Association of serum total bilirubin and plasma 8-OHdG in HIV/AIDS patients

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(Received: May 16, 2017; Revised manuscript received: December 5, 2017; Second revised manuscript received: December 22, 2017; Accepted: December 30, 2017)

Abstract: Oxidative stress is a major contributor in the pathogenesis of insulin resistance (IR) and DNA damage in HIV/AIDS patients. Bilirubin has been shown to have antioxidant effects. In this case-control study, 600 subjects were included. We determined serum total bilirubin and IR in all subjects. We measured 8-hydroxy-2-deoxyguanosine with 8-hydroxy-2-deoxyguanosine enzyme-linked immunosorbent assay kit. IR and oxidative DNA damage were significantly higher in HIV-positive patients with second-line antiretroviral therapy (ART) and first-line ART than ART-naive patients. However, average serum total bilirubin was higher in ART-naive patients than the HIV-positive patients with second-line ART and first-line ART. In a logistic regression analysis, serum total bilirubin was negatively associated with the IR [odds ratio (OR): 0.0127, 95% confidence interval (CI): 0.023–0.070, $p = 0.0000$] and DNA damage (OR: 0.525, 95% CI: 0.351–0.783, $p = 0.0016$). We found that prevalence of IR and DNA damage was less in ART-naive patients compared with ART first-line and ART second-line HIV-positive patients. Larger studies are warranted to determine the molecular mechanisms involved in the negative association of serum bilirubin and DNA damage in ART naive patients.

Keywords: bilirubin, DNA damage, human immunodeficiency virus (HIV) infection, insulin resistance, 8-OHdG, type 2 diabetes

Introduction

The dramatic success of antiretroviral therapy (ART) has allowed people with human immunodeficiency virus (HIV) to live longer, but their chances of getting cancer are also rising. The burden of cancer among people living with HIV in the United States is undergoing notable change, according to new research presented here at the American Association for Cancer Research (AACR) 2017 Annual meeting. The study authors say that only 4.1% of the HIV population was older than 65 years in 2006, but that age group is projected to increase to 21.4% by 2030, according to their new estimates. In this patient population, the number of cancers linked to AIDS and suppressed immunity, such as Kaposi sarcoma and non-Hodgkin's lymphoma, is declining, and cancers related to aging are projected to rise in coming years. Oxidative stress-induced DNA lesions that may contribute to carcinogenesis are suggested by the increased cancer susceptibility of persons with a variety of chronic inflammatory diseases, such as ulcerative colitis, viral hepatitis, prostatitis, Helicobacter pylori infection, parasitic diseases, and others. In these diseases, cancer induction may be a pathological consequence of elevated reactive oxygen species (ROS) levels, which lead to increased steady-state levels of oxidative DNA damage, which in turn leads to a higher risk of mutations that may activate oncogenes or inactivate tumor-suppressor genes [1].

In type 2 diabetes mellitus (T2DM), oxidative stress gives rise to endothelial dysfunction. Recent studies show that oxidative stress plays an important role in insulin resistance (IR) and the development of T2DM [2, 3]. In our previous study, we have shown that exposure to oxidative stress is greater in HIV/AIDS patients undergoing ART than ART-naive HIV/AIDS patients. We have also shown that there was increased oxidative stress-induced IR and DNA damage present in these patients [4].

While hyperbilirubinemia has long been recognized as an ominous sign of liver dysfunction, recent data strongly indicate that mildly elevated bilirubin levels can be

protective against an array of diseases associated with increased oxidative stress [5]. Furthermore, in patients with metabolic syndrome (MS), hyperbilirubinemia is associated with attenuation of IR. Bilirubin, a powerful endogenous antioxidant, significantly attenuates endothelial dysfunction in preclinical experiments [6]. In addition, the modulatory effects of bilirubin on T regulatory cell differentiation were recently reported, further underlining the protective role of bilirubin in the pathogenesis of chronic inflammatory as well as in autoimmune conditions [7, 8].

Material and Methods

Subject selection

A case-control study was carried out on HIV-1 infected patients at the outpatient infectious disease unit (OPD) and ART center of the Sir J J Groups of Hospitals and Grant Government Medical College, Mumbai over a period of 1 year, from February 2014 to March 2015. We have selected 300 subjects from OPD after evaluation of their medical records [negative serial enzyme-linked immunosorbent assay (ELISA)/Western blot for HIV before 3 months of sample collection] as HIV-negative controls and 300 HIV-positive subjects detected by serial ELISA/Western blot method from ART center. We used power analysis method for sample selection at 5% significance level for 95% confidence.

Ethical approval

The protocol study was approved by the institutional ethics committee (no. IEC/Pharm/902/2013) and National AIDS Control Organization, Delhi, India (T-11020/67/2011-NACO).

Inclusion criteria

All participants were 20 years of age or older HIV-positive patients detected by serial ELISA/Western blot method and were included in this study after getting their informed consent. The family history of all subjects was recorded and the subjects without any diabetes history were chosen. Normal control HIV-negative subjects ($n = 300$), who are negative to ELISA/Western blot test for HIV before 3 months of sample collection, were selected from outpatient department of Sir J J Groups of Hospitals, Mumbai, Maharashtra, India.

Exclusion criteria

Exclusion criteria included pregnant women, patients with chronic diseases like hepatitis, diabetes, or family history of diabetes, renal impairment, cardiovascular comorbidities, neurological psychiatric disorders, various malignancies, as well as heavy smokers, alcoholics, and tobacco-chewers, and HIV patients with withdrawal of combination ART. We collected the demographic details from each patient and entered into the pro-forma. Subsequent to this, we have taken detailed history of each patient.

Sample collection

We collected venous blood samples in plain and lithium heparin vacutainers as an anticoagulant. Blood was centrifuged (4,000 × *g*, 10 min, 4 °C) to separate the plasma. The collected plasma was stored at −70 °C with aseptic precautions. We centrifuged plain blood samples 2 h after collection at 3,000 rpm for 5 min; then, we separated the serum and collected it in sterile tubes.

Information on demographic characteristics, physical measurements [waist circumference and blood pressure (BP)], anthropometric and biochemical measurements such as blood glucose and lipid profile was collected from each study subject. Hypertension (high BP) as classified as BP of systolic BP ≥130 mmHg and/or diastolic BP ≥85 mmHg, these two readings were obtained, and the average of the systolic and diastolic BP readings was used.

Different treatment regimens as per NACO guidelines

The list of ART administered to Indian HIV-1 patients is as follows:

The first-line therapy includes Tenofovir, Lamivudine, and Efavirenz.
The second-line therapy includes Tenofovir, Lamivudine, Ritonavir, and Atazanavir.

Biochemical methods

Bilirubin determination

Serum total bilirubin was determined using the colorimetric method of Jendrassik and Gorf [9]. Bilirubin reacts with diazotized sulfanilic acid in alkaline medium to form a blue-colored complex. Bilirubin is determined in the presence of caffeine, which releases albumin-bound bilirubin by reacting with diazotized sulfanilic acid.

Total protein determination

This was determined using the method of Doumas [10]. Cupric ions, in an alkaline medium, react with the peptide bonds of protein molecules, forming a blue–violet-colored complex. The intensity of colored complex produced is proportional to the amount of protein present in the serum.

Determination of blood glucose

We estimated fasting blood glucose levels by the hexokinase (enzymatic) method spectrophotometrically in R × L dimension-automated equipment.

Determination of fasting insulin

Fasting insulin was determined using Immulite 1000 Insulin kit from Siemens using chemiluminescence. We used the homeostasis model assessment – IR (HOMA-IR) index [11], first described by Matthews et al. [12] to determine IR. The formula used to calculate HOMA-IR was:

$$\text{HOMA-IR} = \text{fasting insulin (mU/L)} \times \text{fasting plasma glucose (mmol/L)}/22.5.$$

We assumed a HOMA-IR value of >2.8 to define IR from previous studies on IR [13].

Determination of DNA damage marker 8-hydroxy-2-deoxyguanosine (8-OHdG)

We used plasma levels of the oxidized base, 8-OHdG, as our biomarker of oxidative damage [14]. 8-OHdG was measured with the highly sensitive 8-OHdG check ELISA kit (StressXpress ELA Kit, StressMarq Biosciences Inc., Victoria, BC, Canada). StressMarq's 8-OHdG ELA is a competitive assay that can be used for the quantification of 8-OHdG in urine, cell culture, plasma, and saliva. The ELA utilizes an anti-mouse IgG-coated plate and tracer consisting of an 8-OHdG enzyme conjugate. It is important to note that the OHdG antibody used in this assay recognizes both free 8-OHdG and DNA-incorporated 8-OHdG. Since complex samples such as plasma, cell lysates, and tissues are comprised mixtures of DNA fragments and free 8-OHdG. The assay is based on the competition between 8-OHdG and an 8-OHdG–acetylcholinesterase (AChE) conjugate (8-OHdG Tracer) for a limited amount of 8-OHdG monoclonal antibody. Because the concentration of 8-OHdG Tracer is held constant while the concentration of 8-OHdG varies, the amount of 8-OHdG Tracer that is able to bind to the 8-OHdG monoclonal antibody will be inversely proportional to the concentration of 8-OHdG in the well. This antibody 8-OHdG complex binds to goat polyclonal anti-mouse IgG that was previously attached to the well. The plate was washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 8-OHdG Tracer bound to the well, which is inversely proportional to the amount of free 8-OHdG present in the well during the incubation. We followed all the procedures as manufacturer's instructions.

Preparation of data

Average absorbance reading of the non-specific binding (NSB) well and average absorbance reading of B_0 wells were determined. Then, we subtracted the average NSB readings from average B_0 readings and calculated $\%B/B_0$ (percentage of sample or standard bound/maximum bound). Then, we obtained a standard curve plot $\%B/B_0$ for standards using four parameter logistic equations. The sample concentration was determined using above equation.

Statistical methods

We performed Student's *t* test to assess differences between two means. We used EPI-INFO 07 statistical software for statistical analysis for medical research studies. We used Microsoft® Excel 2007 for production of charts. We compared group means of all parameters using the analysis of variance test. We compared categorical data and the prevalence of MS in HIV-infected patients using the Pearson's χ^2 test. We considered a p value <0.05 statistically significant. We used logistic regression method for correlation of total bilirubin and DNA damage marker 8-OHdG.

Results

A total of 600 (300 HIV-1 positive and 300 HIV negative) subjects were included in this study. All the subjects were divided in two age groups (20–40 years) and (40–60 years). Out of 600 subjects, there were 199 (66.3%) males and 101 (33.7%) females in age group (20–40 years) and 208 (69.3%) males and 92 (30.7%) females in age group (40–60 years). A majority of the ART-naive patients were in the primary clinical stage of infection, while those on second-line treatment [protease inhibitor (PI) containing highly active ART (HAART)] group were at secondary clinical stage of infection. The mean CD4 count was highest among the ART-naive group of patients, followed by the first-line treatment group of patients. The details of the demographic, anthropometric, HIV status, and other characteristics of the 600 participants are shown in *Tables I* and *II*.

Total bilirubin

The average total bilirubin was moderately high in HIV-positive ART-naive patients than HIV-positive with ART patients and controls. The mean serum total bilirubin in HIV-positive and ART not started in age group (20–40 years) was 1.13 mg/dl and in age group (40–60 years) was 1.05 mg/dl ($p < 0.01$). The average total bilirubin for HIV-positive in both age groups (20–40 years) and (40–60 years) with ART (first-line) was 0.92 and 0.87 mg/dl, respectively, and with ART (second-line) was 0.80 and 0.80 mg/dl ($p < 0.01$), respectively (refer *Table II*).

Table I Demographic, HIV status, and other characteristics of the 600 participants

Characteristics	Second-line ART ($n=100$)		First-line ART ($n=100$)		ART naive ($n=100$)		Control ($n=300$)	
Age group (years)	20–40	40–60	20–40	40–60	20–40	40–60	20–40	40–60
HIV status	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative
Gender								
Male	36	43	30	41	28	33	87	92
Female	14	7	20	9	22	17	63	58
Mean age ± SD	35.6 ± 4.3*	47.5 ± 5.3*	34.3 ± 5.2*	48.3 ± 4.8*	31.6 ± 4.2*	45.2 ± 6.1*	34.8 ± 5.1*	47.3 ± 5.4*
Mean CD4 count ± SD (cells/μl)	400 ± 144*	376 ± 183*	502 ± 233*	465 ± 165*	624 ± 202*	602 ± 134*	NA	NA

The data were analyzed by one-way ANOVA. CD4: cluster of differentiation.
*$p < 0.05$ significant when compared with control

Table II Clinical, anthropometric, and biochemical characteristics of study population

Characteristics	Second-line ART ($n=100$)		First-line ART ($n=100$)		ART naive ($n=100$)		Control ($n=300$)	
Age group (years)	20–40	40–60	20–40	40–60	20–40	40–60	20–40	40–60
HIV diagnosed time in months (mean ± SD)	53.8	78.5	68.0	80.4	22.7	34.9	NA	NA
Time on ART in months (mean ± SD)	51.3	76.8	63.6	65.7	NA	NA	NA	NA
Serum total bilirubin (mg/dl) (mean ± SD)	0.80 ± 0.64**	0.80 ± 0.28**	0.92 ± 0.60**	0.87 ± 0.48**	1.13 ± 0.60**	1.05 ± 0.75**	0.34 ± 0.30	0.35 ± 0.30
Total protein (mg/dl) (mean ± SD)	6.16 ± 0.17**	6.10 ± 0.13**	6.07 ± 0.08**	6.07 ± 0.11**	6.23 ± 0.15**	6.34 ± 0.17**	6.14 ± 0.11	6.14 ± 0.11
Insulin resistance (HOMA) ± SD	6.62 ± 5.29**	8.15 ± 7.07**	4.83 ± 3.21**	6.05 ± 4.14**	2.48 ± 1.05**	3.05 ± 1.56**	1.80 ± 0.81	1.91 ± 0.87
8-OHdG (ng/ml) ± SD	2.79 ± 0.95**	3.27 ± 0.99**	2.50 ± 0.91**	3.13 ± 0.92**	1.81 ± 0.66**	1.95 ± 0.56**	1.07 ± 0.25	1.30 ± 0.32

HIV detected time in months, time on ART in months, serum total bilirubin(mg/dl), total protein (mg/dl), insulin resistance (homeostasis model assessments), DNA damage marker 8-hydroxy-2-deoxyguanosine (8-OHdG). Values are mean ± SD of subjects. The data were analyzed by one-way ANOVA.
**$p < 0.01$ significant when compared with control

In a logistic regression analysis, serum total bilirubin was negatively associated with the IR [odds ratio (OR): 0.0127, 95% confidence interval (CI): 0.023–0.070, $p = 0.0000$] and DNA damage (OR: 0.525, 95% CI: 0.351–0.783, $p = 0.0016$) *(Fig. 1)*.

Insulin resistance (IR)

In this study, IR was measured by HOMA-IR. The mean HOMA for HIV-positive patients with ART was higher than that for HIV-positive ART-naive patients. The average HOMA for HIV-positive ART-naive patients in age group (20–40 years) was 2.33 and in age group (40–60 years) was 2.90 ($p < 0.01$). The mean HOMA for HIV-positive in both age group (20–40 years) and (40–60 years) with ART (first-line) was 5.79 and 8.78, respectively, and (second-line) 7.00 and 8.10 ($p < 0.01$), respectively (refer *Table II* and *Fig. 2*). The prevalence of IR in ART-naive HIV-positive patients with age group (20–40 years) was 11 (22%) and in age group (40–60 years) was 19 (38%). The IR which was significant in HIV-positive patients in both age groups (20–40 years) and (40–60 years) with ART (first-line) was 27 (54%) and 34 (68%), respectively, and (second-line) was 31(62%) and 35(70%), respectively. These results show that there was an increase in IR in HIV-positive patients with ART than HIV-positive ART-naive subjects (refer *Table II* and *Fig. 3*).

Under normal physiological conditions in all aerobic organisms, there is a balance maintained between endogenous oxidants and numerous enzymatic and non-enzymatic antioxidant defenses. When an imbalance occurs, oxidants produce extensive oxidative damage to DNA, which, in turn, contributes to aging, malignant tumors, and other degenerative diseases.

DNA damage

DNA damage was significantly higher in HIV-1-positive patients with ART than HIV-positive ART-naive subjects. In this study, DNA damage marker 8-OHdG was measured by ELISA. The mean 8-OHdG for HIV-positive patients with ART was higher than that for HIV-positive ART-naive subjects. The mean 8-OHdG for HIV positive and ART not started in age group (20–40 years) was 1.81 ng/ml and in age group (40–60 years) was 1.95 ng/ml ($p < 0.001$). The mean 8-OHdG for HIV-positive in both age group (20–40 years) and (40–60 years) with ART (first-line) was 2.50 ng/ml and 3.13 ng/ml, respectively, and (second-line) was 2.79 and 3.27 ng/ml ($p < 0.001$), respectively. ART accelerates DNA damage in HIV-positive patients (refer *Fig. 4*). These results show that there was an increase in DNA damage in HIV-positive patients with ART (refer *Table II*).

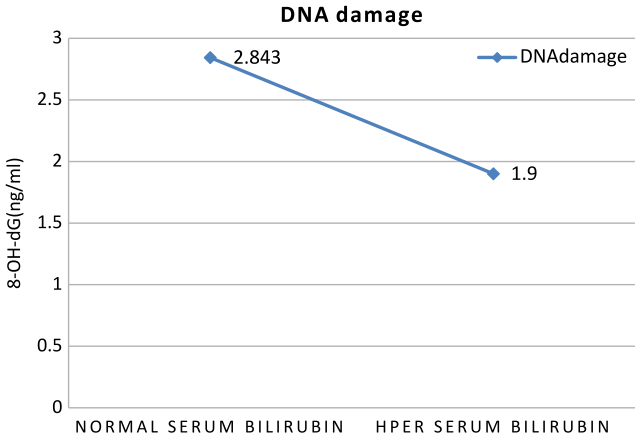

Fig. 1. Oxidative DNA damage on the basis of logistic regression model

Discussion

In our previous study, we have shown increased oxidative stress in HIV patient with ART than ART-naive HIV patients. HAART may increase chemically reactive species

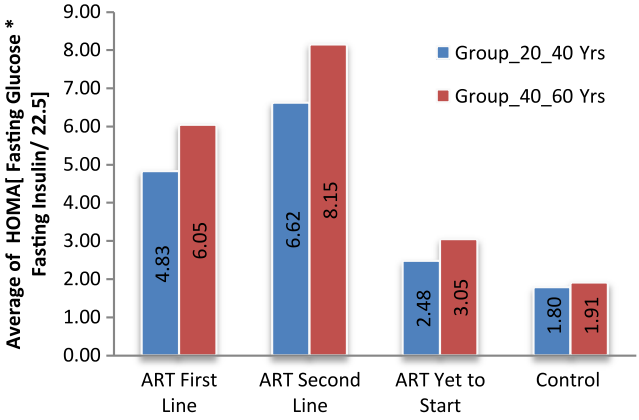

Fig. 2. Average insulin resistance in groups

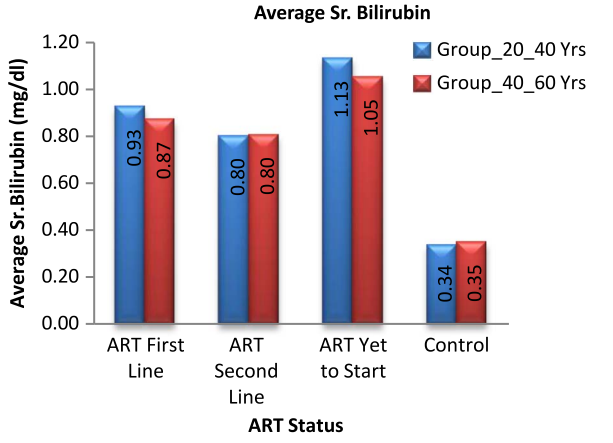

Fig. 3. Average serum total bilirubin

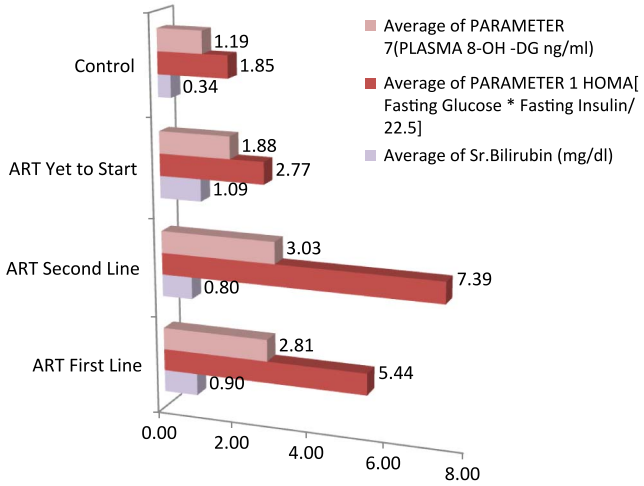

Fig. 4. Correlation of DNA damage, insulin resistance and total bilirubin

in circulation, possibly by producing more oxidized metabolites deriving from the interaction between ROS and infected cell biomolecules. This is supported by several biochemical mechanisms, such as mitochondrial interference, following treatment with HAART-nucleoside reverse transcriptase inhibitors, and activation of the P450 hepatic system by HAART, when comprising PIs [15]. This might be one of the reasons for decreased bilirubin level in HIV/AIDS patients with ART than ART-naive HIV/AIDS patients.

Unconjugated bilirubin (UCB), the principal mammalian bile pigment, is the end product of heme catabolism. Both belong to the super family of tetrapyrrolic compounds that serve multiple biological functions in animals and plants. Its six internal hydrogen bonds give UCB a unique structure responsible for its physicochemical properties and biological effects. Like many weakly polar, poorly soluble compounds, UCB is transported in blood tightly bound to albumin, with less than 0.01% of total bilirubin circulating in an unbound form [free bilirubin (Bf)]. This fraction governs the diffusion of UCB into tissues and therefore Bf is responsible for both its beneficial and toxic effects on cells. Although UCB was long thought to be a non-functional waste product, recent studies have shown that the antioxidant effects of mildly elevated serum bilirubin levels, as well as activation of heme oxygenase (HMOX1), may protect against diseases associated with oxidative stress, such as atherosclerosis [8]. However, in recent years, cytoprotective effects of bilirubin have been reported in a few studies. Frei et al. reported that serum bilirubin significantly contributes to total antioxidant capacity. It was discovered that bilirubin had anti-inflammatory effects as well as acts as scavengers of ROS, as it was mentioned above. In addition to bilirubin, experimental studies have found that enzymes involved in bilirubin metabolism also have several effects. In an experiment conducted with an animal model, it was shown that HMOX1 stimulated insulin products and reduced IR. It was also reported that biliverdin reductase has multiple functions affecting cell signaling and modulating immune system response [16].

ART long-term toxicity is becoming recognized, and a variety of metabolic abnormalities including dyslipidemia, fat redistribution, high BP, and IR have frequently been associated with this therapy, particularly, when it contains protease [17–19]. In particular, free fatty acid and various adipose-derived peptides (adipokines) have been identified as modulators of whole-body insulin sensitivity. The systemic IR seen in patients undergoing HAART also involves impaired insulin responsiveness in skeletal muscle and liver [20].

In the pre-cART era, several cross-sectional studies reported slightly increased or normal insulin sensitivity when compared with uninfected controls. ART introduction led to increases in fasting insulin and decreases in insulin sensitivity, effects dependent both on the class of the antiretroviral used and the different antiretrovirals within each class [21].

In this study, we found that increased IR was present in HIV-positive ART first-line patients and HIV-positive ART second-line patients than HIV-positive ART-naive patients, whereas increased average total bilirubin was present in HIV-positive ART-naive patients than HIV-positive ART first-line patients and HIV-positive ART second-line patients. Therefore, moderately high total bilirubin might act as an antioxidant agent in HIV-positive ART-naive patients. The molecular mechanisms involved in the protective effects of bilirubin on IR are still unclear.

This study demonstrated that the prevalence of DNA damage was dissimilar among the second-line (83%), first-line (75%), and ART-naive (38%) HIV-positive patients. ART-induced increased oxidative stress may be the cause of DNA damage in HIV-positive patients. Bilirubin has been recognized as a substance with potent antioxidant properties. The mean serum total bilirubin was moderately high in ART-naive HIV-positive patients than ART first-line and ART second-line HIV-positive patients. Therefore, moderately high total bilirubin might act as an antioxidant agent against increased oxidative stress in ART-naive HIV-positive patients and reduced the prevalence of IR, and oxidative DNA damage in this group. We also found that the prevalence of oxidative DNA damage and IR was negatively correlated with the serum levels of total bilirubin.

Our findings have a few limitations. First, total bilirubin level was measured only once for each patient, yet it may have transient fluctuation. Second, the data about smoking and drinking may not be very correct, as these data were self-reported. Third, we only measured total bilirubin level, whereas the direct and indirect bilirubin levels were not measured.

To our knowledge, this is the first study addressing the association of serum bilirubin level with the oxidative DNA damage marker 8-OHdG in HIV/AIDS patients. Data from this study suggest that moderately high bilirubin might act as protective agent against IR and DNA damage in ART-naive HIV/AIDS patients. However, additional work must be carried out to understand the full clinical potential of bilirubin as a protective agent for IR and DNA damage in HIV/AIDS patients.

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Funding sources: This study was supported by National AIDS Control Organization (NACO), Delhi, Mumbai District AIDS Control Society (MDACS), Mumbai, and ART Center Sir J J Group of Hospital, Mumbai.

Authors' contribution: VH has done literature search, figures, study design, and carried out all biochemical methods. He also helped in data collection and data analysis. VP helped in data interpretation and manuscript writing.

Conflict of interest: All authors declared no conflict of interest among them.

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