Increased Exhaled Hydrogen Peroxide in Human Immunodeficiency Virus-Infected Patients without Clinical Signs and Symptoms of Opportunistic Lung Disease

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Abstract

Background: HIV-infected subjects present with decreased antioxidant defense and increased activation of inflammatory cells which may lead to overproduction of oxidants. This study determined whether HIV-infected patients without clinical signs and symptoms of opportunistic lung disease (OLD-negative) exhaled more \( \text{H}_2\text{O}_2 \) than healthy controls and whether there was association between the exhalation of \( \text{H}_2\text{O}_2 \) and whole blood chemiluminescence (CL) and clinical variables.

Methods: A cross-sectional study was conducted. \( \text{H}_2\text{O}_2 \) in exhaled breath condensate and CL, resting and agonist-induced with N-formyl-methionyl-leucyl-phenylalanine (fMLP) were measured in 36 OLD-negative patients and 14 healthy controls. Univariate linear regression was used to summarize the average relationship and quantile regression analyzed the relationship at different points of the exhaled \( \text{H}_2\text{O}_2 \) distribution. Multivariate analyses were carried out using multiple linear regressions.

Results: The fold increase of the geometric mean exhaled \( \text{H}_2\text{O}_2 \) against healthy controls was 3.76-times higher in OLD-negative patients than in controls (95% Cl: 2.65-5.33, \( p<0.001 \)), whereas that of either resting or fMLP-induced CL was 1.46 or 1.63, respectively (95%: 1.17-1.83 and 1.27-2.08, \( p<0.01 \)). Exhaled \( \text{H}_2\text{O}_2 \) was not associated with CL, either resting or fMLP-induced. Linear regression detected positive relationship between the exhalation of \( \text{H}_2\text{O}_2 \) and viral load (R-squared 0.23, \( p<0.05 \)). The effects of viral load were best revealed at a higher exhalation of \( \text{H}_2\text{O}_2 \) (quantiles 0.6 and 0.7; both Pseudo R-squared 0.21, \( p<0.05 \)). In a multivariate model, the main independent contributors to the exhalation of \( \text{H}_2\text{O}_2 \), were viral load and highly active antiretroviral therapy (HAART), which together accounted for 35% of the variance in exhaled \( \text{H}_2\text{O}_2 \). If the analysis was limited exclusively to HAART-treated, a better model fit was obtained (R-squared 0.79), confirming that viral load is the main contributor to the exhaled \( \text{H}_2\text{O}_2 \).

Conclusion: Inordinate increase in exhaled \( \text{H}_2\text{O}_2 \) may reflect airway oxidative stress in HIV-1 infection which may be related to viral load.

Keywords: Exhaled hydrogen peroxide; HIV-1; Oxidative stress; Whole blood chemiluminescence

Introduction

Since oxidative stress is implicated in both human immunodeficiency virus, type 1 (HIV-1) expression [1,2] and the pathogenesis of AIDS [3], this pro-oxidant and antioxidant imbalance has been widely described among HIV-infected patients [4-7]. Overproduction of Reactive Oxygen Species (ROS) is thought to be the result of various cell activation and altered redox status, mostly phagocytic [5], inappropriately compensated by antioxidants or antioxidant enzymes [8] and is also augmented by highly active antiretroviral therapy (HAART) [7,9]. Exhaled \( \text{H}_2\text{O}_2 \) belongs to non-invasive markers of ROS production in the airways [10,11]. Since \( \text{H}_2\text{O}_2 \) is a volatile compound, it is easily determined by breath analysis. Exhalation of \( \text{H}_2\text{O}_2 \) is elevated in respiratory tract disorders accompanied by an influx of activated inflammatory cells e.g. bronchial asthma [12,13], COPD [14,15] and pneumonia [16]. To date, this has not been described in HIV-infected patients. They typically show a marked decrease in the concentration of reduced glutathione (GSH) [17,18] and their alveolar macrophages spontaneously produce more superoxide anion [17] which undergo dismutation to \( \text{H}_2\text{O}_2 \). Moreover, progression of HIV-1 infection results in a decreased erythrocyte glutathione peroxidase (GSH-Px) activity and suppression of GSH plasma levels [19]. Since the GSH-GSH-Px system is involved in the decomposition of \( \text{H}_2\text{O}_2 \) one may suspect that HIV-infected subjects have increased \( \text{H}_2\text{O}_2 \) levels in the airways resulting in increased exhalation of \( \text{H}_2\text{O}_2 \). Nevertheless, any concomitant Opportunistic Lung Disease (OLD) can influence such measurements [20].

The mechanisms underlying changes in exhalation of \( \text{H}_2\text{O}_2 \) are likely multifactorial in nature. Exhaled \( \text{H}_2\text{O}_2 \) represents a pool of ROS derived from the NADPH-oxidase system and the mitochondrial chain that avoids decomposition by antioxidant systems, subsequently diffusing into the airway surface which is then blown out as vapor and or aerosolized respiratory fluid droplets released from the respiratory epithelial lining fluid finally collected as exhaled breathe condensate (EBC) [11]. Therefore, the source are credited.

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changes in antioxidant defense in the airway or modifications in the number and activity of pulmonary inflammatory cells may alter the concentration of H$_2$O$_2$ in EBC. In addition, possibly there is a relationship between exhaled H$_2$O$_2$ and the blood oxidant and antioxidant status as measured by luminol enhanced whole blood chemiluminescence. Szkudlarek et al. [21] found an association between increased exhalation of H$_2$O$_2$ and a greater light emission of whole blood in a cross-sectional study of 41 healthy subjects. This associative finding in HIV-infected patients would provide evidence of a shared mechanism in the oxidative response in the blood and the airways. Furthermore, since nuclease reverse transcriptase inhibitors (NRTIs) have been shown to increase intracellular H$_2$O$_2$, treatment with NRTIs may also contribute to exhalation of H$_2$O$_2$ [22].

We conducted a cross-sectional study of HIV-infected men and women without clinical signs and symptoms of concomitant OLD (OLD-negative), accompanied by clinical signs and symptoms of concomitant respiratory tract infection (RTI) without a definite diagnosis of OLD (RTI-positive) and healthy control subjects to determine: 1) the respective amount of exhaled H$_2$O$_2$, 2) luminol enhanced whole blood chemiluminescence, either resting or agonist-induced, 3) whether exhaled H$_2$O$_2$ is associated with whole blood chemiluminescence and finally 4) whether exhaled H$_2$O$_2$ is associated with selected clinical variables, including HIV-infection duration, detectable viral load, treatment with HAART and HAART duration or a history of AIDS.

Materials and Methods

Study population

A total of 310 HIV-infected patients from the Acquired Immune Disorders Outpatient Clinic in Lodz, Poland were screened. The study included 36 OLD-negative subjects, 28 RTI-positive patients and 14 healthy controls. Each patient enrolled had to meet an inclusion criteria: age ≥ 18 and ≤ 60 years, HIV-1 seropositivity, a chest X-ray performed within 10 days prior to the enrollment and a written informed consent. The exclusion criteria included: any episode of alcohol or illicit drug abuse within the last 2 to 6 months before the study, respectively, any history of bronchial asthma, COPD, bronchiectasis, cystic fibrosis, tuberculosis, malignancies, renal or liver damage, pregnancy or breast feeding, of bronchial asthma, COPD, bronchiectasis, cystic fibrosis, tuberculosis, malignancies, renal or liver damage, pregnancy or breast feeding, within the last 2 to 6 months before the study, respectively, any history of bronchial asthma, COPD, bronchiectasis, cystic fibrosis, tuberculosis, malignancies, renal or liver damage, pregnancy or breast feeding, or a history of AIDS.

Study protocol

All subjects enrolled (HIV-1 infected patients and healthy controls) were asked to come to the laboratory between the hours of 8 am to 10 pm for EBC collection. Subsequently, 9 ml blood samples were drawn into EDTAK$_3$ Vacutte tubes (Greiner Labor Technik, Austria) for whole blood chemiluminescence assay, blood cell count and HIV-1 RNA copy number.

Exhaled breathe condensate sampling

2-3 mL of exhaled breath condensate was sampled during 15 min of spontaneous tidal volume breathing (respiratory rate ranged 14-20 bpm), using EcoScreen-1 (Erch Jaeger GmbH, Hoechberg, Germany), with saliva trap. Subjects wore a noseclip and rinsed their mouth with distilled water just before and after 7 min of collection [23]. Immediately after the procedure, EBC specimens were stored at -80ºC [23,24], no longer than 7 days until H$_2$O$_2$ measurement. No amylase activity was detected in EBC specimens (control of salivary contamination) [16]. Subjects who were current smokers refrained from cigarette smoking 12 hrs preceding EBC collection.

Measurement of H$_2$O$_2$

HVA method was used to assess the concentration of H$_2$O$_2$ in EBC [25], as previously described [23,26]. The detection limit of the H$_2$O$_2$ assay was 0.05 µmol/L. The intra-assay variability did not exceed 2.5% for the standard 1 µmol/L of the H$_2$O$_2$ solution. The addition of catalase (30 U) to the EBC specimens of HIV-infected patients (n=4) and healthy subjects (n=3), which previously revealed that detectable exhaled H$_2$O$_2$ levels completely abolished HVA oxidation, demonstrates that the H$_2$O$_2$ assay is specific and other reactive compounds or oxygen species did not contribute to H$_2$O$_2$ readings. Individual results were means from duplicate measurements.

Whole blood chemiluminescence assay

The resting and fMLP-induced luminol enhanced whole blood chemiluminescence (CL) were measured as previously described [21,27]. Two CL parameters were assessed: resting CL prior to the addition of fMLP and peak light emission after the addition of an agonist to a final concentration of 20 µmol/L (∆fMLP-induced peak CL). Resting CL and fMLP-induced peak CL were expressed as mV per 10$^5$ phagocytes in the assayed blood sample. Individual results were obtained as a mean from triplicate measurements.

Other techniques

Blood cell count was performed using the 5-DIFF LH 750 Hematology Analyzer (Beckman-Coulter, Inc. USA). Blood CD$_4$ count was determined with anti-CD$_4$ monoclonal antibodies (Becton Dickinson, NJ, USA), following flow cytometry (Beckman Coulter Epics XI, USA). Serum anti-HIV-1 antibodies were detected with enzyme linked immunosorbent assay (Bio-Rad, USA). HIV-1 seropositivity was confirmed by Western blot analysis (Calyphe Biomedical, USA). HIV-1 RNA copies (viral load) were determined by COBAS Amplicor HIV-1 monitor test (Roche, Branchburg, NJ, USA), with detection limit of 50 copies/mL and expressed as a common logarithm of RNA copies per mL of plasma.

Statistical analysis

Statistical analysis was carried out using the Stata 12 (Stata Corp., College Station, TX, USA). Normally distributed continuous variables and variables of log 10-transformed toward normality were compared between groups using one-way ANOVA with post-hoc Bonferroni adjustment and unpaired Student’s t-test for equal variances; non-normally distributed data were compared using the Kruskall-Wallis rank test and the Wilcoxon rank-sum test. Categorical variables were compared between groups using the Pearson’s chi-squared test and the Fisher’s exact test. The one-way analysis of covariance (ANCOVA) was used for comparison of variables adjusted for covariate. Correlations between continuous variables were determined nonparametrically using Spearman’s rho. Univariate linear regression analyses were carried out with nonparametric bootstrap and 10 000 replications (to avoid transformation of the dependent variables
Results

A total of 36 OLD-negative adults and 14 healthy control subjects matched for gender, age and smoking habits were studied. Moreover, a group of 28 RTI-positive patients, of whom 15 had a cough, 19 had purulent sputum, 6 were dyspneic, 11 had the chest X-ray findings and 12 with a history of past RTI within the last 3 months, were included in the analysis (Table 1). EBC of H$_2$O$_2$ as well as resting and fMLP-induced whole blood chemiluminescence measurements was observed in all patients. Viral load was determined in 28 of OLD-negative patients and 10 of RTI-positive subjects. Viraemia measurement was solely dependent on test availability. Table 1 shows the clinical and demographic findings of HIV-infected subjects and healthy controls. Evaluation of white blood cell (WBC) count and polymorphonuclear leukocytes (PMNs) count revealed significantly lower values in OLD-negative patients as compared to healthy controls (Table 1). Moreover, WBC and lymphocyte counts were higher, estimated duration of HIV-infection was shorter and the number of HAART-treated was lower in RTI-positive subjects as compared to OLD-negative patients.

Exhaled H$_2$O$_2$ and whole blood chemiluminescence

Old-negative vs. RTI-positive subjects: Increased oxidative status defined as elevated exhalation of H$_2$O$_2$ and enhanced whole blood chemiluminescence in comparison to healthy controls was a consistent feature of HIV infection, regardless of concomitant RTI (Table 2, Figures 1A and 1B). The highest significant difference was seen in exhaled H$_2$O$_2$ (Table 2, Figure 1B). Fold increase of the geometric mean exhaled H$_2$O$_2$ was determined in 28 of OLD-negative patients and 10 of RTI-positive patients.

Table 1: Comparison of demographic and clinical variables between healthy controls vs. HIV-infected patients without clinical signs and symptoms of opportunistic lung disease (OLD-negative) or with clinical signs and symptoms of concomitant respiratory tract infection without a definite diagnosis of OLD (RTI-positive).

<table>
<thead>
<tr>
<th>Variable</th>
<th>(1) Control (n=14)</th>
<th>(2) HIV-infected OLD-negative (n=36)</th>
<th>(3) HIV-infected RTI-positive (n=28)</th>
<th>p-value</th>
<th>post-hoc p-value</th>
<th>Difference</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender, n (%)</td>
<td>8 (57%)</td>
<td>20 (55%)</td>
<td>19 (68%)</td>
<td>0.59$^1$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age [yrs], median (95% CI, range)</td>
<td>31 (25.6 to 36.4, 20-52)</td>
<td>28.5 (25.5 to 31.5, 23-54)</td>
<td>29.5 (25.9 to 33.1, 20-56)</td>
<td>0.62$^2$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>7 (50%)</td>
<td>23 (64%)</td>
<td>23 (82%)</td>
<td>0.09$^3$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cough/Sputum/Dyspnea/Chest X-ray/Past RTI, n</td>
<td>0 / 0 / 0 / 0 / 0</td>
<td>0 / 0 / 0 / 0 / 0</td>
<td>0 / 0 / 0 / 0 / 0</td>
<td>0.18$^4$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin [g/dL], mean (95% CI, range)</td>
<td>14.5 (13.8 to 15.3, 12.3-16.5)</td>
<td>14.3 (13.9 to 14.7, 11.4-16.7)</td>
<td>14.9 (14.4 to 15.5, 11.0-17.2)</td>
<td>0.18$^5$</td>
<td></td>
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<tr>
<td>White blood cells count [×10^3 cells/µL], geometric mean (95% CI, range)</td>
<td>6.61 (5.82 to 7.50, 5.10-11.0)</td>
<td>4.75 (4.25 to 5.30, 2.01-8.72)</td>
<td>5.83 (5.24 to 6.49, 4.03-11.40)</td>
<td>&lt;0.001$^6$</td>
<td>0.002$^1$ vs. 2</td>
<td>0.72</td>
<td>0.58 to 0.89 (1.03 to 1.47)</td>
</tr>
<tr>
<td>PMNs count [×10^3 cells/µL], median (95% CI, range)</td>
<td>4.30 (3.65 to 4.95, 3.80-8.90)</td>
<td>3.14 (2.53 to 3.75, 1.06-7.66)</td>
<td>3.58 (2.76 to 4.40, 1.91-8.98)</td>
<td>0.003$^7$</td>
<td>0.001$^1$ vs. 2</td>
<td>-1.45</td>
<td>(-2.24 to -0.59)</td>
</tr>
<tr>
<td>Lymphocyte count [×10^3 cells/µL], mean (95% CI, range)</td>
<td>1.79 (1.55 to 2.04, 1.30-2.30)</td>
<td>1.49 (1.30 to 1.68, 0.31-2.46)</td>
<td>1.86 (1.60 to 2.12, 0.26-3.30)</td>
<td>0.039$^8$</td>
<td>0.043$^2$ vs. 3</td>
<td>0.37</td>
<td>(0.02 to 0.72)</td>
</tr>
<tr>
<td>CD4 count [cells/µL], mean (95% CI, range)</td>
<td>N/A</td>
<td>361.6 (287.1 to 436.0, 6-1063)</td>
<td>391.7 (320.8 to 462.6, 90-822)</td>
<td>0.56$^9$</td>
<td></td>
<td></td>
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<tr>
<td>HIV-infection duration [yrs], median (95% CI, range)</td>
<td>N/A</td>
<td>4.2 (1.9 to 6.5, 0.2-12.0)</td>
<td>2.1 (0.7 to 3.6, 0.1-9.2)</td>
<td>0.003$^a$</td>
<td>-1.8</td>
<td>(-3.3 to -0.7)</td>
<td></td>
</tr>
<tr>
<td>Viral load assays, n (%)</td>
<td>N/A</td>
<td>28 (78%)</td>
<td>10 (36%)</td>
<td>0.001$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detectable viral load*, n (%)</td>
<td>N/A</td>
<td>19 (68%)</td>
<td>6 (60%)</td>
<td>0.71$^c$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral load [×10^4 RNA copies/mL], geometric mean (95% CI, range)</td>
<td>N/A</td>
<td>1.08 (0.26 to 4.42, 0-935)</td>
<td>1.33 (0.07 to 23.71, 0-41.9)</td>
<td>0.88$^d$</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Treatment with HAART, n (%)</td>
<td>N/A</td>
<td>23 (64%)</td>
<td>7 (25%)</td>
<td>0.003$^e$</td>
<td>-39%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment duration [mths], geometric mean (95% CI, range)</td>
<td>N/A</td>
<td>17.4 (10.9 to 28.0, 1-84)</td>
<td>7.9 (1.7 to 36.0, 1-62.5)</td>
<td>0.15$^f$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of AIDS*, n (%)</td>
<td>N/A</td>
<td>14 (39%)</td>
<td>5 (18%)</td>
<td>0.10$^g$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p-values: $^1$Pearson’s chi-squared test; $^2$Fisher’s exact test; $^3$one-way ANOVA; $^4$post hoc ANOVA test with Bonferroni adjustment; $^5$unpaired Student’s t-test (equal variances); $^6$Kruskal-Wallis rank test; $^7$Wilcoxon rank-sum test with Bonferroni adjustment; $^8$Wilcoxon rank-sum test.

HAART, highly active anti-retroviral therapy; PMNs, polymorphonuclear leukocytes.

*Detectable viral load defined as ≥50 copies of HIV-1 RNA/mL. AIDS defined as a history of CD4 count <200 cells/µL or AIDS-defining illness.
against healthy controls was about 2 to 3-times higher than that of either resting or fMLP-induced CL, respectively (post hoc ANOVA against healthy controls; all p<0.05) (Table 2, Figure 1B). Moreover, there were no significant differences between OLD-negative patients and RTI-positive subjects (post hoc ANOVA against OLD-negative; all p>0.05) (Table 1, Figure 1A). Adjustment of CL variables for the significant difference on PMNs count with ANCOVA changed the conclusion concerning the significant difference in resting and fMLP-induced CL (Table 2, Figure 1B). While controlling for the effect of PMNs count in OLD-negative patients, there were no significant differences in the PMNs-adjusted means of CL variables (ANCOVA testing against healthy controls with PMNs as covariate; all p>0.05 in OLD-negative patients). For the exhaled H\textsubscript{2}O\textsubscript{2} the assumptions for ANCOVA with PMNs as a covariate were not met. Regardless of that, the strong increase in exhaled H\textsubscript{2}O\textsubscript{2} was also highly significant in terms of 95% CI (the entire 95% confidence interval for the ratio of geometric means was well over 2-times that of HIV-infected to control geometric means ratio) (Figure 1B).

To recapitulate, there exist a highly significant difference in the exhalation of H\textsubscript{2}O\textsubscript{2} between HIV-infected patients and healthy controls; what is more, exhaled H\textsubscript{2}O\textsubscript{2} was the most prominent marker of oxidative stress in HIV-infected individuals, regardless of concomitant RTI.

**HAART-naive vs. HAART-treated subjects:** A total of 23 OLD-negative patients commenced aggressive antiretroviral treatment regimens with HAART and 13 OLD-negative subjects remained off therapy until clinically indicated (Table 3). Viral load assays confirmed a significant decrease in the number of HIV-1 RNA copies associated with antiretroviral treatment (p<0.05) (Table 3). Along with suppression of viral load no further differences occurred between HAART-naive and HAART-treated arms (post hoc ANOVA against HAART-naive; all p>0.05) (Table 3, Figure 2A). The analysis confirmed an elevated exhalation of H\textsubscript{2}O\textsubscript{2} in comparison to healthy controls, regardless of HAART and no significant difference in resting and fMLP-induced CL after adjustment of CL variables for a difference in PMNs with ANCOVA (Table 3, Figures 2A and 2B).

**Relationship between exhaled H\textsubscript{2}O\textsubscript{2} and whole blood chemiluminescence:** Spearman’s rank correlations were calculated between exhaled H\textsubscript{2}O\textsubscript{2} and CL variables. Exhaled H\textsubscript{2}O\textsubscript{2} did not significantly correlate with resting CL and fMLP-induced peak CL in OLD-negative patients (all Spearman’s rho p>0.05; detailed data not shown). This was in agreement with no significant associations between exhaled H\textsubscript{2}O\textsubscript{2} and either resting CL or fMLP-induced peak CL in healthy controls (all Spearman’s rho p>0.05; detailed data not shown).

**Factors determining exhaled H\textsubscript{2}O\textsubscript{2} in OLD-negative subjects**

**Univariate analyses:** When exhaled H\textsubscript{2}O\textsubscript{2} was established as dependent variable linear regression by nonparametric bootstrap did not find any evidence of significant association with any demographic or clinical variables except a detectable viral load being revealed as a significant and positive predictor for exhaled H\textsubscript{2}O\textsubscript{2} (R-squared=0.23, p=0.014) (Table 4, Figure 3A). On the contrary, the linear regression by nonparametric bootstrap did not show any significant relations between CL variables, either resting CL or fMLP-induced peak CL and viral load (all p>0.05, detailed data not shown).

Moreover, quantile regression was employed to estimate the relationships between exhaled H\textsubscript{2}O\textsubscript{2} and viral load for a large part of the exhaled H\textsubscript{2}O\textsubscript{2} distribution. We present results by simultaneous bootstrap analysis narrowed to a range from 0.2 to 0.8 quantiles, as justified by the small sample (n=19) and large sampling variation for upper quantiles (Figure 3B). Quantile regression estimates indicated some significant and positive relations between exhaled H\textsubscript{2}O\textsubscript{2} and viral load. The effects of viral load on exhaled H\textsubscript{2}O\textsubscript{2} were best revealed at higher H\textsubscript{2}O\textsubscript{2} concentration, as shown for quantiles from 0.6 to 0.7 (Figure 3B). There was a significant increase in exhaled H\textsubscript{2}O\textsubscript{2} in response to viral load at quantile 0.6 (Pseudo R-squared=0.21, p=0.043) and at quantile 0.7 (Pseudo R-squared=0.21, p=0.042).

Moreover, quantile regression was employed to estimate the relationships between exhaled H\textsubscript{2}O\textsubscript{2} and viral load for a large part of the exhaled H\textsubscript{2}O\textsubscript{2} distribution. We present results by simultaneous bootstrap analysis narrowed to a range from 0.2 to 0.8 quantiles, as justified by the small sample (n=19) and large sampling variation for upper quantiles (Figure 3B). Quantile regression estimates indicated some significant and positive relations between exhaled H\textsubscript{2}O\textsubscript{2} and viral load. The effects of viral load on exhaled H\textsubscript{2}O\textsubscript{2} were best revealed at higher H\textsubscript{2}O\textsubscript{2} concentration, as shown for quantiles from 0.6 to 0.7 (Figure 3B). There was a significant increase in exhaled H\textsubscript{2}O\textsubscript{2} in response to viral load at quantile 0.6 (Pseudo R-squared=0.21, p=0.043) and at quantile 0.7 (Pseudo R-squared=0.21, p=0.042).

Insofar as it can be ascertained, the estimated effects of viral load were well represented by changes in exhaled H\textsubscript{2}O\textsubscript{2}.

**Multivariate analysis:** In order to determine the factors contributing to exhaled H\textsubscript{2}O\textsubscript{2} (to generate hypotheses regarding the causes in variation of the exhalation of H\textsubscript{2}O\textsubscript{2}), a multivariate analysis was carried out with exhaled H\textsubscript{2}O\textsubscript{2} as the dependent variable together with smoking habits, duration of HIV-infection (in years), a detectable viral load (in log\textsubscript{10} of RNA copies/...
treatment with HAART and a history of AIDS as possible explanatory factors. In this model, the main contributors to exhaled H$_2$O$_2$ as described by a multiple linear regression equation were viral load and treatment with HAART. The standardized coefficients indicated that viral load contributed considerably to the model, followed by treatment with HAART. The standardized coefficients indicated that viral load contributed most considerably to the model, followed by treatment with HAART.

Table 2: Comparison of exhaled H$_2$O$_2$ and whole blood chemiluminescence between healthy controls vs. HIV-infected patients without clinical signs and symptoms of opportunistic lung disease (OLD-negative) or with clinical signs and symptoms of concomitant respiratory tract infection without a definite diagnosis of OLD (RTI-positive).

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<th>post-hoc p-value</th>
<th>Difference</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exhaled breath H$_2$O$_2$ (µmol/L), geometric mean (95% CI)</td>
<td>0.21 (0.15 to 0.31, 0.05-0.63)</td>
<td>0.80 (0.67 to 0.95, 0.30-2.20)</td>
<td>0.90 (0.75 to 1.09, 0.31-2.20)</td>
<td>&lt;0.001$^1$</td>
<td>&lt;0.001$^2$ 1 vs. 2</td>
<td>3.76</td>
<td>(2.81 to 5.58)</td>
</tr>
<tr>
<td>Resting CL [mV/10$^6$ cells], geometric mean (95% CI, range)</td>
<td>0.59 (0.50 to 0.70, 0.28-0.84)</td>
<td>0.84 (0.74 to 0.95, 0.48-1.35)</td>
<td>0.71 (0.61 to 0.83, 0.35-1.28)</td>
<td>0.008$^1$</td>
<td>0.008$^2$ 1 vs. 2</td>
<td>1.46</td>
<td>(1.02 to 1.83)</td>
</tr>
<tr>
<td>fMLP-induced peak CL [mV/10$^6$ cells], geometric mean (95% CI, range)</td>
<td>0.92 (0.74 to 1.13, 0.42-2.24)</td>
<td>1.49 (1.30 to 1.71, 0.62-2.97)</td>
<td>1.67 (1.34 to 2.09, 0.63-4.71)</td>
<td>&lt;0.001$^1$</td>
<td>0.004$^2$ 1 vs. 2</td>
<td>1.63</td>
<td>(1.20 to 2.19)</td>
</tr>
</tbody>
</table>

Adjusted variables for significant difference on PMNs count:
- Exhaled breath H$_2$O$_2$ (µmol/L), geometric mean (95% CI)
- Resting CL [mV/10$^6$ cells], geometric mean (95% CI)
- fMLP-induced peak CL [mV/10$^6$ cells], geometric mean (95% CI)

p-values: $^1$one-way ANOVA; $^2$post hoc ANOVA test with Bonferroni adjustment; $^3$one-way ANCOVA (assumptions not met); $^4$one-way ANCOVA (assumptions met); $^5$post hoc ANCOVA test with Bonferroni adjustment.

Figure 2: Exhaled H$_2$O$_2$ as marker of oxidative stress in HIV-infected patients without signs and symptoms of opportunistic lung disease (OLD-negative) and either HAART-naive or HAART-treated. (A - top) Dot plot of individual results in healthy controls and HIV-1 infected patients. Box plot shows the geometric mean as a solid line and 95% confidence intervals as a rectangle. (B - bottom) Fold increase of geometric mean exhaled H$_2$O$_2$ in HAART-naive or HAART-treated. (A - top) Dot plot of individual results in healthy controls vs. HIV-infected patients without clinical signs and symptoms of opportunist lung disease (OLD-negative) or with clinical signs and symptoms of concomitant respiratory tract infection against healthy controls. Raw data vs. adjusted (adj.) for differences in resting CL and fMLP-induced peak whole blood chemiluminescence (pCL) in OLD-negative patients either HAART-naive or HAART-treated against healthy controls.Adjusted variables for significant difference on PMNs count:
- Exhaled breath H$_2$O$_2$ (µmol/L), geometric mean (95% CI)
- Resting CL [mV/10$^6$ cells], geometric mean (95% CI)
- fMLP-induced peak CL [mV/10$^6$ cells], geometric mean (95% CI)

p-values: $^1$one-way ANOVA; $^2$post hoc ANOVA test with Bonferroni adjustment; $^3$one-way ANCOVA (assumptions not met); $^4$one-way ANCOVA (assumptions met).

Discussion
Whereas systemic oxidative stress is a common feature of HIV-1 infection, the lungs are one of the major targets of HIV-1 attack. Accumulation of ROS induces airway inflammation that can be deteriorated by opportunistic lung diseases. Exhaled H$_2$O$_2$ is a known noninvasive inflammatory marker of the respiratory tract which has not been previously reported in HIV-1-infected patients. Moreover, the relationship between exhaled H$_2$O$_2$ and whole blood chemiluminescence has not been established. In this cross-sectional study, we found a high
level of exhaled H$_2$O$_2$ among HIV-infected patients as compared to healthy controls, regardless of concomitant respiratory tract infection and despite treatment with HAART. This was accompanied by greater luminol enhanced light emission of the whole blood, either resting or agonist-induced, even though an increase in the exhalation of H$_2$O$_2$ was more evident. Nevertheless, elevated exhalation of H$_2$O$_2$ and appreciable

<table>
<thead>
<tr>
<th>Variable</th>
<th>(1) Control (n=14)</th>
<th>(2) HIV-infected OLD-negative HAART-naive (n=13)</th>
<th>(3) HIV-infected OLD-negative HAART-treated (n=23)</th>
<th>p-value</th>
<th>post-hoc p-value</th>
<th>Difference</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral load assays, n (%)</td>
<td>N/A</td>
<td>5 (39%)</td>
<td>23 (100%)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detectable viral load, n(%)</td>
<td>N/A</td>
<td>5 (100%)</td>
<td>14 (61%)</td>
<td>0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral load [x10$^3$ RNA copies/mL], geometric mean (95% CI, range)</td>
<td>N/A</td>
<td>13.65 (0.43 to 436.13, 0.84-935)</td>
<td>0.53 (0.11 to 2.47, 0-625)</td>
<td>0.047</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exhaled breath H$_2$O$_2$ [µmol/L], geometric mean (95% CI, range)</td>
<td>0.21 (0.15 to 0.31, 0.05-0.63)</td>
<td>0.77 (0.54 to 1.10, 0.42-2.20)</td>
<td>0.81 (0.66 to 1.00, 0.30-1.80)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting CL [mV/10$^4$ cells], geometric mean (95% CI, range)</td>
<td>0.59 (0.50 to 0.70, 0.28-0.84)</td>
<td>0.79 (0.66 to 0.94, 0.47-1.22)</td>
<td>0.87 (0.73 to 1.04, 0.43-2.14)</td>
<td>0.004</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fMLP-induced peak CL [mV/10$^4$ cells], geometric mean (95% CI, range)</td>
<td>0.92 (0.74 to 1.13, 0.42-2.24)</td>
<td>1.61 (1.29 to 2.02, 0.90-2.97)</td>
<td>1.42 (1.19 to 1.70, 0.62-2.87)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adjusted variables for significant difference on PMNs count:

<table>
<thead>
<tr>
<th>Variable</th>
<th>(1) Control (n=14)</th>
<th>(2) HIV-infected OLD-negative HAART-treated (n=23)</th>
<th>(3) HIV-infected OLD-negative HAART-treated (n=23)</th>
<th>p-value</th>
<th>post-hoc p-value</th>
<th>Difference</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exhaled breath H$_2$O$_2$ [µmol/L], geometric mean (95% CI)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting CL [mV/10$^4$ cells], geometric mean (95% CI)</td>
<td>0.75 (0.68 to 0.83)</td>
<td>0.73 (0.67 to 0.80)</td>
<td>0.77 (0.72 to 0.83)</td>
<td>0.63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fMLP-induced peak CL [mV/10$^4$ cells], geometric mean (95% CI)</td>
<td>1.13 (0.95 to 1.35)</td>
<td>1.52 (1.28 to 1.79)</td>
<td>1.30 (1.14 to 1.47)</td>
<td>0.078</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p-values: 1Fisher’s exact test; 2unpaired Student’s t-test (equal variances); 3one-way ANOVA; 4post hoc ANOVA test with Bonferroni adjustment; 5one-way ANCOVA (assumptions not met); 6one-way ANCOVA (assumptions met).

CL, whole blood chemiluminescence; fMLP, N-formyl-methionyl-leucyl-phenylalanine; HAART, highly active anti-retroviral therapy; N/A, not applicable; PMNs, polymorphonuclear leukocytes.

Table 4: Summary of regression models in HIV-infected patients without clinical signs and symptoms of opportunistic lung disease (OLD-negative).
Multiple linear regression models in HIV-infected patients without signs and symptoms of opportunistic lung disease (OLD-negative). Linear regression (A - top) to estimate changes in exhaled H₂O₂ (y) as a function of log10-transformed viral load (x), containing sequences required for the initiation of HIV-1 transcription via a post-translational control of NF-kappaB [2,31-33]. Moreover, alveolar macrophages are susceptible to HIV-1 virus infection and can be recognized as latent viral reservoir [34]. These cells isolated from asymptomatic HIV-1 positive subjects exhibited a constitutive activation of phosphatidylinositol 3-kinase pathway [35]. The Nef (Negative Regulatory Factor) protein of the HIV-1 virus could be one of the activators in the signal transduction pathway leading to stimulation of the NADPH oxidase complex [36] and increased oxidants release from macrophages [37]. Additionally, Tat protein has been shown to induce the release of cytokines, thereby enhancing the production of H₂O₂ in a variety of cells, including macrophages [38,39]. In fact, a study by Buhl showed that alveolar macrophages isolated from the lungs of HIV-infected subjects presented with an increased spontaneous chemiluminescence result from enhanced activity of phagocytes and may be a compensatory mechanism in response to the underlying immunodeficiency [29]. We found that the observed differences in CL variables were explained by an adjustment to a lower whole blood PMNs count. Elbim et al. confirmed that PMNs counts in HIV-infected patients were significantly decreased, though circulating PMNs were activated producing more H₂O₂ [30]. Interestingly, there was no association between exhaled H₂O₂ and whole blood chemiluminescence in HIV-infected patients. This associative finding had been previously reported in a study of 41 healthy subjects using the same methods to measure both the exhalation of H₂O₂ and light emission of blood phagocytes [21]. These results indicate that exhalation of H₂O₂ in HIV-infected patients does not depend on ability of blood phagocytes to generate ROS, to a higher extent, this may involve phagocytes within the lungs.

In addition, increased exhalation of H₂O₂ in OLD-negative patients was associated with detectable viral load. The association was more evident at higher levels of exhaled H₂O₂ as revealed by quantile regression analysis. The mechanism underlying the observed associations aims to uncover direct casual pathway between viral load and exhaled H₂O₂. Numerous studies have shown that H₂O₂ strongly activates HIV long terminal repeat (LTR), containing sequences required for the initiation of HIV-1 transcription via a post-translational control of NF-kappaB [2,31-33]. Moreover, alveolar macrophages are susceptible to HIV-1 virus infection and can be recognized as latent viral reservoir [34]. These cells isolated from asymptomatic HIV-1 positive subjects exhibited a constitutive activation of phosphatidylinositol 3-kinase pathway [35]. The Nef (Negative Regulatory Factor) protein of the HIV-1 virus could be one of the activators in the signal transduction pathway leading to stimulation of the NADPH oxidase complex [36] and increased oxidants release from macrophages [37]. Additionally, Tat protein has been shown to induce the release of cytokines, thereby enhancing the production of H₂O₂ in a variety of cells, including macrophages [38,39]. In fact, a study by Buhl showed that alveolar macrophages isolated from the lungs of HIV-infected subjects presented with an increased spontaneous chemiluminescence result from enhanced activity of phagocytes and may be a compensatory mechanism in response to the underlying immunodeficiency [29]. We found that the observed differences in CL variables were explained by an adjustment to a lower whole blood PMNs count. Elbim et al. confirmed that PMNs counts in HIV-infected patients were significantly decreased, though circulating PMNs were activated producing more H₂O₂ [30]. Interestingly, there was no association between exhaled H₂O₂ and whole blood chemiluminescence in HIV-infected patients. This associative finding had been previously reported in a study of 41 healthy subjects using the same methods to measure both the exhalation of H₂O₂ and light emission of blood phagocytes [21]. These results indicate that exhalation of H₂O₂ in HIV-infected patients does not depend on ability of blood phagocytes to generate ROS, to a higher extent, this may involve phagocytes within the lungs.

When the analysis was narrowed exclusively to HAART-treated, we found other positive associations with CD₄ count and HIV-1 infection duration. Bucy et al. showed evidence that increase in CD₄ lymphocytes after HIV antiretroviral therapy reflects redistribution from lymphoid tissues [42]. The link between the increased exhalation of H₂O₂ and the duration of HIV-infection is likely complex, resulting from disease factors, such as decrease in the GSH concentration over time in the alveolar lining.
fluid [18], decreased erythrocyte GSH-Px activity [43], interactions with HAART [9] and patient factors including genetically based susceptibility. For example, Delanghe et al. reported that HIV-seropositive patients with the antioxidant protein haptoglobin 2-2 phenotype, known to bind free hemoglobin more slowly, had a higher mortality and worse prognosis than patients with other phenotypes, suggesting enhanced hemoglobin-driven oxidative stress [44].

Our study has several limitations. Given our small sample size, which necessitates testing in larger groups, we were unable to fully explore all the hypotheses. Secondly, the cross-sectional study design makes the assessment of causal relationships difficult. Finally, we enrolled OLD-negative patients, which included normal chest X-rays, to avoid opportunistic infections, though asymptomatic presentations of OLD could not be excluded. Despite these limitations, the findings in our study should encourage an answer to the question of whether or not the increased exhalation of H2O2 in HIV-1 infected subjects evinces clinical significance. Implications for further studies are HIV-associated pulmonary emphysema [45], since H2O2 is linked to breakdown of elastic fibers [46] and Kaposi’s sarcoma [47], as H2O2 mediates herpesvirus reactivation from latency. Therefore, it is possible that determination of exhaled H2O2 can be helpful in the selection of patients with a higher risk of some HIV-1 associated diseases.

Acknowledgment

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References