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Association of Circulating Cytochrome C with Clinical Manifestations of Antiretroviral-Induced Toxicity

Allison Langs-Barlow, MD^a, Shanmugapriya Selvaraj, MS^b, Onyema Ogbuagu, MD^b,
Veronika Shabanova^c, Eugene D. Shapiro, MD^{a,d}, and Elijah Paintsil, MD^{a,d,e}

Allison Langs-Barlow: Allison.Langs-Barlow@yale.edu; Shanmugapriya Selvaraj: ss_vel28@yahoo.com; Onyema Ogbuagu: Onyema.Ogbuagu@yale.edu; Veronika Shabanova: Veronika.Shabanova@yale.edu; Eugene D. Shapiro: Eugene.Shapiro@yale.edu; Elijah Paintsil: Elijah.Paintsil@yale.edu

^aYale School of Medicine, Department of Pediatrics, P.O. Box 208064, New Haven, CT 06520-8064, USA

^bYale School of Medicine, Department of Medicine, 330 Cedar St, Boardman 110, P.O. Box 208056, New Haven, CT 06520-8056, USA

^cYale School of Public Health, Center for Analytical Sciences, 300 George Street, Suite 555, New Haven, CT 06520, USA

^dYale School of Public Health, Department of Epidemiology of Microbial Diseases, 60 College Street, P.O. Box 208034, New Haven, CT 06520-8034, USA

^eYale School of Medicine, Department of Pharmacology, Sterling Hall of Medicine, 333 Cedar Street, SHM B-208, New Haven, CT 06520-8066, USA

Abstract

Diagnosis of antiretroviral therapy (ART) toxicity is complicated. Apoptosis has been implicated in ART toxicity. Cytochrome C (Cyt-C) is a mitochondrial protein found in plasma during pro-apoptotic states. We conducted a study of HIV-infected individuals on ART with (cases, n=21) and without (controls, n=21) clinical evidence of toxicity to determine if elevated plasma Cyt-C is associated with ART toxicity. When corrected for CD4 count, viral load, and duration of HIV infection, cases are 7.86 times more likely than controls to have plasma Cyt-C > 0.216 ng/mL. Cyt-C could be a useful clinical tool to guide treatment decisions in this population.

Keywords

antiretroviral therapy; Cytochrome C; mitochondrial toxicity

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Correspondence and Reprints to: Elijah Paintsil, MD, Departments of Pediatrics & Pharmacology, Yale School of Medicine, 464 Congress Avenue, New Haven, CT 06529; Tel: 203 785 6101; Fax: 203 785 6961; Elijah.Paintsil@yale.edu.

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1.1 INTRODUCTION

Though antiretroviral therapy (ART) has decreased morbidity and mortality for Human Immunodeficiency Virus (HIV), long-term therapy is associated with toxicity [1]. As ART use increases worldwide, the need for toxicity monitoring has become imperative. The 2013 UNAIDS report on the global AIDS epidemic highlights ART pharmacovigilance as a priority for HIV care programs. The prevalence of all ART adverse events is reported to be 47% for clinical and 27% for laboratory manifestations, with 9%–16% of these leading to changes in medication, hospitalization, chronic disability, or death [2]. These side effects are due in part to ART-induced mitochondrial toxicity.

Clinical manifestations of ART-induced mitochondrial toxicity are protean and can affect nearly any organ system [1]. Examples of well known manifestations are lactic acidosis, lipodystrophy, and peripheral neuropathy [2], although more subtle manifestations, such as anemia [3] may also have lasting implications, especially for children receiving ART. The underlying mechanism for mitochondrial toxicity, especially in the case of nucleoside/tide reverse transcriptase inhibitors (NRTIs), is attributed to inhibition of polymerase gamma, the key enzyme responsible for mitochondrial DNA replication [4]. In addition NRTIs as well as other classes of antiretroviral agents, such as non-nucleoside reverse transcriptase inhibitors and protease inhibitors, have been reported to increase apoptosis in various cell types [5–7], whether as a result of or in addition to mitochondrial damage has been suggested [7] though not established. Outside of clearly defined clinical manifestations, diagnosis of mitochondrial toxicity is difficult given that there is no gold standard test. A combination of clinical symptoms, laboratory testing, and imaging studies, culminating in tissue biopsy to look for mitochondrial damage is deemed to be the most accurate method [8, 9], though it is cost-prohibitive and invasive. Thus, a “trial and error” approach to ART toxicity, in which medications are stopped or switched to see if the clinical manifestations resolve, has become commonplace. This practice favors the emergence of drug-resistant strains of HIV [10] and could lead to inappropriate use of second-line medications, especially in resource-limited settings. There is a need for a non-invasive, cost-effective biomarker for ART-induced toxicity to prevent unnecessary interruptions in ART and to guide use of second-line regimens.

Given the association between exposure to various classes of ART and apoptosis, we investigated whether apoptotic indicators could be used as diagnostic markers of ART-induced toxicity. The mitochondrial protein Cytochrome C (Cyt-C) acts as a pro-apoptotic signal if released into cytosol [11]. Cyt-C is detectable extracellularly during pro-apoptotic states *in vivo* [12] as well as during *in vitro* models of drug induced renal and hepatic toxicity [13, 14]. Cyt-C can be detected in plasma using a commercial enzyme linked immunosorbant assay (ELISA) [10]. We hypothesized that concentrations of plasma Cyt-C would be higher in HIV-infected patients on ART with clinical or laboratory evidence of toxicity compared with ART exposed, HIV-infected controls without toxicity.

1.2 MATERIAL AND METHODS

We conducted a pilot, case-control study. Participants were enrolled at the Adult HIV Service at Yale-New Haven Hospital from April, 2011 to March, 2013. Potential cases of ART-induced toxicity were identified by their HIV care providers as having symptoms consistent with possible mitochondrial toxicity that could not be attributed to alternative diagnoses. For the purposes of our study, a case was defined as HIV infected, treated with an NRTI based ART regimen for > 12 months, and with clinical and/or laboratory evidence of one of the following ART toxicities [9, 15, 16]: pancreatitis, peripheral neuropathy, lactic acidosis (lactate > 2 mmol/L), anemia (hemoglobin < 11 g/dL), thrombocytopenia (platelets < 100,000 per mL), lipodystrophy, creatinine >1.2 mg/dl, cholesterol >199 mg/dl, and/or transaminitis (alanine transaminase/aspartate transaminase each > 100 U/L). Each case was matched for age, race, and gender with an HIV-infected control (also recruited from the Adult HIV Service), also treated with an NRTI based ART regimen for > 12 months, but without ART toxicity. The study protocol was approved by the Institutional Review Board of the Yale School of Medicine. Participants provided written informed consent prior to enrollment in the study.

Each participant donated 20 cc of whole blood at the time of enrollment. Samples were centrifuged at 2000 rpm for 15 minutes. Plasma was removed and stored at -80°C until use. Plasma Cyt-C concentrations were measured using the Human Cyt-C ELISA from Bender MedSystems (Vienna, Austria) according to manufacturer's instructions with slight modification [17, 18]. Samples were run in triplicate. The ELISA kit provided purified Human Cyt-C, which was freshly reconstituted and serially diluted (1:2) for each experiment and subjected to a 5-parameter curve fit. Final Cyt-C concentrations were read at 450nm using a microplate reader (SpectraMAX, Molecular Devices, Sunnyvale, CA). Levels of Cyt C are not affected by up to five freeze/thaw cycles per manufacturer report, which was verified in house with a positive control sample. The limit of detection of the ELISA is 0.04 ng/mL.

Spearman correlation coefficients were used to describe the association between patient characteristics and plasma Cyt-C concentrations. Differences between the groups were described using Wilcoxon Signed Rank tests. A receiver operator characteristics (ROC) curve was drawn to compare the sensitivity and 1-specificity of various Cyt-C concentration cutoffs for identifying cases. A generalized estimating equations approach (GEE) was used to evaluate the statistical significance of the difference in the proportion of cases and controls with plasma Cyt-C > 0.216 ng/mL adjusted for duration of HIV infection, viral load, and CD4 count.

1.3 RESULTS AND DISCUSSION

Twenty-one pairs of cases and controls were enrolled. Each group contained 38% females, with a mean age of 53 (+/- 7) years and was predominantly African American (71%), with 24% white non-Hispanic, and 5% Hispanic individuals. There was no difference between the groups with regards to CD4 count or viral load. Viral load was undetectable (<20 copies/mL) for 71% of cases and 86% of controls (p = 0.45). In the GEE model, viral load

was not correlated with Cyt-C levels. Past and present nucleoside/tide analogue exposure time was similar between the two groups [Table 1]. Current nucleoside/tide analogues in use were Truvada (tenofovir/emtricitabine), Atripla (efavirenz/tenofovir/emtricitabine), Combivir (lamivudine/zidovudine), Epivir (lamivudine), and Trizir (abacavir/lamivudine/zidovudine). Use of an integrase inhibitor, protease inhibitor(s), and/or non-nucleoside/tide reverse transcriptase inhibitor was not correlated with plasma Cyt-C concentration (Mann Whitney U test, $p=0.99$, 0.14 , and 0.15 respectively). Duration of HIV infection was the only variable to differ significantly between the two groups, with controls having had the diagnosis of HIV for a longer period of time in years (15.4 ± 7.3) than cases (8.4 ± 4.6) ($p<0.001$); there was no significant correlation with plasma Cyt-C level (Spearman rho = -0.14 , 95% CI 0.43 , 0.18).

The average duration of clinical and or laboratory abnormalities in the cases prior to attribution to ART was 4 years (± 3.56 years). Forty-eight percent of the cases had two or more manifestations of toxicity. There was no correlation between the presence of more than one manifestation of toxicity and higher Cyt-C levels ($p=0.65$). The cases had the following documented clinical manifestations of ART toxicity: hyperlipidemia (38%), elevated liver function tests (33%), thrombocytopenia (19%), elevated blood urea nitrogen/creatinine (19%), anemia (14%), lipodystrophy (14%), peripheral neuropathy (10%), lactic acidosis (5%), and pancytopenia (5%).

The difference in the median plasma concentration of Cyt-C was statistically significant between cases (0.65 ng/mL, range <0.04 – 3.85) and controls (<0.04 ng/mL, range <0.04 – 0.95 , $p < 0.01$) [Figure 1A]. Using the ROC approach, plasma Cyt-C concentration of >0.216 ng/mL had a sensitivity of 76.2% (95% CI 0.52 , 0.91) and a specificity of 71.4% (95% CI 0.47 , 0.88) to detect the cases [Figure 1B]. In addition, when we adjusted for the duration of HIV infection, viral load, and CD4 count using the GEE approach, we were able to demonstrate an odds ratio of 7.86 (95% CI 1.57 , 39.20) for cases to have a plasma level of Cyt-C > 0.216 ng/mL compared with controls ($p=0.01$). When our single outlier case and its matched control were removed from analysis, the difference in Cyt-C plasma concentration remained significant between the two groups ($p < 0.02$).

This is the first study to examine the utility of plasma Cyt-C as a biomarker of ART-induced mitochondrial toxicity in HIV infected patients on ART. We analyzed various concentration cutoffs of Cyt-C to determine which might have the best combinations of sensitivity and specificity, should it be used as a clinical test. No study to date has reported these test characteristics for plasma Cyt-C in any disease state. A mouse model of drug induced hepatic toxicity demonstrated serum levels of 50 to 150 ng/mL of Cyt-C, with the saline treated controls demonstrating levels of 0 to 50 ng/mL, as measured by ELISA [14]. In this and other drug toxicity studies, medications were being given with the intention of inducing high levels of cellular death, which may explain why the Cyt-C concentrations were higher than those seen in our study.

One limitation of our study was the inability to confirm the diagnosis of ART toxicity through biopsy, thus some of the clinical symptoms of the cases could have been misclassified. Nevertheless, given that primary HIV-care providers often “diagnose” ART

toxicity without use of the biopsy, we feel that our method of identifying cases was justified. Potential cases were recruited by their HIV care providers, and we used our case definition to ensure that each case currently had a manifestation of possible ART toxicity. Furthermore, we recently published results of additional mitochondrial studies looking at this same cohort of patients demonstrating that cases have significantly increased expression of polymerase gamma as well as other mitochondrial enzymes necessary for DNA replication, without a corresponding rise in mitochondrial DNA. We concluded that cases with possible ART-induced toxicity may be undergoing replicative stress, in that they require up-regulation of enzymes in order to maintain normal rates of mitochondrial DNA replication [19]. Well designed prospective studies are needed to further elucidate the effect of ART on the mitochondria of HIV-infected patients, especially in those who develop clinical and laboratory evidence of toxic effects.

Given the small sample size, correlation of types of toxicity and specific nucleoside analogs with plasma Cyt-C levels was not possible, nor were we able to evaluate the effect of co-morbidities on plasma Cyt-C levels in HIV-infected individuals. Given that we know Cyt-C can be elevated in other disease states, such as cancer [10], a thorough medical history would be important for future studies of plasma Cyt-C.

1.4 CONCLUSION

We have demonstrated that elevated plasma Cyt-C could serve as a biomarker for ART-induced toxicity in HIV-infected patients on ART. Collection of plasma for ELISA is less invasive, inexpensive, and technically simple, especially compared with tissue biopsy. Therefore, plasma Cyt-C is an excellent candidate for a larger, prospective investigation to assess its utility as a biomarker for ART toxicity.

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Abbreviations

ART	antiretroviral therapy
Cyt-C	Cytochrome C
HIV	human immunodeficiency virus
AIDS	acquired immunodeficiency syndrome
NRTI	nucleoside/tide reverse transcriptase inhibitors
ELISA	enzyme linked immunosorbant assay
ROC	receiver operator characteristics

GEE generalized estimating equations

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Highlights

- There is no gold standard for diagnosing antiretroviral-induced toxicity
- HIV treatment-experienced with toxicity had elevated plasma cytochrome C levels
- Plasma cytochrome C could serve as a biomarker of antiretroviral-induced toxicity
- Plasma cytochrome C assay is less invasive, inexpensive, and easily accessible

Figure 1.

Figure 1A: Absolute Plasma Cyt-C Concentration in HIV-infected Individuals with and without ART-Induced Mitochondrial Toxicity. Solid gray horizontal lines represent the median Cyt-C concentrations for the cases and controls respectively. The dotted horizontal line represents the limit of detection for the ELISA: 0.04 ng/mL. Significance of the difference between the two groups evaluated using Wilcoxon Signed Rank test. When analysis is run omitting the outlier from the cases, along with its matched counterpart in the HIV positive control group, the significance remains with $p < 0.02$.

Figure 1B: Receiver Operator Characteristic Curve for Plasma Cyt-C in HIV-infected Individuals. For Cyt-C Concentration > 0.216 ng/mL: Sensitivity 95% CI 0.52 to 0.91; Specificity 95% CI 0.47 to 0.88; PPV: positive predictive value with 95% CI 0.50 to 0.89; NPV: negative predictive value with 95% CI 0.51 to 0.90; LR: Likelihood Ratio. When the analysis is run without the outlier case and its matched control, the sensitivity and specificity of plasma Cyt-C >0.216 ng/mL to detect cases becomes 75.0% (95% CI 0.51, 0.91) and 75.0% (95% CI 0.51, 0.91) respectively. Area under the curve: 0.72 (p=0.01)

Table 1

Characteristics of study participants

	HIV-infected with Toxicity (n=21)	HIV-infected without Toxicity (n=21)	P Values
Variables	% or mean (SD)	% or mean (SD)	
CD4+ T-cell Count	602 (+/- 388)	750 (+/- 257)	0.16
Viral Load			
% undetectable	71.4%	85.7%	0.45
average viral load (copies/mL)	5296 (+/- 11769) ^a	5966 (+/- 10248) ^a	0.69
Duration of HIV infection (years)	8.4 (+/- 4.59)	15.4 (+/- 7.30)	0.0005 ^b
Current nucleoside/tide analog	100.00%	100.00%	1.00
Truvada (tenofovir/emtricitabine)	57.1%	38.1%	
Atripla (efavirenz/tenofovir/emtricitabine)	14.3%	42.9%	
Combivir (lamivudine/zidovudine)	4.8%	0.0%	
Epivir (lamivudine)	23.8%	14.3%	
Trizir (abacavir/lamivudine/zidovudine)	0.0%	4.8%	
Previous nucleoside/nucleotide analog exposures	71.4%	66.7%	0.73
More than one previous nucleoside/tide	33.3%	52.4%	1.00
No previous nucleoside/tide exposure	28.6%	33.3%	1.00
Total duration of nucleoside/tide exposure (years)	7.2 (+/- 4.69)	7.9 (+/- 3.55)	0.62
Current exposure to Integrase Inhibitor	19.0%	4.8%	0.25
Current exposure to Protease Inhibitor	66.7%	33.3%	0.09
Current exposure to NNRTI [¶]	38.1%	61.9%	0.23

P values calculated using paired t-test for continuous variables and McNemar's test for categorical variables. SD: standard deviation

^a HIV-infected with toxicity, n=6; HIV-infected without toxicity, n=3

^b statistically significant

[¶] NNRTI, non-nucleoside reverse transcriptase inhibitor