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Metabolic events in HIV-infected patients using abacavir are associated with erythrocyte inosine triphosphatase activity.

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ABSTRACT

Objectives

Abacavir use has been associated with an increased risk of cardiovascular disease (CVD) and metabolic events in HIV-infected patients, although this finding was not consistently found. It is unclear whether abacavir only increases this risk in subpopulations of HIV-infected patients. It may be hypothesized that inosine 5'-triphosphate pyrophosphohydrolase (ITPase), an enzyme involved in the metabolism of purine analogues used in HIV treatment, plays a role in the risk of CVD and metabolic events in HIV-infected patients.

Methods

ITPase activity and *ITPA* genotype were determined in 393 HIV-infected patients. ITPase activity <4 mmol IMP/mmol Hb/h was considered decreased. *ITPA* polymorphisms tested were: c.94C>A (rs1127354) and c.124+21A>C (rs7270101). ORs were determined using generalized estimating equation models for developing CVD in patients who had ever been exposed to abacavir, tenofovir or didanosine and for developing metabolic events in patients currently using these drugs.

Results

In patients using abacavir, metabolic events were associated with ITPase activity. No association was demonstrated for tenofovir or didanosine. The OR for metabolic events was 3.11 in patients using abacavir with normal ITPase activity (95% CI 1.34–7.21; p=0.008) compared with patients with decreased ITPase activity (adjusted for age, BMI, cumulative duration of combination antiretroviral therapy (cART) use and the use of PI and NNRTI). CVD was not associated with ITPase activity or *ITPA* genotype.

Conclusions

This study shows, for the first time, that ITPase activity is associated with the occurrence of metabolic events in patients using abacavir. Further studies are needed to confirm this association and to elucidate the possible mechanism.

INTRODUCTION

The life expectancy of patients infected with HIV has improved substantially in recent decades[1] and in developed countries has been approaching that of the non-HIV-infected general population.[2] In the Netherlands, currently, 42% of HIV patients are older than 50 years of age[3] and in the USA, at year-end in 2015, the group of patients aged 50–54 years made up the largest percentage of persons living with HIV (18%).[4] With increasing age, non-communicable comorbidities such as cardiovascular disease (CVD) increase, especially among HIV-infected patients using combination antiretroviral therapy (cART).[5–7] For CVD it is unclear what the contribution of the HIV infection itself is compared with the drugs that are part of the cART regimens. In a meta-analysis the pooled relative risk of CVD among HIV-infected patients without treatment was 1.61 compared with people not infected with HIV.[8] The pooled relative risk for HIV-infected patients on treatment was 2.00 compared with non-HIV-infected people.[8] Different antiretroviral medication classes have been associated with a higher risk of CVD. The relative risk of CVD was 1.41 (95% CI 1.2–1.65) for patients on PI-based cART compared with those on non-PI-based cART.[8] An increased rate of myocardial infarction in patients recently treated with abacavir and didanosine was first reported in the D:A:D study.[9] Since then, several cohort studies and randomized clinical trials have confirmed the association of abacavir use and increased risk for CVD[10–12] and in a meta-analysis the pooled relative risk was 1.8 (95% CI 1.43–2.26; $p<0.001$) for patients treated with abacavir.[8] Currently this association is still debated, as other studies have found either no effect or no significant effect of abacavir use on the risk of CVD.[13,14]

The pathogenic mechanism of the potential association between abacavir use and CVD is not clear. Which factors predispose patients using abacavir to a higher risk of CVD, apart from the classic risk factors such as dyslipidaemia, hypertension (HT) and smoking, has not yet been elucidated. It may be hypothesized that differences in the metabolism of abacavir predispose to a higher risk for CVD. The active metabolite of abacavir is carbovir triphosphate, which is a guanosine analogue. Guanosine 5'-triphosphate (GTP) is a low-affinity substrate for the enzyme inosine 5'-triphosphate pyrophosphohydrolase (ITPase) compared with the natural substrate inosine 5'-triphosphate (ITP).[15] ITPase is one of the scavenger enzymes eliminating the potentially cytotoxic or genotoxic non-canonical nucleoside triphosphates from the nucleotide pool[16] and is encoded by the *ITPA* gene on chromosome 20p (OMIM #147520). The SNPs c.94C>A (p.Pro32Thr, NCBI rs1127354) and c.124+21A>C (NCBI rs7270101) in the *ITPA* gene can cause a decrease in ITPase activity.[17] In HIV-infected patients, ITPase was found to have decreased expression in leukocytes, which was not associated with *ITPA* genotype,[18] and decreased activity in erythrocytes in

patients with *ITPA* genotype wt/wt or c.94C>A compared with a control population with the same genotype.[19]

The natural role of ITPase, the different ITPase activity in HIV-infected patients and the potential role of ITPase in the metabolism of the purine analogues used in HIV treatment, suggest that ITPase activity may play a role in the risk of CVD in patients using abacavir.

We therefore determined whether ITPase activity and *ITPA* genotype are associated with the occurrence of CVD and with risk factors such as HT, hypercholesterolaemia and diabetes mellitus (DM) during abacavir use in a cohort of HIV-infected patients.

PATIENTS AND METHODS

Patients

HIV-infected patients at the outpatient clinic of the Maastricht University Medical Center in Maastricht, The Netherlands, who were treated with cART, were included in this study after providing written informed consent. The data used for this study were collected as described previously.[20] The study was performed according to the Helsinki Declaration and approved by the Medical Ethics Committee of the Maastricht University Medical Center, Maastricht, The Netherlands.

Endpoints

The endpoints of the study were CVD and metabolic events. CVD was defined as: reported cerebrovascular accident (CVA) (transient ischaemic attack, haemorrhagic/ischaemic/unspecified CVA), arterial occlusion, peripheral stent or bypass, coronary event (coronary artery bypass graft, percutaneous intervention or myocardial infarction) or anamnestic complaints of peripheral claudication, during the use of cART. Metabolic events were defined as: dyslipidaemia as the reported reason for stopping the cART regimen, laboratory-confirmed dyslipidaemia (total cholesterol >6.5 mM, triglycerides >2.3 mM, LDL >4.5 mM), use of lipid-lowering therapy (statins and/or fibrates), DM or HT. DM was defined as fasting glucose ≥7 mM (126 mg/dL) measured on two or more consecutive occasions within 3 months, or glucose ≥11.1 mM (200 mg/dL) measured once and in combination with symptoms of hyperglycaemia, or if the patient was reported as being diabetic, or if the patient had glucose-lowering therapy.[21] HT was defined as mean systolic pressure >140 mmHg, mean diastolic pressure >90 mmHg or if the patient used anti-hypertensive medication.[22]

ITPase activity

Erythrocyte ITPase activity was determined as described previously[23] and assessed by formation of inosine 5'-monophosphate (IMP) from ITP. ITPase activity was expressed as millimoles (mmol) of IMP formed from ITP in 1 h per mmol hemoglobin (mmol IMP/mmol Hb/h). ITPase activity of ≥4 mmol IMP/mmol Hb/h was considered normal, which is the lowest value within the 95% CI for *ITPA* wild-type (wt/wt) carriers.[19,24]

ITPA genotype analysis

ITPA genotype was analysed as described previously by Bierau et al.[19] The Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) was used to isolate genomic DNA from whole blood. The DNA was genotyped using Sanger sequencing for the two *ITPA* polymorphisms; c.94C>A (p.Pro32Thr, rs1127354) and c.124+21A>C (rs7270101). The genotype was considered to be wild-type (wt/wt) when neither of the polymorphisms was detected. All sequences were evaluated by two independent laboratory experts.

Statistical analysis

All analyses were performed with IBM SPSS Statistics 21 (IBM Corporation, NY, USA) and the statistical software package R (free download from www.rproject.org) version 3.4.3. Pearson's χ^2 tests, Fisher's exact test, independent samples T-tests and Mann–Whitney U tests were used to determine significant differences.

The occurrence of CVD was analysed using generalized estimating equation models to account for repeated statement and adjusted for differences in CVD risk profile: age at the time of changing the cART regimen, the last measured BMI, smoking status (current or former smoker), pre-existing CVD before the start of cART, HT, DM, dyslipidaemia and gender. Further adjustment for cumulative total duration of cART and use of a PI or an NNRTI at the time of the event was performed. Numerical variables were standardized in order to have the same scale.

The occurrence of a metabolic event was analysed using generalized estimating equation models to account for repeated statement and adjusted for cumulative total duration of cART, age at the time of changing the cART regimen, the last BMI measured, use of a PI and use of an NNRTI at the time of the event. Numerical variables were standardized in order to have the same scale.

Since tenofovir disoproxil fumarate (further referred to as tenofovir) and didanosine are also purine analogues, their active metabolites being adenine nucleotide analogues instead of guanosine nucleotide analogues such as carbovir, the association of ITPase activity, *ITPA* genotype and CVD in patients using these drugs in their cART regimen was determined in

addition. For exposure to either abacavir, tenofovir or didanosine, in the analysis of CVD we used current (which means use at the time of the event) and past exposure. Only current exposure was used in the analysis of metabolic events.

When abacavir, tenofovir or didanosine were used concomitantly in one cART regimen, this regimen was excluded from the analysis. P-values <0.05 were considered to be statistically significant. We did not correct for multiple testing because our analyses were hypothesis driven.

RESULTS

Patient characteristics

After excluding regimens containing a combination of didanosine, tenofovir or abacavir, 1422 regimens in 393 patients were used in the analysis.[20] Of these patients, 52.2% had an ITPase activity of <4 mmol IMP/mmol Hb/h (Table 1). No statistically significant difference was found between patients with decreased ITPase activity and with normal ITPase activity with regard to age, gender, race, CD4 nadir counts and PI and NNRTI use. In 60.1% of regimens containing no purine, a PI was part of the cART regimen; this was, respectively, 34.5%, 22.9% and 60.2% in regimens containing tenofovir, abacavir or didanosine. Current exposure to the purine analogues tenofovir, abacavir and didanosine was frequent (n=601 (42.3%), n=244 (17.2%) and n=128 (9.0%) respectively). The number of regimens with current plus past exposure to a purine analogue was 699 (49.2%) for tenofovir, 465 (32.7%) for abacavir and 365 (25.7%) for didanosine. CVD was present in 12.5% of the patients (n=49) and reported to be CVA (n=15), arterial occlusion (n=2), peripheral stent or bypass (n=6), coronary arterial bypass graft (n=6), percutaneous intervention (n=12), myocardial infarction (n=7) or anamnestic complaints of peripheral claudication (n=1). In patients with current or past exposure to abacavir, 24 CVD events occurred; CVA (n=7), coronary artery bypass grafting (n=3), percutaneous coronary intervention (n=7), myocardial infarction (n=2), arterial occlusion (n=2) and peripheral stent or bypass (n=3). In 749 regimens (52.7%) a metabolic event was found. An overview of different components of the combined endpoint metabolic event is presented in Table 2.

Effect of ITPase activity and *ITPA* genotype on CVD

No significant difference in CVD between the regimens used in patients with decreased versus normal ITPase activity was found, irrespective of cART regimen used (22 versus 27 events, respectively; crude p=0.34). ITPase activity in combination with current or past exposure to abacavir was not associated with a higher number of CVD events compared with tenofovir or didanosine exposure in our cohort (Table 3).

Table 1. Demographic and clinical characteristics of the patients (n=393) with ITPase activity <4 and ≥4 mmol IMP/mmol Hb/hour.

Characteristic	ITPase activity[a]		P-value
	<4 (n=205)	≥4 (n=188)	
Age*; mean years ± SD[b]	50.1 ± 11.1	49.7 ± 11.9	0.78
Male gender,* n (%)	164 (80.0%)	155 (82.4%)	0.53
Race, n (%)*			0.81
Caucasian	164 (80.0%)	147 (78.2%)	
Hispanic	5 (2.4%)	4 (2.1%)	
African	22 (10.7%)	27 (14.4%)	
Asian or other	14 (6.9%)	10 (5.3%)	
Mean ITPase activity* ± SD	2.44 ± 1.12	5.24 ± 1.09	<0.001
ITPA* genotype, n (%)			<0.001
Wt/wt	90 (43.9%)	175 (93.1%)	
Wt/c.124+21A>C	59 (28.8%)	9 (4.8%)	
Wt/c.94C>A or other[c]	53 (25.9%)	-	
Unknown	3 (1.5%)	4 (2.1%)	
Alcohol use*, n (%)			0.51
<2 (IU/day)	157 (76.6%)	133 (70.7%)	
≥2 (IU/day)	35 (17.1%)	36 (19.1%)	
Unknown	13 (6.3%)	19 (10.1%)	
Smoking, n (%)			0.65
Never	18 (8.8%)	21 (11.2%)	
Current or former	130 (63.4%)	116 (61.7%)	
Not current, unknown if ever	55 (26.8%)	46 (24.5%)	
Unknown	2 (1.0%)	5 (2.7%)	
Body mass index, n (%)			0.09
Underweight (<18.5)	14 (6.8%)	3 (1.6%)	
Normal (≥18.5-<25)	102 (49.8%)	95 (50.5%)	
Mild/Moderate obesity (≥25-<30)	59 (28.8%)	52 (27.7%)	
Severe obesity (≥30)	10 (4.9%)	15 (8.0%)	
Unknown	20 (9.8%)	23 (12.2%)	
Median CD4 nadir*, x10^6 cells/L (range)	207 (1-1022)	209 (3-612)	0.40
Median year of start cART* (range)	2006 (1987-2013)[c]	2006 (1987-2013)[d]	0.25
Total number of cART regimens*	734	688	
Type cART, n (%)			0.09
NNRTI[d]	342 (46.6%)	295 (42.9%)	
PI[e]	239 (32.6%)	267 (38.8%)	
NNRTI[d] and PI[e]	59 (8.0%)	45 (6.5%)	
Other	94 (12.8%)	81 (11.8%)	

* previously published; [a] mmol IMP/mmol Hb/hour; [b] SD, standard deviation; [c] Other = homozygous c.124+21A>C or homozygous c.94C>A or heterozygous c.124+21A>C/c.94C>A; [d] NNRTI, nucleoside/nucleotide analog reverse-transcriptase inhibitor; [e] PI, Protease inhibitor

Table 2. Diabetes mellitus, dyslipidemia and use of blood pressure medication or cholesterol lowering therapy for regimens used in patients with decreased and normal ITPase activity.

	All regimens		P-values	Regimens currently containing abacavir		P-values
	ITPase activity[a]			ITPase activity[a]		
	<4 (n=734)	≥4 (n=688)		<4 (n=131)	≥4 (n=113)	
Diabetes, n (%)			0.03			0.04
Yes	28 (3.8)	45 (6.5)		3 (2.3)	11 (9.7)	
Unknown	-	18 (2.6)		-	-	
Hypertension, n (%)			0.03			0.16
Yes	146 (19.9)	171 (24.9)		26 (19.8)	31 (27.4)	
Unknown	-	-		-	-	
Dyslipaemia, n (%)			0.01			0.17
Yes	195 (26.6)	226 (32.8)		40 (30.5)	44 (38.9)	
Unknown	-	-		-	-	
Cholesterol lowering therapy, n (%)			0.014			0.68
Yes	86 (11.7)	117 (17.1)		20 (15.3)	23 (20.4)	
Unknown	10 (1.4)	9 (1.3)		1 (0.8)	1 (0.9)	

[a] mmol IMP/mmol Hb/hour

Table 3. CVD and metabolic events for regimens used in patients with decreased and normal ITPase activity and different *ITPA* genotypes.

	ITPase activity[a]		Crude p	Adjusted p	*ITPA* genotype		Crude p	Adjusted p
	<4	≥4			Other[b]	wt/wt		
	n / Regimens	n / Regimens			n / Regimens	n / Regimens		
CVD								
Total[c] n=1422	22/734	27/688	0.34	0.82	13/435	35/960	0.72	0.86
Tenofovir[d] n=699	12/354	17/345	0.31	0.50	7/211	21/478	0.35	0.77
Abacavir[e] n=465	11/245	13/220	0.49	0.40	6/132	18/328	0.86	0.41
Didanosine[f] n=365	2/165	6/200	0.30	0.45	0/91	8/272	0.24	n.a.
Metabolic events								
Total[c] n=1422	356/734	393/688	0.001	0.12	202/435	537/960	0.001	0.14
Tenofovir[g] n=601	155/306	168/295	0.12	0.55	89/186	231/409	0.13	0.53
Abacavir[e] n=244	63/131	82/113	<0.0001	0.008	39/75	106/164	0.003	0.24
Didanosine[h] n=128	25/51	37/77	0.92	0.75	12/31	49/96	0.26	0.61

[a] mmol IMP/mmol Hb/hour; [b] heterozygous wt/c.124+21A>C or wt/c.94C>A or homozygous c.124+21A>C or homozygous c.94C>A or compound heterozygous c.124+21A>C/c.94C>A; [c] Genotype unknown in 27 regimens; [d] Genotype unknown in 10 regimens; [e] Genotype unknown in 5 regimens; [f] Genotype unknown in 2 regimens; [g] Genotype unknown in 6 regimens; [h] Genotype unknown in 1 regimen

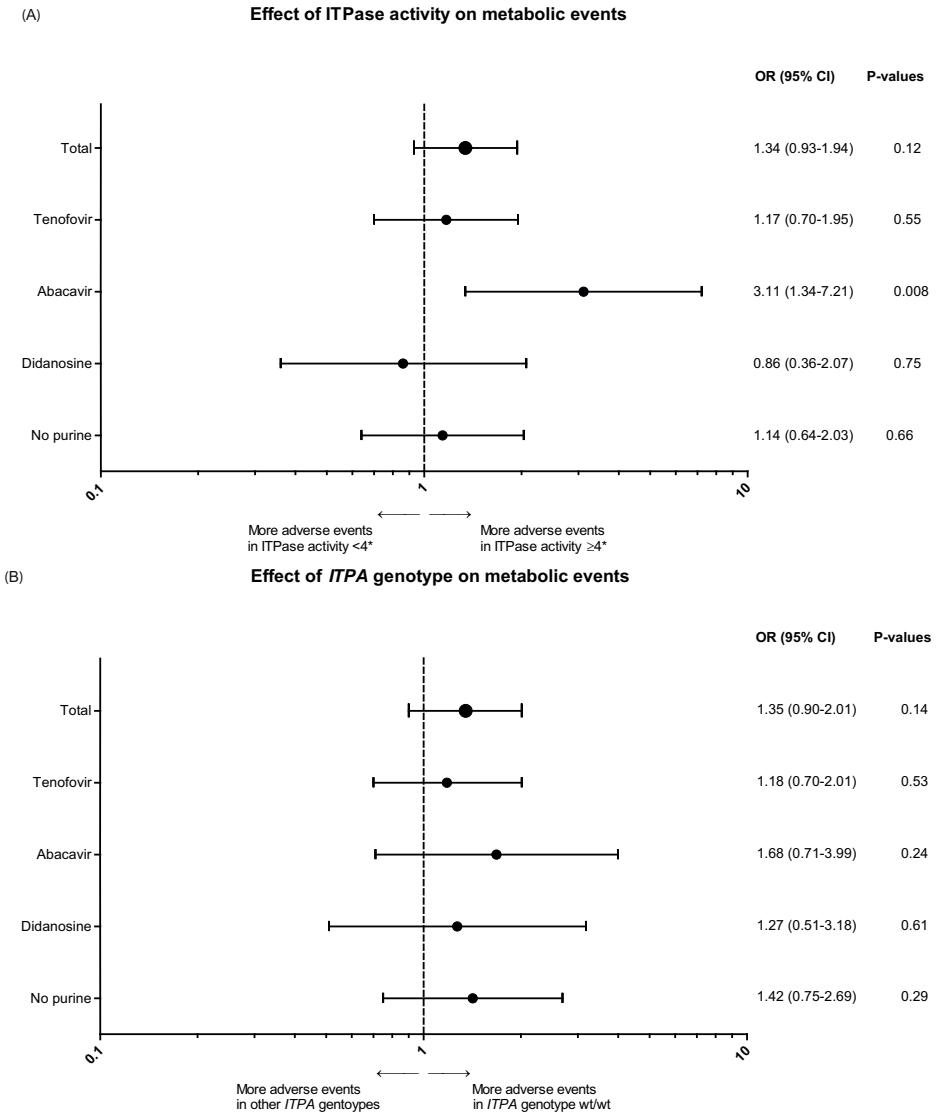

Figure 1: Effect ITPase activity (A) and *ITPA* genotype (B) on metabolic events. The effect of decreased versus normal ITPase activity (A) and of other *ITPA* genotypes versus *ITPA* genotype wt/wt (B) on the occurrence of metabolic events are plotted for all regimens (Total), and for regimens containing tenofovir, abacavir, didanosine or no purine. Odds ratio (OR) with 95% confidential interval and matching p-values are displayed. * mmol IMP/mmol Hb/hour

Regardless of cART regimen, CVD more often occurred in patients with *ITPA* genotype wt/wt versus the other *ITPA* genotypes [CVD: n=35 (71.4%) versus n=13 (26.5%) respectively; although this difference was not statistically significant (p=0.72)] (Table 3). One CVD oc-

curred in a patient with unknown *ITPA* genotype. Adjusting the data for CVD risk profiles, cumulative cART use and PI and NNRTI use did not change the outcome for CVD.

Effect of ITPase activity and *ITPA* genotype on metabolic events

Of all the metabolic events, 52.5% occurred during regimens prescribed to patients with normal ITPase activity (crude p=0.001) (Table 3). In 72.6% of the regimens currently containing abacavir used by patients with normal ITPase activity, a metabolic event occurred, compared with 48.5% in patients with decreased ITPase activity (p<0.0001; Table 3). After adjusting for age, BMI, cumulative duration of cART use and the use of PI and NNRTI, the OR for developing a metabolic event was statistically significantly higher in patients with normal versus decreased ITPase activity (3.11, 95% CI 1.34–7.21; p=0.008) in abacavir-containing regimens. In regimens containing tenofovir or didanosine, no association between ITPase activity and the occurrence of metabolic events could be demonstrated (Figure 1a). Also in regimens without a purine analogue but in 60% containing a PI no association was found (Figure 1a).

In regimens prescribed in patients with wt/wt genotype, metabolic events were more frequently found compared with the other genotypes (537/960 regimens versus 202/435 regimens; p=0.001) (Table 3). Metabolic events occurred more frequently in patients using abacavir and having the wt/wt genotype (p=0.003). This increase could not be found in patients using tenofovir or didanosine (Table 3). However, after adjusting for age, BMI, cumulative cART use and the use of PI or NNRTI, no significant difference in metabolic events between patients using abacavir with wt/wt genotype and the other *ITPA* genotypes could be demonstrated (Figure 1b).

DISCUSSION

In this study we show, for the first time, that ITPase activity may play a role in the association of abacavir use and the occurrence of metabolic events in HIV-infected patients using cART. In regimens containing abacavir decreased ITPase activity was associated with fewer metabolic adverse events. We could not confirm the hypothesis that decreased ITPase activity decreased the risk of CVD. No association between *ITPA* genotype and CVD or metabolic events was found after adjustment for other CVD risk factors.

In multiple studies it was reported that the use of abacavir increased the risk of developing CVD or myocardial infarction.[9–11] Other studies, however, could not confirm this association.[14,25] We found no association between abacavir use, ITPase activity and CVD events, possibly owing to the relatively small number of CVD events found in our study. Like oth-

ers, we found that metabolic events were associated with abacavir, but not with tenofovir use.[26–30] A possible explanation may be the difference in chemical structure since tenofovir is an adenosine analogue and abacavir is a guanosine analogue.

How ITPase might be involved in the occurrence of metabolic events during abacavir use is not known. Carbovir triphosphate was found not to be a direct substrate for ITPase.[20] Potentially the answer lies in cellular signal transduction. Guanosine 3',5'-cyclic monophosphate (cGMP) is an important secondary messenger that modulates multiple cellular processes, such as platelet aggregation, neurotransmission, blood pressure, lipolysis and gut peristalsis.[31,32] cGMP is produced from GTP by the enzyme soluble guanylate cyclase (sGC)[33,34] and stimulation of sGC was found to be protective against obesity.[35] However, in the presence of nitric oxide (NO) and magnesium ions (Mg^{2+}) sGC shifts its substrate specificity to ITP to form inosine 3',5'-cyclic monophosphate (cIMP) and also, in the absence of hypoxaemia, addition of exogenous ITP to intact porcine arteries led to higher cIMP levels.[36,37] In contrast to cGMP, which induces vasodilatation, cIMP induces vasoconstriction.[36] This vasoconstriction has been well studied by measurement of flow-mediated dilatation (FMD) of the brachial artery and is strongly associated with the risk of CVD.[38–40] Patients using abacavir were found to have lower FMD than patients not using abacavir,[41] whereas didanosine and tenofovir use were not associated with lower FMD. Support for the hypothesis that abacavir shifts sGC to use ITP instead of GTP, leading to higher cIMP levels, can be found in the study by Baum et al.[42] Formation of cGMP was decreased in human platelets incubated with carbovir triphosphate (the active metabolite of abacavir) and the authors concluded that carbovir triphosphate inhibited sGC. The question remains whether sGC was inhibited, carbovir triphosphate competed with GTP or a shift in substrate specificity of sGC occurred. New research is warranted to investigate under which circumstances ITP is a substrate for sGC, what the consequences of this shift in substrate specificity are and what the impact of carbovir triphosphate is on sGC activity in human cells.

In 2010 Fellay et al.[17] showed that the SNPs 94C>A and 124+21A>C in the *ITPA* genotype were associated with the protection against hemolytic anemia during treatment with ribavirin for hepatitis C infection. We found ITPase might be a more accurate predictor of the development of hemolytic anemia in these patients than *ITPA* genotype.[43] In HIV-infected patients both ITPase activity and *ITPA* genotype were associated with adverse events; however, *ITPA* genotype correlated less well with adverse events compared with ITPase activity.[20] In the present study again, we found *ITPA* genotype and ITPase activity were both crudely associated with adverse events; however, after adjusting with the logistic mixed effects model, only ITPase activity remained statistically significant. The difference in the effect of ITPase activity versus *ITPA* genotype in HIV-infected patients may be explained by the finding that in these patients the wt/wt and the c.94C>A carriers have decreased

erythrocyte ITPase activity compared with non-HIV-infected patients carrying the same genotypes.[19]

Here, we report a lower risk of metabolic events in HIV-infected patients that use abacavir and have decreased ITPase activity. In a previous study, in tenofovir-containing regimens, decreased ITPase activity was also associated with a lower risk of adverse events.[20] In contrast, the use of abacavir in this previous study was crudely associated with an increase in adverse events in patients having decreased ITPase activity. The adverse events in that study were different from the current study and included gastro-intestinal, neurological, renal, skin and liver-related adverse events, and potentially this explains why decreased ITPase activity may lead to an increase in adverse events in one study, but a decrease in adverse events in the other study. In other studies the association between decreased ITPase activity or a SNP in the *ITPA* genotype and adverse events was shown to be dependent on the kind of adverse event and the drug that was used, leading to, for instance, an increase in hepatic toxicity (during azathioprine or 6-mercaptopurine use) but a decrease in hemolytic anemia (during ribavirin use).[44–47] We hypothesize that the effect of ITPase activity in patients using abacavir is also dependent on the nature of the adverse events.

There are limitations that need to be mentioned. Our study was retrospective with all the possible caveats of retrospective studies. For example, factors other than the cART regimen could have contributed to alterations in metabolic events. This is, however, not different for patients with decreased or patients with normal ITPase activities, and thus the influence of this factor seems limited. Although our results could be mechanistically made plausible, further prospective studies are still warranted to confirm our results. Owing to the number of patients included, a low frequency of cardiovascular events was found. This may explain the lack of association of CVD with ITPase activity. Also, the clinical relevance of our findings is to be further established by larger prospective trials.

In conclusion, we showed that ITPase activity may be associated with a risk of metabolic events in HIV-infected patients using abacavir. Using ITPase activity as a potential biomarker to predict adverse metabolic events may be a further step towards more patient-tailored medicine in the future. However, elucidation of the pathogenic mechanism needs further studies and our results need to be confirmed in a prospective trial.

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