005 and 2009 were retrospectively selected with at least 1 plasma TG level assessment. TG levels were examined before and after 1 year of HAART. Hypertriglyceridemia was defined as TG > 150 mg/dL. *Hind III* (H+/H−), *Pvu II* (P+/P−) and S447X (S/X) were determined by polymerase chain reaction and restricted fragment length polymorphism. The Wilcoxon sum-rank test was used to compare median plasma TG among groups. Also, allelic frequencies were estimated for these variants in an Argentinean population.

Results: Allelic frequencies for human immunodeficiency virus-1-infected children were: H−, 0.21; P−, 0.53; and X, 0.05 with no significant differences to controls. After 1 year of HAART, median TG levels were significantly lower in P−/P− (98.5 mg/dL) when compared with P+/P+ (180 mg/dL) ($P = 0.039$). The presence of the P− allele was associated with an 11-fold lower risk of hypertriglyceridemia. *Hind III* and S447X were not associated with TG at the selected time points.

Conclusions: Our findings suggest a protective effect of lipoprotein lipase polymorphisms against hypertriglyceridemia in children after 1 year of HAART. These results could endorse a prompt nutritional or pharmacological intervention in patients lacking the P− allele.

Key Words: lipoprotein lipase, triglycerides, highly active antiretroviral therapy, children, human immunodeficiency virus-1 infection

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Highly active antiretroviral therapy (HAART) reduces clinical symptoms of human immunodeficiency virus-1 (HIV-1) infection, delays the onset of acquired immunodeficiency syndrome and improves the prognosis and quality of life in HIV-1-infected individuals.[1–3] Given the long-term exposure to these drugs and in addition to several short-term and medium-term side-effects, a considerable number of patients experience metabolic complications such as insulin resistance, redistribution of body fat and dyslipidemia. These metabolic abnormalities occur in both adults[4–8] and children.[9–11] Lipid alterations include hypertriglyceridemia, decreased high-density lipoprotein cholesterol, increased total cholesterol (TC) and increased low-density lipoprotein cholesterol.[12–16] These abnormalities may vary according to the antiretroviral (ARV) regimen received. For example, hypertriglyceridemia frequently occurs with the use of ritonavir used as a single drug or protease inhibitor (PI) booster;[12,13] high low-density lipoprotein cholesterol or TC is associated with the use of PIs, nonnucleoside reverse transcriptase inhibitors, such as efavirenz and nucleoside reverse transcriptase inhibitors, such as stavudine (d4T).[12,14–16] The same combination of ARV drugs, however, does not lead to the same extent and severity of dyslipidemia in all HIV-infected individuals; and these differences can be partially explained by the genetic background of the host, along with environmental and nutritional factors.

Among the many genes involved in lipid metabolism, the one coding for lipoprotein lipase (LPL) has been thoroughly studied. This is a key enzyme in lipid metabolism, responsible for the hydrolysis of circulating triglycerides (TGs), and has a bridging function that favors clearance of lipoproteins.[17] Complete loss of enzyme activity results in the accumulation of chylomicrons and is mainly due to homozygosity or compound heterozygosity for missense, nonsense mutations, deletions or insertions in the LPL gene.[18,19] Also, several single-nucleotide polymorphisms (SNPs) have been described in both exonic and intronic sequences,[20] and some have been studied in association with lipids, lipoproteins and risk for atherosclerosis. Three of the most explored SNPs are *Hind III* (T→G transversion in intron 8), *Pvu II* (T→C transition in intron 6) and Ser447X (a T→G mutation in exon 9 that introduces a premature stop codon) mainly due to their relatively high frequency among a Caucasian population as opposed to other coding SNPs, such as D9N and N291S.[21]

In an HIV-1-infected adult population, 2 genetic association studies assessing the impact of SNPs on lipid levels evaluated just 1 LPL variant (S447X or 1595 G→C), among other genes,[22,23] yet the results were not conclusive. There are no studies in HIV-1-infected children. There is great concern about the metabolic abnormalities occurring early in the life of HIV-1 perinatally infected children,[9] since abnormal lipid levels in childhood are associated with increased evidence of early atherosclerotic lesions.[24–31] Thus, we aimed to characterize 3 genetic variants in the LPL gene (*Hind III*, *Pvu II* and S447X) in a group of HIV-1-infected children receiving highly antiretroviral therapy HAART and to evaluate their impact on plasma TG levels.

MATERIALS AND METHODS

Study Population

A retrospective study was performed in a white-Hispanic population of Argentina composed of 52 HIV-1 perinatally infected children and 86 unrelated blood donors who were seronegative

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for blood-borne viruses, including HIV-1. All patients were diagnosed with HIV-1 according to the 1994 criteria of the US Centers for Disease Control and Prevention[32] and were followed up at the Hospital de Pediatría "J.P. Garrahan" of Buenos Aires, Argentina. Inclusion criteria for the study were 1) HIV-1 diagnosis between 2005 and 2009, 2) age under 18 years and 3) at least one blood lipid assessment between 2005 and 2010. The Ethics Committee and Institutional Review Board of the hospital approved the study. Written informed consent was obtained from blood donors and from parents or legal guardians of the children involved in this study.

TGs and Cholesterol Assessment

Lipid blood levels were determined on plasma samples obtained throughout patient follow-up. Parents received instructions so that children would fast at least 8 hours (in children older than 12 months). However, fasting status could not be confirmed during TG data acquisition through the electronic hospital database.

TC and TG plasma levels were assessed using an automated analyzer (Hitachi 912 analyzer, Roche Diagnostics, Indianapolis, IN) at the central laboratory of the hospital. TG levels were measured using enzyme-based colorimetric techniques (Triglycerides GPO-PAP, Roche Diagnostics). Results were recorded in a database and data from 2005 to 2010 were retrospectively collected. Since there was no fixed time schedule for lipid assessment in this retrospective study, each patient had a different number of lipid results. We selected lipid values before HAART initiation (baseline) (n = 28); when more than 1 value was available we chose the nearest to therapy start. And as close to 1 year after HAART initiation (±3 months), as these results were available in the majority of ourpatients (n = 39). For longer exposure times, the number of patients with available TG values dropped significantly (data not shown). Hypertriglyceridemia was defined as TG > 150 mg/dL according to the NCEPIII guidelines.[33]

LPL Genotyping

LPL genotypes were identified using polymerase chain reaction and restricted fragment length polymorphism assays. *Hind III* (rs320) (T/G), *Pvu II* (rs285) (T/C) and S447X (rs328) (T/G) genotypes were determined on DNA samples based on previous reports of Kosaka et al.[34] Briefly, PCR amplicons of 350 bp, 431 bp and 315 bp were subjected to 4 hours digestion with restriction enzymes *Hind III*, *Pvu II* and *Mnl I*, respectively. Afterwards, digested products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining under UV light with the El Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY).

Statistical Analysis

Genotypic frequencies were estimated by direct allele counting. The fit to Hardy–Weinberg equilibrium was tested with Pearson's χ^2. Also, to analyze *Hind III* and S447X genotype distribution among groups, the Pearson test for 2×2 contingency tables was used. The Fisher–Freeman–Halton test was used for *Pvu II* in 2×3 contingency tables. Haplotype frequencies were estimated using the expectation maximization algorithm, and linkage disequilibrium was evaluated with the D' statistic. Also, odds ratios for haplotypes and risk of hypertriglyceridemia were obtained by logistic regression; the alternative hypothesis for testing each locus was "any genotype shows a difference or any genotype is different from the rest (ie, codominant genetic model)." Additional genotypic and haplotypic analyses were carried out with the web tools available at www.bioinfo.iconcologia.net/snpstats

The Wilcoxon signed test was used to compare between median TG blood concentration at 2 times and the Wilcoxon sum-rank test was used to compare differences among groups. Linkage disequilibrium was tested with Pearson's χ^2, and P values were corrected for multiple testing with the Bonferroni method.

RESULTS

Study Population

The study included 52 patients diagnosed with HIV-1 between 2005 and 2009, with a fairly equal distribution between boys (n = 24) and girls (n = 28). The median age at diagnosis was 11 months (IQR 3.9–37.1). Except for 1 patient who remained naïve to treatment at the study end point, all started HAART between 2005 and 2010. We observed a wide range in the age of therapy initiation with a median age of 12 months (IQR 5.8–46.5). ARV treatment administered in the whole group included 2 nucleoside reverse transcriptase inhibitors as a backbone, mainly zidovudine and lamivudine (n = 41); plus (a) PI (n = 40) or (b) nonnucleoside reverse transcriptase inhibitors (n = 10) or (c) a third nucleoside reverse transcriptase inhibitor (n = 1). Most PI regimens contained lopinavir/ritonavir (n = 35) and the remaining 5 included nelfinavir. Efavirenz was used in 8 and nevirapine in 2 patients. TG values closest to 1 year of HAART (n = 39) were selected; similarly, at baseline (n = 28) a single TG value was considered. TG data were available at baseline and after 1 year of HAART for only 20 patients. Hypertriglyceridemia was present in 14 out of 28 children at baseline and in 13 out of 39 after 1 year of HAART; only 3 patients had TG values over 150 mg/dL both before and after 1 year of HAART (Table 1).

TABLE 1. Characteristics of the Studied Population

Characteristics		Total of Patients With Available Data
Girls (%)	28 (53.8)	52
Median age in months at HIV-1 diagnosis (IQR)	11 (3.9–37.1)	52
Median age in months when HAART started (IQR)	12 (5.8–46.5)	51
Median $CD4^+$ T cells percentage after 1 year of HAART (IQR)	35 (27–42)	38
Median HIV-1 copies per mL after 1 year of HAART (IQR)	4040 (356–43300)	39
Median of TG assessment after HAART initiation (IQR)	6 (4–8)	48
Hypertriglyceridemia episodes before HAART (%)	14 (50)	28
Hypertriglyceridemia episodes after 1 year of HAART (%)	13 (33.3)	39
HAART regimens after 1 year of HAART		39
AZT + 3TC + LPVr (%)	20 (51.3)	
3TC + ABC + LPVr (%)	3 (7.7)	
3TC + D4T + LPVr (%)	3 (7.7)	
D4T + DDI + LPVr (%)	1 (2.6)	
AZT + 3TC + NFV (%)	2 (5.1)	
3TC + D4T + NFV (%)	1 (2.6)	

AZT, zidovudine; 3TC, lamivudine; NFV, nelfinavir.

TABLE 2. Genotype and Allele Frequencies of LPL *Hind III*, *Pvu II* and S447X SNPs in HIV-1-infected Children and Seronegative Blood Donors

	LPL Alleles and Genotypes	HIV-1-infected [n (%)]		Blood Donors [n (%)]		*P*
Hind III	H+/H+	33	(63)	47	(55)	0.26*
rs320	H+/H−	16	(31)	35	(41)	
T/G	H−/H−	3	(6)	4	(5)	
	n	52		86		
	Allele H− frequency		0.21		0.25	
Pvu II	P+/P+	10	(19)	24	(28)	0.39†
rs285	P+/P−	29	(56)	38	(45)	
T/C	P−/P−	13	(25)	23	(27)	
	n	52		85		
	Allele P− frequency		0.53		0.49	
S447X	S/S	46	(90)	73	(85)	0.46*
rs328	S/X	5	(10)	12	(14)	
T/G	X/X	0	(0.)	1	(1)	
	n	51		86		
	Allele X frequency		0.05		0.08	

All genotypic frequencies fitted to Hardy–Weinberg equilibrium.
*Pearson test for contingency 2×2 tables.
†Fisher–Freeman–Halton test for contingency 2×3 tables.

LPL Genotypes Distribution in Healthy Donors and HIV-1-infected Patients

First we investigated whether the distribution of the 3 SNPs in the LPL gene, *Hind III* (H+/H−), *Pvu II* (P+/P−) and S447X, differed in HIV-1-infected children from the general population. Thus, the distribution of genotypes was studied in unrelated HIV-1 seronegative blood donors (n = 86). *Pvu II* was the most frequent polymorphism (P− allele ~ 50%), followed by *Hind III* (H− allele 21–25%), while the 447X SNP had the lowest allelic frequency (5–8%). The only homozygote X/X was found in the control group; interestingly, this subject also carried H−/H− and P−/P− genotypes. Allelic frequencies for *Hind III*, *Pvu II* and S447X polymorphisms were similar between blood donors and HIV-1-infected children (Table 2). All genotypes fit Hardy–Weinberg equilibrium, suggesting that there was no population bias in the groups analyzed.

Out of the 8 possible haplotype combinations, we observed only 5 in HIV-1-infected children and 7 in healthy blood donors, as shown in Figure 1. The H+/P+/X haplotype was not observed in any of the groups. This stems from the fact that there was linkage disequilibrium among the SNPs in each group; *Hind III* and *Mnl I* were linked in HIV-1-infected children and in blood donors, $D' = 0.9986$ ($P < 0.0001$) and $D' = 0.7482$ ($P < 0.0001$), respectively; and *Pvu II* was linked to *Hind III* and *Mnl I* in the first group, $D' = 0.7123$ ($P = 0.0004$) and $D' = 0.9977$ ($P = 0.0308$). The most frequent haplotypes were H+/P+/S and H+/P−/S; however, H−/P−/S and H−/P+/S haplotypes were more common in HIV-1-infected patients and blood donor groups, respectively.

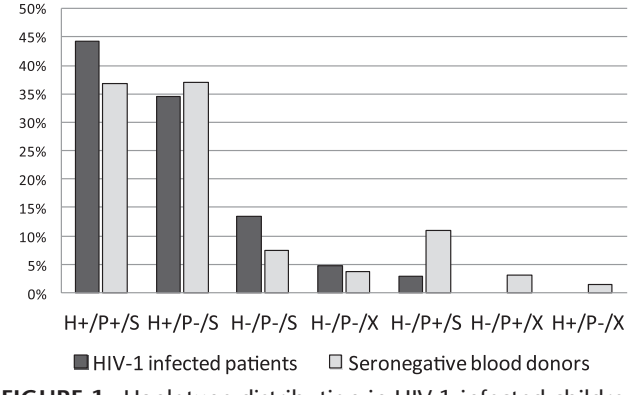

FIGURE 1. Haplotype distribution in HIV-1-infected children and blood donors.

LPL Genotypes Influence on TG Levels After 1 Year of HAART

We first compared TG levels at baseline and after 1 year of HAART in the 52 HIV-1-infected children. The median TG level was 136 mg/dL (IQR: 90–209) at baseline (n = 28) and not statistically different from the median of 129 mg/dL (IQR: 83–168) after 1 year of HAART (n = 39) ($P = 0.48$) (Fig. 2). There was no significant association among LPL polymorphisms and TG levels at baseline (data not shown). However, a statistically significant global level of association (Bonferroni corrected $P = 0.0022$) was found between TG values and *Pvu II* polymorphism after 1 year of HAART. Median TG levels were significantly lower in P−/P− [98.5 mg/dL (IQR: 60.8–122.3)] carriers when compared with P+/P+ [180 mg/dL (IQR: 137.8–220.3)] ($P = 0.039$), with the lowest median TG values observed in the P−/P− group. Even though there was no significant difference between P+/P− [131 mg/dL (IQR: 82–164)] patients compared with P+/P+ ($P > 0.05$), there was a trend towards lower TG values when the P− allele appeared. The P− allele showed a gene dose-dependent effect on plasma TG. Interestingly, none of the 10 HIV-1-infected children carrying the P−/P− genotype had TG values over 150 mg/dL; conversely, 7 out of 10 patients that were P+/P+ had TG values over 150 mg/dL after 1 year of HAART (Fig. 3B). *Hind III* and S447X polymorphisms

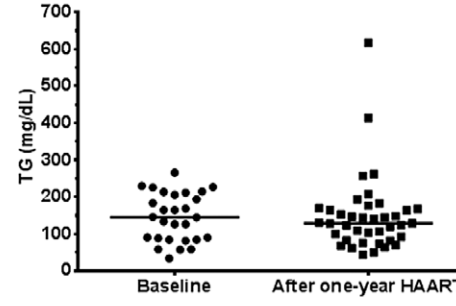

FIGURE 2. Plasma triglyceride values before and after 1 year of HAART. No difference was found between median TG values before (n = 28) and after 1 year of HAART (n = 39).

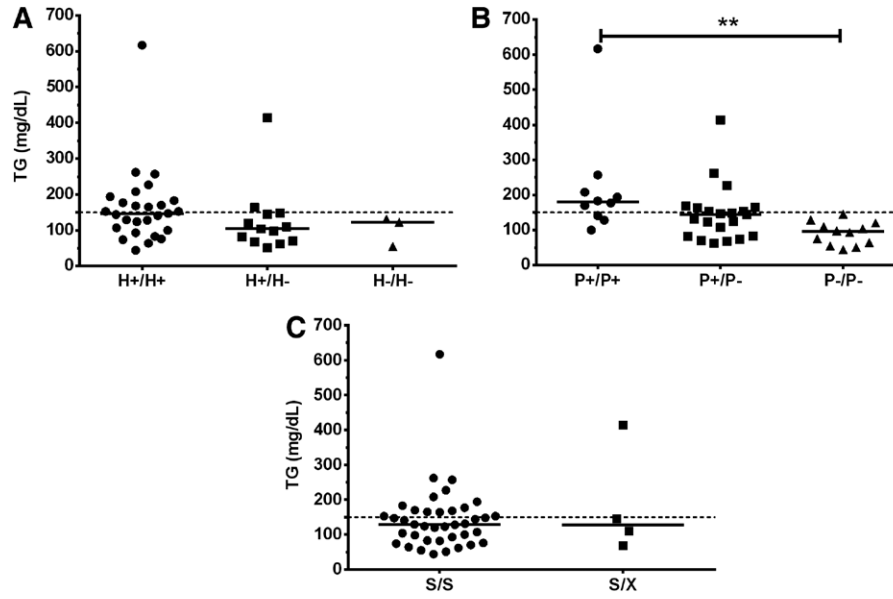

FIGURE 3. TG levels after 1 year of HAART according to LPL genotypes. Panel A represents the *Hind* III genotype. Panel B represents the *Pvu* II genotype. Panel C represents the S447X genotype. The discontinuous line is placed at TG levels of 150 mg/dL. Only significant contrasts were depicted ($P < 0.05$).

were not significantly associated with TG levels after 1 year of HAART ($P > 0.05$) (Fig. 3A and 3C). Nevertheless, a trend towards lower TG levels was detected in children carrying the H− allele. Notably, while almost 50% of H+/H+ carriers had TG levels over 150 mg/dL, just 18% of H+/H− patients and none of the H−/H− carriers had hypertriglyceridemia. Given that ARV regimens containing PI were the most frequent in this group of children (n = 31), we further analyzed whether the association between *Pvu II* and plasma TG levels after 1 year of HAART remained significant when excluding patients receiving non-PI regimens (n = 8). We confirmed that *Pvu II* was indeed associated with TG values only in patients receiving PI regimens ($P = 0.012$). Also, the influence of the P− allele in TG values remained significant when corrected for viral load ($P = 0.01$; CI 95%:−102.65 to −14.6).

Given the findings of a significant protective role of the P− allele against hypertriglyceridemia and a trend towards lower TG levels in carriers of the H− allele, we further explored if LPL haplotypes explained any of the variation in the risk of developing hypertriglyceridemia after 1 year of HAART (Table 3). Remarkably, the presence of the P− allele was associated with up to an 11-fold lower risk of hypertriglyceridemia; additionally, when H− and P− were both present, this effect increased to over 16-fold. However, the additional existence of the X allele was not significantly associated with any protection against TG values over 150 mg/dL.

We further assessed if HIV-1 viral loads and $CD4^+$ T cells percentage in our study group were influenced by the LPL genotypes studied; no significant association was observed among these characteristics and the polymorphisms (data not shown).

TABLE 3. Risk of Developing Hypertriglyceridemia After 1 Year of HAART According to LPL Haplotype

LPL Haplotypes	OR (95% CI)	*P*
H+/P+/S	1.00	—
H+/P−/S	0.09 (0.02–0.43)	**0.004**
H−/P−/S	0.06 (0.01–0.74)	**0.033**
H−/P−/X	0.25 (0.02–3.58)	0.310

Global haplotype association *P* value: 0.0018.
Bold values are statistically significant.

DISCUSSION

In this study, we explored the role of 3 LPL genetic variants in plasma TG values in a group of HIV-1-infected children receiving HAART. We found that the P− allele had a diminishing effect on TG levels after 1 year of HAART in a gene dose-dependent manner. The presence of the P− allele alone significantly protected patients from developing hypertriglyceridemia, and the risk was even lower when the H− allele was also present.

Hypertriglyceridemia frequently occurred both before and after HAART initiation in this group of children. Hypertriglyceridemia at baseline was related to HIV-1 infection itself, consistent with prior reports establishing that HIV-1 infection alone increases plasma TG values.[35,36] Hypertriglyceridemia after receiving HAART for 1 year was associated with low median viral loads and a high percentage of $CD4^+$ T cell count, proxy indicators of medication adherence in children.

As there are no reports to date of the distribution of LPL variants in an Argentinean population, we first assessed it in a group of HIV-1 seronegative blood donors and found that the P− allele was very frequent (~50%), while H− (~25%) and 447X (~8%) were present in a smaller proportion. When compared with HIV-1-infected patients, no differences amid them were evident. Two of the SNPs, *Hind III* and S447X, were in linkage disequilibrium in both groups, as previously reported.[37–39] The Argentinean population is considered to be white-Hispanic due to mostly European ancestry; accordingly we found no difference for the French population[40] or Brazilians of European descendants[41,42] in any of the 3 SNPs analyzed. In Spanish[38] and Italian[43] populations there was no difference in *Hind III* and S447X distribution. Also, no difference was found for *Hind III* variants when compared with Saudi[44] or Chinese[45] populations; conversely, there was a significant difference ($P < 0.05$) regarding *Pvu II* distribution, with a higher proportion of the P− allele in our population in both cases.

The role of LPL genetic variants has been explored in many pathological settings, such as cardiovascular disease, dyslipidemia and type two diabetes;[46,47] however, in adult HIV-1-infected patients receiving HAART, data are scarce, and to our knowledge this is the first report in children. Our findings strongly indicate a protective effect of the P− allele against high plasma TG values in children receiving HAART. Moreover, this occurred in a gene dose-dependent manner; thus, children carrying the P−/P− genotype had the lowest TG values and none rose above 150 mg/dL. In a different setting, Sepetiba et al[42]

reported, in a group of pregnant women (n = 88), that those carrying the P−/P− genotype had the lowest TG levels during puerperium and none above 150 mg/dL. Also, Wang et al[48] found in an adult population at risk of coronary artery disease (CAD) (n = 475) a dose-dependent relationship between the presence of the P− allele and low TG levels. However, there are several reports indicating that *Pvu II* does not have an association with plasma lipid levels[49–52] or CAD[53] in adults.

In our study an increase in the protective effect of the P− allele was encountered when H− appeared in the same haplotype; this is explained by an additive effect of both loci, since a trend towards lower TG values after 1 year of HAART in H− carriers was also observed, even though the association between *Hind III* and TG values neither at baseline nor after 1 year of HAART was statistically significant. Several studies found a relationship between *Hind III* and hypertriglyceridemia[40,41,54] and hypercholesterolemia,[55] but others failed to encounter an association with lipid levels or CAD.[43,44,56]

In a recent study, Marzocchetti et al[23] recruited 174 HIV-1-infected adults receiving HAART and evaluated the impact of S447X in the LPL gene, among other SNPs, in plasma lipid levels. A trend towards a lower risk of reaching high TG levels was associated when comparing the S447X mutant against the wild type. Nonetheless, they defined hypertriglyceridemia as TG > 500 mg/dL. Also, a considerable number of reports in adults have shown significantly lower plasma TG levels and higher plasma HDL cholesterol levels among 447X carriers when compared with noncarriers.[46,57–63]

In contrast, we did not find an overall association between the S447X genotype and plasma TG levels, either before or after 1 year of HAART. Studies that included healthy children by Chen et al, and children with or without a paternal history of CAD by Talmud et al have suggested that the effect of the S447X polymorphism in TG levels is more pronounced in adults and less perceptible in children.[64,65] Changes in the penetrance of underlying genes as well as the effect of cumulative exposure to certain environmental factors are considered to affect variability in lipoprotein levels with age;[66,67] still how aging affects the expression of this particular polymorphism remains to be determined. Additionally, the low number of patients carrying the 447X variant, only 4 S/X and no X/X, may not be enough to detect the presence of a true association between this SNP and plasma TG levels in this group of HIV-1-infected children.

Summarizing, there is growing evidence that these LPL polymorphisms have an effect on plasma lipids and risk to CAD. Whether they affect TG levels at baseline cannot be ruled out immediately in our group of patients, given the low number of children with data at this point. Thus, the association found between *Pvu II* and TG levels after 1 year of HAART may be due to an effect that was present before HAART started and enhanced after. Yet, the results point to an influence of this SNP when HAART has been present for at least a year. Various in vitro studies have addressed the impact of antiretroviral drugs in the expression of several genes that control the differentiation and lipid metabolism of adipocytes, such as the LPL gene.[68,69] It is likely that polymorphisms in intronic sequences, like *Pvu II*, may act as regulatory elements or be in LD with such elements, probably having a different response to the effect that some PIs, such as nelfinavir, ritonavir and saquinavir, have on the expression of LPL mRNA.[70]

This study group was fairly homogeneous with a high percentage of children under the PI regimen. The use of PIs has been associated with hypertriglyceridemia and hypercholesterolemia, in both healthy volunteers[71] and HIV-1-infected patients[72]; yet, we were able to distinguish a group of patients carrying a particular SNP in the LPL gene (P−/P− genotype) that in spite of having the same kind and time of exposure to ARV drugs had significantly lower plasma TG values. This approach allowed us to evaluate the interaction between ARV treatment and the genetic background involved in lipid metabolism for the first time in children under HAART.

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