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The Role of Oxidative Stress in HIV

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The Role of Oxidative Stress in HIV

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Abstract

The Role of Oxidative Stress in HIV

By Sushma K. Cribbs, M.D.

Objective: Despite the advent of anti-retroviral therapy (ART), lung infections continue to be a leading cause of death in HIV-infected individuals. There is substantial evidence that HIV infection causes oxidative stress, both in pre-clinical and clinical studies. Oxidative stress can be generated through several mechanisms including the oxidation of extracellular thiol disulfide pairs such as glutathione/glutathione disulfide (GSH/GSSG) and cysteine/cystine (Cys/CySS). Measuring redox in HIV-infected individuals may identify those with chronic oxidative stress who are at increased risk for lung infection. Therefore, we sought to estimate the association between HIV infection and oxidative stress in the lung, as reflected by decreased levels of GSH and Cys in the epithelial lining fluid.

Study design: Cross-sectional study of subjects with and without HIV.

Methods: Subjects with and without HIV infection were enrolled at Grady Memorial Hospital, Atlanta GA. Individuals were excluded if they had evidence of major medical co-morbidities, were malnourished or smoked cigarettes. Exhaled breath condensate (EBC) and bronchoscopy with bronchoalveolar lavage (BAL) were performed to assess for airway oxidant stress. Primary outcomes were GSH and Cys levels in the BAL.

Results: 26 HIV and 28 non-HIV subjects were enrolled. There were no significant differences in median BAL fluid GSH and Cys levels between HIV and non-HIV-infected subjects. HIV infection without ART was associated with an estimated OR of 2.12 for low BAL GSH. Although not statistically significant, there was a trend showing that HIV infection with ART was associated with increased BAL GSH. There was no association observed between BAL Cys, HIV infection, age, BMI and use of ART.

Conclusion: HIV infection without ART was associated with increased oxidative stress in the lung, as measured by BAL GSH. There was a trend showing that HIV infection with ART was associated with decreased oxidative stress, suggesting that the use of ART is protective. Further study needs to be done to address the role of antioxidants, particularly GSH supplementation, to mitigate HIV-induced oxidative stress and enhance lung health in HIV-infected individuals.

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INTRODUCTION

Human immunodeficiency virus (HIV) infection remains an important problem worldwide. The Centers for Disease Control (CDC) estimates that approximately 1 million people are living with HIV in the United States (1) and approximately 20% are unaware that they are infected. Additionally, in the past decade, the number of people living with HIV has increased considerably. Prior to the widespread use of anti-retroviral therapy (ART), pulmonary diseases, especially lung infections, were among the most frequent complications in individuals with HIV and were associated with significant morbidity and mortality (2).

Although anti-retroviral therapy (ART) has reduced the risk of opportunistic infections, pneumonias from common pathogens such as pneumococcus and influenza, remain a leading cause of morbidity and mortality in HIV-infected individuals, resulting in tremendous healthcare costs (3, 4). It is unclear at this time what the mechanism is which renders these individuals susceptible to lung infection.

One possible mechanism is the generation of oxidative stress within the lung. Chronic HIV infection alone has been known to cause significant oxidative stress systemically, both in pre-clinical (5-7) and clinical studies (8-10). The mechanisms by which HIV infection causes oxidative stress are still unclear, but may involve alveolar macrophage immune responses and inflammatory pathways (11, 12). Oxidative stress can be generated from a number of different mechanisms including oxidation of extracellular thiol disulfide pairs such as glutathione/glutathione disulfide (GSH/GSSG) and cysteine/cystine (Cys/CySS), redox potential (Eh Cys/CySS or Eh GSH/GSSG), dysfunction of NADPH oxidase, and generation of reactive oxygen species (13, 14). Extracellular redox potential in the lung can serve as a biomarker of alveolar immune function, which can be quantified by an invasive bronchoalveolar lavage (BAL) procedure or non-invasively via exhaled breath condensate (EBC). Measuring alveolar redox

potential in HIV-infected individuals may identify those with chronic oxidative stress who are at increased risk for lung infection. Although BAL samples the alveolar space, its use is limited in a clinic setting. However, EBC is a non-invasive technique for sampling the epithelial lining fluid. It can be assayed for GSH/GSSG and Cys/CySS concentrations and other oxidative stress-related metabolic pathways (15).

The primary objective of this study was to estimate the association between HIV infection and oxidative stress in the lung, as reflected by decreased levels of GSH and CYS in the BAL fluid and EBC. We hypothesized that HIV infection would be associated with increased oxidative stress in the lung, as reflected by decreased levels of GSH and Cys in both BAL fluid and EBC sampling,

BACKGROUND

Human immunodeficiency virus (HIV) infection remains an important problem worldwide. The Centers for Disease Control (CDC) estimates that 1,148,200 people older than 13 years of age are living with HIV infection in the United States, including 207,600 who are unaware they are infected (1). Thousands of new people continue to be infected and the CDC estimates that approximately 17,000 people with Acquired Immunodeficiency Syndrome (AIDS) died in 2009 (16). Before the widespread use of combination anti-retroviral therapy (ART), pulmonary diseases were among the most common complications of HIV infection and were frequently associated with significant morbidity and mortality (2, 17). The multicenter Pulmonary Complications of HIV Infection Study, conducted approximately 15 years prior to ART, found that the most common complications were lung infections including *Pneumocystis* Pneumonia and bacterial pneumonia (18).

Community-acquired pneumonia is a leading cause of death worldwide (19, 20). It affects approximately 4 million people in the United States per year resulting in more than 1 million hospital admissions and 40,000 deaths (21). *Streptococcus Pneumoniae* (pneumococcus) is associated with more severe forms of pneumonia and is one of the main organisms leading to hospitalization in all groups (22). However, pneumococcal disease has been shown to be higher in persons with underlying medical conditions (such as HIV/AIDS), low socioeconomic status or those who engage in high-risk behaviors such as cigarette smoking, intravenous drug use and alcohol abuse (23, 24).

Although ART has substantially decreased opportunistic infections associated with HIV infection, pneumonias from these 'routine' pathogens such as pneumococcus and influenza continue to cause significant morbidity and mortality in these susceptible individuals (25-29). A recent study in the Veterans Administration (VA) HIV cohort found that bacterial pneumonia was

the most common pulmonary disease in HIV-infected individuals, with an incidence of 28.0 per 1,000 person years (95% confidence interval [CI], 27.2–28.8) compared with 5.8 per 1,000 person years (95% CI, 5.6–6.0) among HIV-uninfected individuals (p=0.001) (17). Advanced immunosuppression is a known risk factor and even single episode of pneumonia is associated with increased morbidity and mortality (30). In fact, recurrent bacterial pneumonia has been considered a cause of AIDS since the expanded European definition was adopted in 1993 (30, 31).

It is unclear at this time the exact mechanism by which HIV infection renders individuals susceptible to lung infections. One possible mechanism is the generation of reactive oxygen species, resulting in increased oxidative stress and impairment of lung immunity. Glutathione (GSH), a cysteine-containing tripeptide, is the most abundant non-protein thiol in living organisms and essential for viability of nearly all cells (12, 32). GSH is able to scavenge reactive oxygen species and provides the principal intracellular defense against oxidative stress (33). Cysteine (Cys), an essential amino acid, is a rate-limiting component of GSH synthesis and plays an important role in maintaining the detoxification of free radicals and reactive oxygen species (13, 14). Oxidative stress can be generated through several mechanisms including the oxidation of extracellular thiols, in particular glutathione (GSH) to glutathione disulfide (GSSG) and cysteine (Cys) to cystine (CySS) (25).

Chronic HIV infection alone has been known to cause significant oxidative stress systemically, both in pre-clinical (5-7) and clinical studies (8-10). GSH levels were found to be decreased greater than 90% in HIV transgenic rats compared to wild-type and GSSG:GSH ratios were increased 3-fold (6). Post-lipopolysaccharide treatment, HIV-infected animals also have decreased GSH, increased nitric oxide metabolites and superoxide (5). Clinically, reduced plasma Cys was significantly lower in HIV-positive patients compared to control subjects (10)

and several studies have shown that HIV-infected patients have disturbances in GSH redox balance (34-36) . GSH deficiency has even been associated with impaired survival in HIV-infected subjects (32). However, few studies have been done evaluating whether HIV infection results in increased alveolar oxidative stress. Our group identified that chronic HIV transgene expression in animal models causes significant alveolar oxidative stress, as reflected by a greater than 90% decrease in GSH levels and a three-fold increase in GSSG:GSH ratios (6). Others have also shown similar results (37, 38). In clinical studies, the results have been conflicting. Pacht et al. reported that the concentration of GSH in the epithelial lining fluid was similar between HIV and non-infected subjects (39); however, these same authors demonstrated, in a small study of 33 HIV-infected subjects, that GSH levels in the epithelial lining fluid were found to be significantly decreased over time (12). Others have also shown that HIV-infected subjects had a deficiency of GSH in the lung (40-42), but these studies have involved small numbers of subjects.

Therefore, we sought to explore the potential mechanisms by which HIV infection results in impaired lung immunity, thereby extending our preliminary experimental investigations. In a population where lung disease continues to be a significant cause of morbidity and mortality despite ART, further study needs to be done to gain a better understanding of mechanisms of lung immunity and to discover innovative therapies to improve the lung immune response and reduce the burden of lung disease.

METHODS

Hypothesis: HIV infection is associated with oxidative stress in the lung, as reflected by decreased levels of glutathione (GSH) and cysteine (Cys) in both bronchoalveolar lavage (BAL) fluid and exhaled breath condensate (EBC) sampling.

Study Design: To test this hypothesis, we performed a cross-sectional study of HIV-infected subjects without other medical problems and compared them to healthy (non-HIV-infected) subjects.

Characteristics of study population: This project was conducted within the Grady Health System in Atlanta, GA. Subjects were recruited using flyers and clinician referrals from an infectious disease clinic in Atlanta, Grady Medicine Clinics, and the Atlanta community. Informed consent was obtained from all subjects. Inclusion criteria included all subjects with and without HIV infection. Exclusion criteria included active liver disease (known cirrhosis and/or total bilirubin > 2.0 mg/dL), heart disease (ejection fraction < 50%, h/o acute myocardial infarction, New York Heart Association (NYHA) II-IV cardiac symptoms, severe valvular dysfunction), current renal disease (dialysis dependent or creatinine > 2.0 mg/dL), current lung disease (spirometry revealing a forced vital capacity (FVC) or forced expiratory volume in 1 second (FEV1) < 80% of predicted), diabetes, current pregnancy, malnutrition (body mass index < 17), current tobacco use and age < 25 years.

Study Protocol: Informed consent was obtained from all subjects. All study procedures were done at Grady Memorial Hospital in Atlanta, GA. The study coordinator entered the date, patient's name, medical record number, and study ID number in the patient enrollment log. Data was initially collected using our established case report forms (CRFs) and then entered into a HIPAA-compliant, secure database. All subjects completed a pre-enrollment evaluation (visit 1)

which included: 1) complete history and physical exam, 2) routine blood chemistries (basic chemistry, liver function tests, complete blood count, coagulation parameters, hemoglobin A1C), 3) CD4+ count and viral load (if not done in past 30 days) 4) urine pregnancy test (qualitative beta-HCG) for women of child-bearing potential, 5) urine dipstick for cotinine, 6) spirometry (FEV1, FVC), 7) Short Michigan Alcohol Screening Test (SMAST) and Alcohol Use Disorders Identification Test (AUDIT) alcohol use questionnaires, 8) 24 hour food record documentation to estimate glutathione and cysteine intake and analysis (performed by a registered dietician) and 9) Body mass index (BMI). Demographic data was collected. Subjects with exclusions identified at the time of the pre-enrollment evaluation were excluded from further participation. Subjects who were eligible after visit 1 completed visit 2.

After completing the pre-enrollment evaluation to confirm eligibility, subjects presented to the Grady Memorial Hospital (GMH) Clinical Interaction Site after an overnight fast. Enrolled volunteers had the following interventions on visit 2: 1) systemic markers of oxidative stress (GSH/GSSG and Cys/CySS) and CD4+ count and viral load for HIV-infected subjects only; 2) 3-day food record documentation and analysis (performed by a registered dietician), 3) Exhaled breath condensate (EBC) using the R-tube, a non-invasive breath condensate collection device (Respiratory Research, Inc.), and 4) Flexible fiberoptic bronchoscopy with standardized bronchoalveolar lavage (BAL) technique performed using standard conscious sedation techniques. The BAL was performed by installing 30mL aliquots of 0.9% non-bacteriostatic normal saline solution, followed by withdrawal with low-pressure hand suction. The BAL procedure was continued until a total of 180mL had been administered. Subjects were contacted by phone 24 hours after completing the study to ensure patient safety. A Data Safety Monitoring Representative was appointed to review each patient's enrollment procedures and bronchoscopy report on a monthly basis.

Outcome variables: The primary outcome variables included GSH and Cys levels in the BAL. Secondary outcomes variables include GSH and Cys levels in the EBC.

Predictor variables: The primary variable of interest was HIV infection. Potential confounders included age, BMI and use of ART because they have been shown to be associated with the outcome and HIV infection in previous studies.

Biological Specimens: BAL fluid was transported immediately to a research lab at Emory University. All lab personnel were blinded to grouping of sample. BAL samples were preserved immediately after collection in 5% perchloric acid solution containing iodoacetic acid (6.7 μM) and boric acid (0.1M). The EBC was immediately treated with iodoacetic acid (13.4 mM final) for GSH/GSSG and Cys/CySS determinations of concentrations, oxidations and redox potentials. This step was completed within 30 min of collection to prevent degradation or oxidation of GSH. After protein removal, samples were derivatized with dansyl chloride and separated on a 10-μm Ultrasil amino column by high performance liquid chromatography (HPLC) (Waters 2690; Waters Corp., Milford MA).

Fluorescence detection was recorded by two detectors. This procedure has been done before by this lab (43). GSH, GSSG, Cys, and CySS were quantified relative to ɣ-glutamyl-glutamate, an internal standard. BAL dilution was corrected by urea (44).

Sample size calculations: In our cohort of HIV and non-HIV-infected subjects, we conducted a study with GSH and Cys, two continuous response variables from independent HIV and non-HIV subjects with 1 non-HIV per HIV subject. Consistent with the results of a previous study (45), we assume that the response within each subject group for BAL GSH will be normally distributed with a standard deviation of 2754.9. If the true difference in the HIV and non-HIV means is 6200, we need to study 5 HIV-infected subjects and 5 non-HIV-infected subjects to be able to

reject the null hypothesis that the population means of the HIV and non-HIV groups are equal with probability of 0.9 at a significance level of 0.05.

Statistical Analyses: Univariate comparisons between HIV subjects and non-HIV subjects were calculated and evaluated for a significance level of 0.05 using a chi-squared test for categorical variables and a two-sample t-test for continuous variables. The data was log-transformed or a Wilcoxon Rank-Sum Test was used when the data was not normally distributed. To examine BAL GSH and CYS levels among those with and without HIV, multi-variable linear regression analysis was used with log-transformed BAL GSH and BAL Cys levels as the outcome controlling for age, BMI and use of ART. Three exposure groups were analyzed – those with HIV on ART, those with HIV without ART and those without HIV. We also examined the relationship between BAL and EBC levels of GSH and Cys using linear regression.

RESULTS

Baseline Characteristics of HIV-infected Subjects and non-HIV-infected Subjects

A total of 26 HIV and 28 non-HIV subjects were enrolled. 22 HIV and 20 non-HIV subjects had bronchoalveolar lavage (BAL) and exhaled breath condensate (EBC) oxidative stress markers. Forty-two subjects total had both BAL and EBC measures to assess the relationship between both methods. **Table 1** shows the demographic characteristics of HIV and non-HIV-infected subjects enrolled in the study. The mean age of the HIV-infected subjects was significantly greater than the mean age of the non-HIV-infected subjects (47.9 years +/- 7.2 vs. 40.8 years +/- 10.3, p = 0.01). The majority of subjects, both HIV and non-HIV-infected, were African American. There were no statistically significant differences in gender or body mass index (BMI) between the groups. Among those with HIV, the median CD4 count was 432 (IQR 278-612) with a median viral load of 0 (IQR 0-3.2). 72.7% of the HIV-infected subjects were on anti-retroviral therapy (ART).

Glutathione and Cysteine BAL and EBC Levels in HIV versus non-HIV-infected Subjects

There was no difference detected in median BAL fluid glutathione (GSH) levels between HIV-infected and non-HIV-infected subjects [1958.57 nM (IQR 839.11- 4873.87) vs. 1641.37 nM (IQR 515.33- 4015.73), respectively, p=0.53] (**Figure 1a**). However, median GSH levels in the EBC were significantly higher in the HIV-infected subjects compared to the non-HIV-infected subjects [11.24 nM (IQR 3.84- 101.67) vs. 3.50 nM (IQR 0.70- 14.74) respectively, p=0.03] (**Figure 1b**). There was no difference in median BAL fluid cysteine (Cys) levels between HIV-infected and non-HIV-infected subjects [1439.38 nM (IQR 311.56- 5365.14) vs. 1459.10 nM (IQR 852.47- 3941.66) respectively, p=0.84] (**Figure 2a**). There was also no difference in median Cys levels between HIV-infected and non-HIV-infected subjects [0.28 nM (IQR 0.14-4.98) vs. 0.28 nM (IQR 0.0054-1.53) respectively, p=0.48] (**Figure 2b**).

Association of HIV with Glutathione and Cysteine BAL levels

The association of HIV infection with BAL fluid GSH and Cys levels was determined using multiple linear regression with age, BMI and use of ART entered as potential confounders into the model. HIV infection without ART was associated with an estimated OR of 2.12 for low BAL GSH (**Table 2**). Although not statistically significant, there was a trend showing that HIV infection with ART was associated with increased BAL GSH. There was no association observed between BAL Cys, HIV infection, age, BMI and use of ART (**Table 3**). Also, there was no interaction detected between predictor variables in either model.

Association between BAL Glutathione and Cysteine with EBC Glutathione and Cysteine Levels

The association between BAL GSH with EBC GSH and BAL Cys with EBC Cys was assessed using linear regression analysis. There was a very poor correlation between the two methods for both GSH (r^2=0.02, p= 0.33) and Cys (r^2=0.001, p=0.83) (**Figure 3**). There were significant differences in the amount of GSH between BAL and EBC in both HIV and non-HIV-infected subjects (1958.57 nM vs. 11.24 nM, $p < 0.01$ and 1459.10 nM vs. 0.28 nM, $p < 0.01$ respectively) (**Figures 4a and 4b**). There were also significant differences in the amount of Cys between BAL and EBC in both HIV and non-HIV-infected subjects (1439.38 nM vs. 0.28 nM, $p < 0.01$ and 1641.37 nM vs.3.49 nM, $p < 0.01$ respectively) (**Figures 5a and 5b**).

DISCUSSION/CONCLUSIONS

The results from this cross-sectional study show that HIV infection without anti-retroviral therapy (ART) was associated with increased oxidative stress in the lung, as reflected by low bronchoalveolar lavage (BAL) glutathione (GSH) levels, when adjusted for age and body mass index (BMI). Additionally, there was also a trend showing that HIV infection with ART was associated with increased BAL GSH, suggesting that the use of ART is protective. These associations were not seen with BAL cysteine (Cys). Although several studies have looked at various systemic oxidative stress biomarkers in HIV-infected individuals on ART (46-48), the impact of ART on alveolar oxidative stress is still unknown. There were no significant differences in BAL GSH or Cys levels between HIV and non-HIV-infected subjects. Interestingly, exhaled breath condensate (EBC) GSH levels were greater in HIV-infected subjects compared to non-HIV-infected subjects. Finally, there was a very poor correlation between BAL and EBC measurements of both GSH and Cys.

GSH is the most abundant non-protein thiol in living organisms and essential for many biologic functions including cell defense, T and B cell differentiation, and cytotoxic T-cell activation (49, 50). It also functions as an antioxidant, scavenging reactive oxygen species and reducing hydrogen peroxide (51, 52). Not just systemically, but GSH is also considered a primary antioxidant in the lung (53) and the alveolar space. Several studies have shown that HIV-infected subjects have disturbances in GSH redox balance (34-36). Clinically relevant, HIV-associated disturbances in GSH have even been associated with impaired survival (32). Alveolar GSH is likely to be very important in HIV-infected individuals as well. Alveolar macrophages, the primary host immune defense cells in the lung, have been shown to release exaggerated amounts of superoxide anion even in asymptomatic HIV-infected individuals (54). Previous investigations have shown that GSH protects alveolar macrophages from hyperoxia-

induced injury and injury to Type II alveolar epithelial cells (55, 56). Therefore, GSH may serve as a critical antioxidant in this environment of oxidative stress.

Although ART has resulted in dramatic clinical and immunological improvements in HIV-infected patients, not all individuals experience a favorable response (57, 58). ART has also resulted in the emergence of drug-resistant strains of the virus and serious long-term side effects (59). Few studies have examined the effect of ART on oxidative stress parameters and these studies remain conflicting. Aukrust and colleagues found that not only did ART decrease viral load and increase CD4+ T cell count, but there was an improvement in the abnormal GSH redox status (46) as well. Others, however, have shown that ART is associated with increased oxidative stress (47). However, both studies measured plasma biomarkers of oxidative stress and not biomarkers in the lung. In this study, we demonstrated that HIV infection without ART was associated with lower GSH levels in the lung as opposed to HIV infection with ART, suggesting that ART may be protective in the lung. However, although ART has been shown to improve oxidative stress, previous studies have shown that ART did not fully normalize the abnormal redox status (46). This suggests that a therapeutic intervention aimed at reducing oxidative stress may be beneficial to improve lung immunity, even for those individuals on ART.

Extracellular redox potential in the lung can be quantified by an invasive BAL procedure or non-invasively via EBC. Collection of EBC has several advantages over traditional BAL sampling of the lower airways. It is easy to perform, non-invasive, does not introduce foreign substances into the lung and has been standardized (60). Furthermore, it can be performed repeatedly on sick patients. Cytokines and markers of oxidative stress have been quantified by EBC previously (15, 61, 62). Specifically, GSH measurements in EBC were optimized and standardized in our lab (15). Previous studies have shown approximately 1000-fold dilution in EBC when compared to BAL (15, 63). The range of EBC GSH concentrations in our study was

also diluted 1000-fold compared to BAL GSH levels. In assessing the correlation between BAL and EBC, some studies have found a strong association when measuring certain inflammatory biomarkers such as eicosanoids (64, 65) and TNF-α (66); whereas other did not find a correlation at all between BAL and EBC when measuring other inflammatory biomarkers (66, 67). Therefore, there is still considerable controversy as to whether EBC may be used as a substitute for bronchoscopy. In this study, we found that there was a very poor correlation between measurements of GSH and Cys in the BAL and EBC. This may be secondary to dilutional factors as well as sampling. EBC samples a greater proportion of the respiratory tract compared to BAL and the relative contribution of each part is unknown. Additionally, EBC is collected via the mouth so the sample may be affected by oral components.

The strengths of this study include the restricted study population. By focusing on healthy subjects without other co-morbidities, we were able to minimize confounding and study a more homogenous population. An additional strength includes our ability to collect BAL fluid and EBC on all the subjects. All of these procedures were done utilizing the same method and personnel, minimizing measurement error. Finally, all lab personnel were blinded to the HIV status of the samples collected which minimizes bias. GSH and Cys levels have also been measured routinely in this lab, which also minimizes measurement error.

There are several limitations with this study. One, given that this is an observational cross-sectional study it is subject to confounding by known and unknown measures. However, we did attempt to minimize this by restricting our study population and adjusting for known confounders such as age in our model. Two, approximately 27% of the HIV-infected subjects were not on ART for varied reasons. Some of the subjects did not require ART clinically, whereas others were simply non-compliant with it. This could have influenced our results; however, we were able to analyze three exposure groups – those with HIV on ART, those with

HIV without ART and those without HIV allowing us to assess the effect of ART on alveolar oxidative stress markers which has not been done before. Three, the cross-sectional nature of the study did not allow us to determine whether HIV infection truly *causes* oxidative stress in the lung. A longitudinal design would allow for a better approach to model the relationship between longitudinal response and covariates. Four, we did not check HIV status on all the subjects so it is possible that the non-HIV subjects were actually infected HIV. Given that we did not demonstrate any difference in BAL GSH or Cys levels between HIV and non-HIV-infected subjects, it is possible that it was due to this misclassification, which would bias our results towards the null. Five, although all lab personnel were blinded to the HIV status of the sample, the PI and research staff were not. Finally, although these markers have been routinely measured in this lab, they have not routinely been measured in HIV-infected subjects.

In conclusion, our study demonstrates that HIV infection without ART is associated with increased oxidative stress in the lung, as measured by BAL GSH. There was a trend showing that HIV infection with ART maybe associated with lower oxidative stress in the lung. This suggests that ART in this population of otherwise healthy HIV-infected individuals is protective in the lung and may improve lung immunity. However, previous studies have shown that ART does not completely restore the disruption in redox pathways. Therefore, further study needs to be done to address the role of antioxidants, particularly GSH supplementation to mitigate HIV-induced oxidative stress and enhance lung health in HIV-infected individuals. Also, additional investigation of alveolar macrophage immune functions in this population will allow us to study the impact of HIV and oxidative stress on lung health. In a population where lung disease continues to be a significant cause of morbidity and mortality despite ART, further study needs to be done to discover new innovative therapies to improve the lung immune response and reduce the burden of lung disease. Finally, our study has confirmed that EBC biomarkers can be readily measured in HIV-infected patients. However, we did not show a correlation between BAL fluid

and EBC which could be secondary to differences in dilution. Although EBC has an advantage over BAL being less invasive, the discrepancy in measurements demonstrates that more investigation is needed before EBC can be used in a clinical setting as a substitute for bronchoscopy.

REFERENCES

1. CDC. Monitoring selected national HIV prevention and care objectives by using HIV surveillance data—United States and 6 U.S. dependent areas—2010. HIV Surveillance Supplemental Report 2012;17(No. 3, part A). 2012.
2. Murray JF, Mills J. Pulmonary infectious complications of human immunodeficiency virus infection. Part I. Am Rev Respir Dis 1990; 141(5 Pt 1): 1356-72
3. Grau I, Pallares R, Tubau F, et al. Epidemiologic changes in bacteremic pneumococcal disease in patients with human immunodeficiency virus in the era of highly active antiretroviral therapy. Arch Intern Med 2005; 165(13): 1533-40
4. Sullivan JH, Moore RD, Keruly JC, et al. Effect of antiretroviral therapy on the incidence of bacterial pneumonia in patients with advanced HIV infection. Am J Respir Crit Care Med 2000; 162(1): 64-7
5. Jacob BA, Porter KM, Elms SC, et al. HIV-1-induced pulmonary oxidative and nitrosative stress: exacerbated response to endotoxin administration in HIV-1 transgenic mouse model. Am J Physiol Lung Cell Mol Physiol 2006; 291(4): L811-L819
6. Lassiter C, Fan X, Joshi PC, et al. HIV-1 transgene expression in rats causes oxidant stress and alveolar epithelial barrier dysfunction. AIDS Res Ther 2009; 6: 1
7. Louboutin JP, Agrawal L, Reyes BA, et al. HIV-1 gp120-induced injury to the blood-brain barrier: role of metalloproteinases 2 and 9 and relationship to oxidative stress. J Neuropathol Exp Neurol 2010; 69(8): 801-16
8. Bilbis LS, Idowu DB, Saidu Y, et al. Serum levels of antioxidant vitamins and mineral elements of human immunodeficiency virus positive subjects in Sokoto, Nigeria. Ann Afr Med 2010; 9(4): 235-9
9. Coaccioli S, Crapa G, Fantera M, et al. Oxidant/antioxidant status in patients with chronic HIV infection. Clin Ter 2010; 161(1): 55-8
10. Naisbitt DJ, Vilar FJ, Stalford AC, et al. Plasma cysteine deficiency and decreased reduction of nitrososulfamethoxazole with HIV infection. AIDS Res Hum Retroviruses 2000; 16(18): 1929-38
11. Balamayooran G, Batra S, Fessler MB, et al. Mechanisms of neutrophil accumulation in the lungs against bacteria. Am J Respir Cell Mol Biol 2010; 43(1): 5-16
12. Pacht ER, Diaz P, Clanton T, et al. Alveolar fluid glutathione decreases in asymptomatic HIV-seropositive subjects over time. Chest 1997; 112(3): 785-8
13. Jones DP. Redefining oxidative stress. Antioxid Redox Signal 2006; 8(9-10): 1865-79
14. Jones DP. Radical-free biology of oxidative stress. Am J Physiol Cell Physiol 2008; 295(4): C849-C868
15. Yeh MY, Burnham EL, Moss M, et al. Non-invasive evaluation of pulmonary glutathione in the exhaled breath condensate of otherwise healthy alcoholics. Respir Med 2008; 102(2): 248-55
16. CDC. HIV Surveillance Report, 2010; vol. 22. http://www.cdc.gov/hiv/topics/surveillance/resources/reports/. 2012.

Ref Type: Generic

17. Crothers K, Huang L, Goulet JL, et al. HIV infection and risk for incident pulmonary diseases in the combination antiretroviral therapy era. Am J Respir Crit Care Med 2011; 183(3): 388-95
18. Wallace JM, Hansen NI, Lavange L, et al. Respiratory disease trends in the Pulmonary Complications of HIV Infection Study cohort. Pulmonary Complications of HIV Infection Study Group. Am J Respir Crit Care Med 1997; 155(1): 72-80
19. Kozak LJ, Owings MF, Hall MJ. National Hospital Discharge Survey: 2002 annual summary with detailed diagnosis and procedure data. Vital Health Stat 13 2005; (158): 1-199

20. Lim WS, Macfarlane JT, Boswell TC, et al. Study of community acquired pneumonia aetiology (SCAPA) in adults admitted to hospital: implications for management guidelines. Thorax 2001; 56(4): 296-301
21. Park DR, Sherbin VL, Goodman MS, et al. The etiology of community-acquired pneumonia at an urban public hospital: influence of human immunodeficiency virus infection and initial severity of illness. J Infect Dis 2001; 184(3): 268-77
22. Marrie TJ, Durant H, Yates L. Community-acquired pneumonia requiring hospitalization: 5-year prospective study. Rev Infect Dis 1989; 11(4): 586-99
23. Harrison LH, Dwyer DM, Billmann L, et al. Invasive pneumococcal infection in Baltimore, Md: implications for immunization policy. Arch Intern Med 2000; 160(1): 89-94
24. Loeliger AE, Rijkers GT, Aerts P, et al. Deficient antipneumococcal polysaccharide responses in HIV-seropositive patients. FEMS Immunol Med Microbiol 1995; 12(1): 33-41
25. El-Sadr WM, Lundgren JD, Neaton JD, et al. CD4+ count-guided interruption of antiretroviral treatment. N Engl J Med 2006; 355(22): 2283-96
26. Gordin FM, Roediger MP, Girard PM, et al. Pneumonia in HIV-infected persons: increased risk with cigarette smoking and treatment interruption. Am J Respir Crit Care Med 2008; 178(6): 630-6
27. Hirschtick RE, Glassroth J, Jordan MC, et al. Bacterial pneumonia in persons infected with the human immunodeficiency virus. Pulmonary Complications of HIV Infection Study Group. N Engl J Med 1995; 333(13): 845-51
28. Jordano Q, Falco V, Almirante B, et al. Invasive pneumococcal disease in patients infected with HIV: still a threat in the era of highly active antiretroviral therapy. Clin Infect Dis 2004; 38(11): 1623-8
29. Lin JC, Nichol KL. Excess mortality due to pneumonia or influenza during influenza seasons among persons with acquired immunodeficiency syndrome. Arch Intern Med 2001; 161(3): 441-6
30. Caiaffa WT, Graham NM, Vlahov D. Bacterial pneumonia in adult populations with human immunodeficiency virus (HIV) infection. Am J Epidemiol 1993; 138(11): 909-22
31. Madeddu G, Porqueddu EM, Cambosu F, et al. Bacterial community acquired pneumonia in HIV-infected inpatients in the highly active antiretroviral therapy era. Infection 2008; 36(3): 231-6
32. Herzenberg LA, De Rosa SC, Dubs JG, et al. Glutathione deficiency is associated with impaired survival in HIV disease. Proc Natl Acad Sci U S A 1997; 94(5): 1967-72
33. Shan XQ, Aw TY, Jones DP. Glutathione-dependent protection against oxidative injury. Pharmacol Ther 1990; 47(1): 61-71
34. Aukrust P, Svardal AM, Muller F, et al. Increased levels of oxidized glutathione in CD4+ lymphocytes associated with disturbed intracellular redox balance in human immunodeficiency virus type 1 infection. Blood 1995; 86(1): 258-67
35. Aukrust P, Muller F. Glutathione redox disturbances in human immunodeficiency virus infection: immunologic and therapeutic consequences. Nutrition 1999; 15(2): 165-7
36. Roederer M, Staal FJ, Osada H, et al. CD4 and CD8 T cells with high intracellular glutathione levels are selectively lost as the HIV infection progresses. Int Immunol 1991; 3(9): 933-7
37. Cota-Gomez A, Flores AC, Ling XF, et al. HIV-1 Tat increases oxidant burden in the lungs of transgenic mice. Free Radic Biol Med 2011; 51(9): 1697-707
38. Jacob BA, Porter KM, Elms SC, et al. HIV-1-induced pulmonary oxidative and nitrosative stress: exacerbated response to endotoxin administration in HIV-1 transgenic mouse model. Am J Physiol Lung Cell Mol Physiol 2006; 291(4): L811-L819
39. Pacht ER, Diaz P, Clanton T, et al. Alveolar fluid glutathione is not reduced in asymptomatic HIV-seropositive subjects. Am J Respir Crit Care Med 1997; 155(1): 374-7

40. Buhl R, Jaffe HA, Holroyd KJ, et al. Systemic glutathione deficiency in symptom-free HIV-seropositive individuals. Lancet 1989; 2(8675): 1294-8
41. Diaz PT, Wewers MD, King M, et al. Regional differences in emphysema scores and BAL glutathione levels in HIV-infected individuals. Chest 2004; 126(5): 1439-42
42. Holroyd KJ, Buhl R, Borok Z, et al. Correction of glutathione deficiency in the lower respiratory tract of HIV seropositive individuals by glutathione aerosol treatment. Thorax 1993; 48(10): 985-9
43. Moss M, Guidot DM, Wong-Lambertina M, et al. The effects of chronic alcohol abuse on pulmonary glutathione homeostasis. Am J Respir Crit Care Med 2000; 161(2 Pt 1): 414-9
44. Rennard SI, Basset G, Lecossier D, et al. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. J Appl Physiol 1986; 60(2): 532-8
45. Yeh MY, Burnham EL, Moss M, et al. Chronic alcoholism alters systemic and pulmonary glutathione redox status. Am J Respir Crit Care Med 2007; 176(3): 270-6
46. Aukrust P, Muller F, Svardal AM, et al. Disturbed glutathione metabolism and decreased antioxidant levels in human immunodeficiency virus-infected patients during highly active antiretroviral therapy--potential immunomodulatory effects of antioxidants. J Infect Dis 2003; 188(2): 232-8
47. Hulgan T, Morrow J, D'Aquila RT, et al. Oxidant stress is increased during treatment of human immunodeficiency virus infection. Clin Infect Dis 2003; 37(12): 1711-7
48. Mandas A, Iorio EL, Congiu MG, et al. Oxidative imbalance in HIV-1 infected patients treated with antiretroviral therapy. J Biomed Biotechnol 2009; 2009: 749575
49. Droge W, Pottmeyer-Gerber C, Schmidt H, et al. Glutathione augments the activation of cytotoxic T lymphocytes in vivo. Immunobiology 1986; 172(1-2): 151-6
50. Staal FJ, Ela SW, Roederer M, et al. Glutathione deficiency and human immunodeficiency virus infection. Lancet 1992; 339(8798): 909-12
51. el-Hag A, Lipsky PE, Bennett M, et al. Immunomodulation by neutrophil myeloperoxidase and hydrogen peroxide: differential susceptibility of human lymphocyte functions. J Immunol 1986; 136(9): 3420-6
52. Stagnaro R, Pierri I, Piovano P, et al. Thiol containing antioxidant drugs and the human immune system. Bull Eur Physiopathol Respir 1987; 23(4): 303-7
53. Cantin AM, North SL, Hubbard RC, et al. Normal alveolar epithelial lining fluid contains high levels of glutathione. J Appl Physiol 1987; 63(1): 152-7
54. Buhl R, Jaffe HA, Holroyd KJ, et al. Activation of alveolar macrophages in asymptomatic HIV-infected individuals. J Immunol 1993; 150(3): 1019-28
55. Pacht ER, Abernathy F. Prevention of intracellular adenosine triphosphate depletion after sublethal oxidant injury to rat type II alveolar epithelial cells with exogenous glutathione and N-acetylcysteine. Am J Med Sci 1995; 310(4): 133-7
56. Simon RH, DeHart PD, Nadeau DM. Resistance of rat pulmonary alveolar epithelial cells to neutrophil- and oxidant-induced injury. Am J Respir Cell Mol Biol 1989; 1(3): 221-9
57. Engsig FN, Gerstoft J, Kronborg G, et al. Long-term mortality in HIV patients virally suppressed for more than three years with incomplete CD4 recovery: a cohort study. BMC Infect Dis 2010; 10: 318
58. Pacheco YM, Jarrin I, Del AJ, et al. Risk factors, CD4 long-term evolution and mortality of HIV-infected patients who persistently maintain low CD4 counts, despite virological response to HAART. Curr HIV Res 2009; 7(6): 612-9
59. Hartman TL, Buckheit RW, Jr. The Continuing Evolution of HIV-1 Therapy: Identification and Development of Novel Antiretroviral Agents Targeting Viral and Cellular Targets. Mol Biol Int 2012; 2012: 401965
60. Silkoff PE, Erzurum SC, Lundberg JO, et al. ATS workshop proceedings: exhaled nitric oxide and nitric oxide oxidative metabolism in exhaled breath condensate. Proc Am Thorac Soc 2006; 3(2): 131-45

61. Horvath I, Donnelly LE, Kiss A, et al. Exhaled nitric oxide and hydrogen peroxide concentrations in asthmatic smokers. Respiration 2004; 71(5): 463-8
62. Kostikas K, Papatheodorou G, Psathakis K, et al. Oxidative stress in expired breath condensate of patients with COPD. Chest 2003; 124(4): 1373-80
63. Effros RM, Peterson B, Casaburi R, et al. Epithelial lining fluid solute concentrations in chronic obstructive lung disease patients and normal subjects. J Appl Physiol 2005; 99(4): 1286-92
64. Ciebiada M, Gorski P, Antczak A. Eicosanoids in exhaled breath condensate and bronchoalveolar lavage fluid of patients with primary lung cancer. Dis Markers 2012; 32(5): 329-35
65. Piotrowski WJ, Antczak A, Marczak J, et al. Eicosanoids in exhaled breath condensate and BAL fluid of patients with sarcoidosis. Chest 2007; 132(2): 589-96
66. Kowalska A, Puscinska E, Czerniawska J, et al. [Markers of fibrosis and inflammation in exhaled breath condensate (EBC) and bronchoalveolar lavage fluid (BALF) of patients with pulmonary sarcoidosis -- a pilot study]. Pneumonol Alergol Pol 2010; 78(5): 356-62
67. Jackson AS, Sandrini A, Campbell C, et al. Comparison of biomarkers in exhaled breath condensate and bronchoalveolar lavage. Am J Respir Crit Care Med 2007; 175(3): 222-7

TABLES/GRAPHS

Table 1: Demographic characteristics of HIV-infected Subjects and Non-HIV-infected Subjects Enrolled in the Study

Patient Characteristics	Non-HIV N=20	HIV N=22	*P*-value
Age (mean, SD)	40.8 (10.3)	47.9 (7.2)	0.01
Gender (% male)	10 (50)	8 (36.4)	0.37
Race			0.05
White n (%)	6 (30)	2 (9.1)	
Black n (%)	12 (60)	20 (90.9)	
Other n (%)	2 (10)	0	
BMI (kg/m^2, mean, SD)	33.3 (10.4)	34.6 (7.8)	0.64
SMAST (median, IQR)	0 (0-0)	0 (0-3)	0.19
AUDIT (median, IQR)	0.5 (0-2.8)	1.5 (0-2.5)	0.46
CD4 (median, IQR)		432 (278-612)	
Viral load (log copies/mL, median, IQR)		0 (0-3.2)	
Use of ART (%)		72.7%	

BMI = Body Mass Index

SMAST = Short Michigan Alcohol Screening Test

AUDIT = Alcohol Use Disorders Identification Test

ART = Anti-retroviral medications

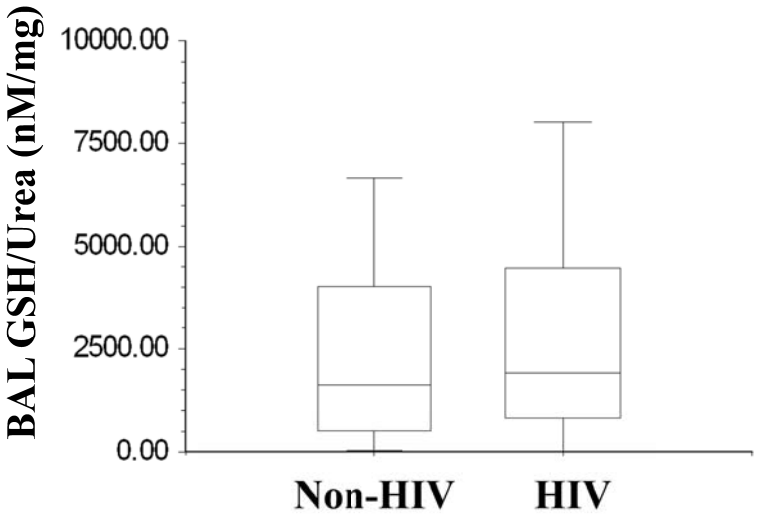

Figure 1a: Box plots of GSH levels measured in BAL fluid. Median GSH levels in non-HIV vs. HIV subjects were 1641.37 nM vs. 1958.57 nM, respectively (p=0.53).

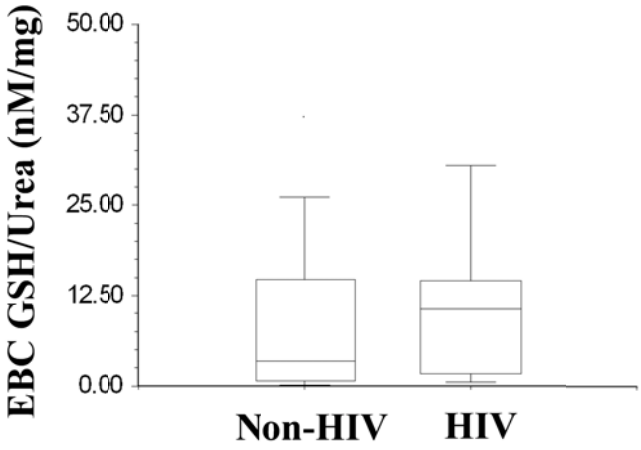

Figure 1b: Box plots of GSH levels measured in EBC. Median GSH levels in non-HIV vs. HIV subjects were 3.50 nM vs. 11.24 nM respectively (p=0.03).

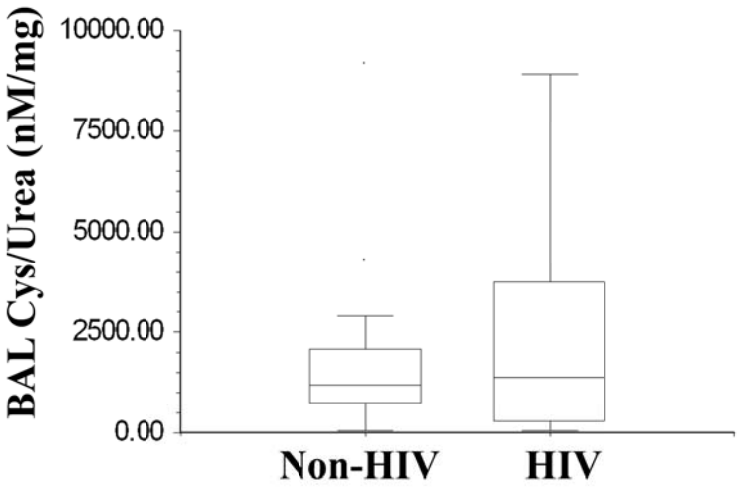

Figure 2a: Box plots of CYS levels measured in BAL fluid. Median CYS levels in non-HIV vs. HIV subjects were 1459.10 nM vs. 1439.38 nM respectively, (p=0.84).

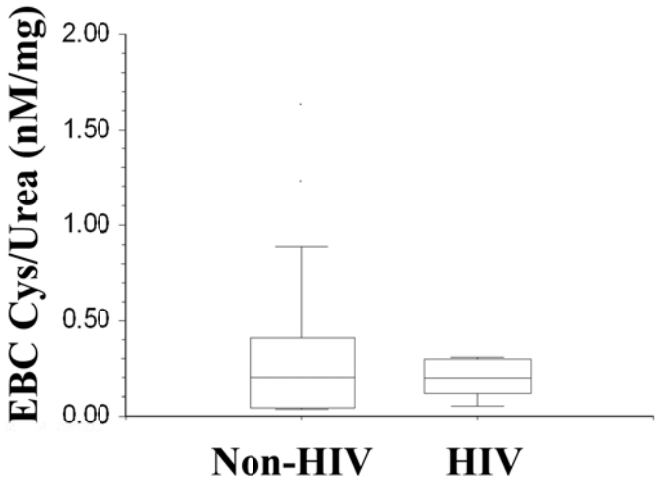

Figure 2b: Box plots of CYS levels measured in EBC. Median CYS levels in non-HIV vs. HIV subjects were 0.28 nM vs. 0.28 nM respectively, (p=0.48).

Table 2: Multiple Linear Regression Model for Log-transformed BAL Glutathione Levels

Variable	Slope	% Change in BAL GSH	95% CI	p-value
HIV on ART (vs. no HIV)	0.3422	41% increase	[-12%,126%]	0.17
HIV not on ART (vs. no HIV)	-0.7524	53% decrease	[-75%, -12%]	0.02
Age (per year)	0.0033	0%	[-2%, 3%]	0.78
BMI (kg/m^2)	0.0059	0%	[-2%, 3%]	0.62
p=0.03 Adjusted R^2 =0.17				

Table 3: Multiple Linear Regression Model for Log-transformed BAL Cysteine Levels

Variable	Slope	% Change in BAL Cys	95% CI	p-value
HIV on ART (vs. no HIV)	-0.3583	30% decrease	[-57%, 15%]	0.16
HIV not on ART (vs. no HIV)	0.3791	46% increase	[-24%, 180%]	0.26
Age (per year)	0.0059	0%	[-2%, 3%]	0.64
BMI (kg/m^2)	-0.0001	0%	[-2%, 2%]	0.99
p=0.29 Adjusted R^2 =0.03				

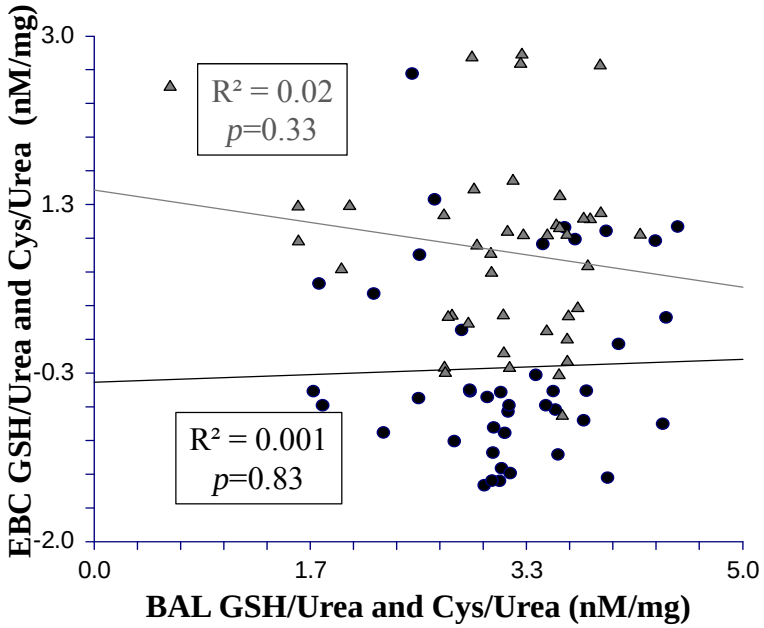

Figure 3: Scatter Plot of Log-transformed GSH (gray triangle) and Cys (black circle) levels in EBC (nM/mg) as a function of GSH and Cys levels in BAL (nM/mg).

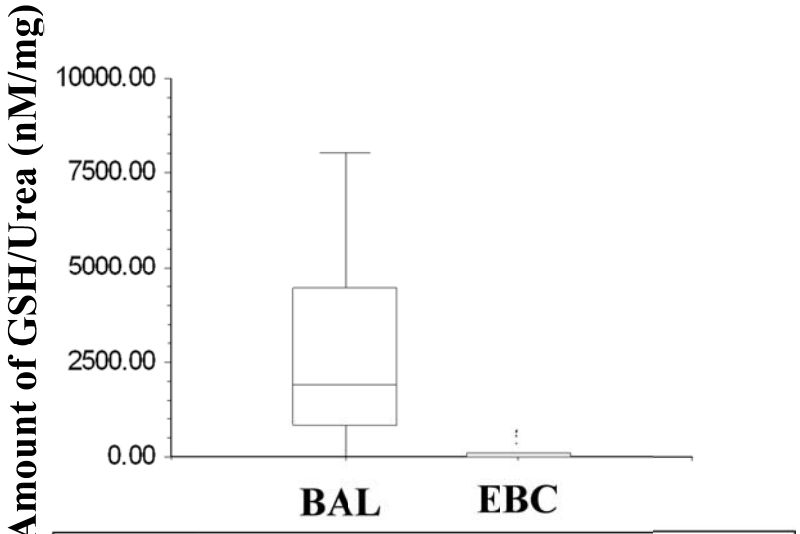

Figure 4a: Box plots of GSH levels measured in BAL fluid and EBC in HIV-infected subjects. Median GSH levels in BAL vs. EBC were 1958.57 nM vs. 11.24 nM respectively ($p < 0.01$).

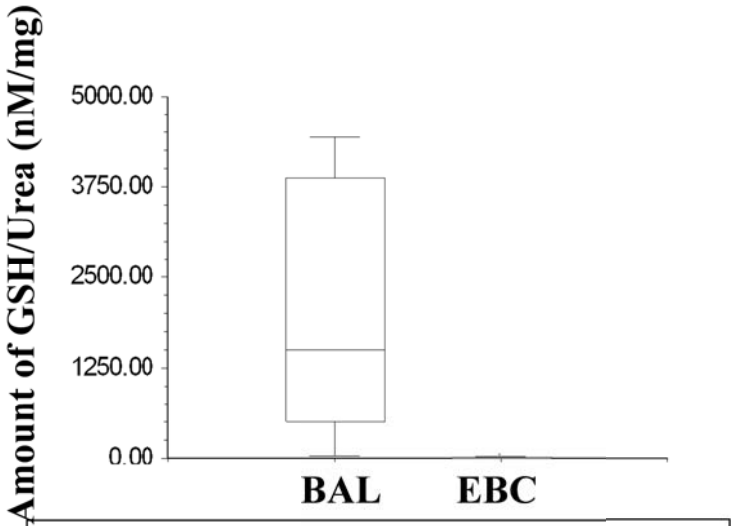

Figure 4b: Box plots of GSH levels measured in BAL fluid and EBC in non-HIV-infected subjects. Median GSH levels in BAL vs. EBC were 1459.10 nM vs. 0.28 nM respectively ($p < 0.01$).

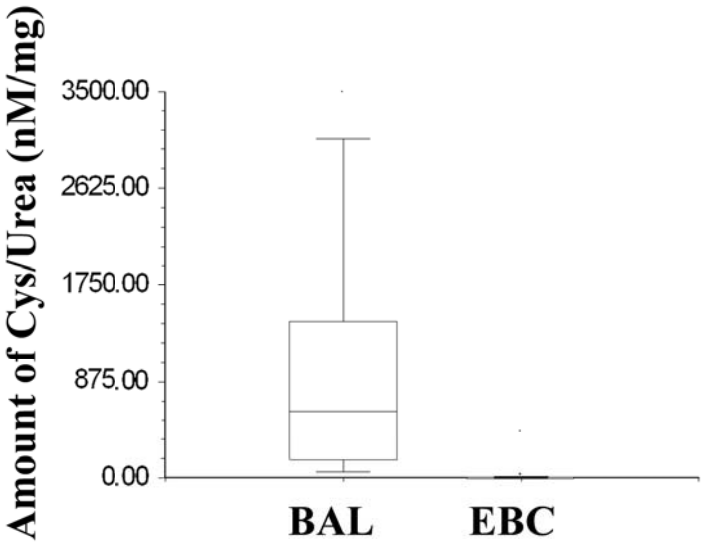

Figure 5a: Box plots of CYS levels measured in BAL fluid and EBC in HIV-infected subjects. Median CYS levels in BAL vs. EBC were 1439.38 nM vs. 0.28 nM respectively ($p < 0.01$).

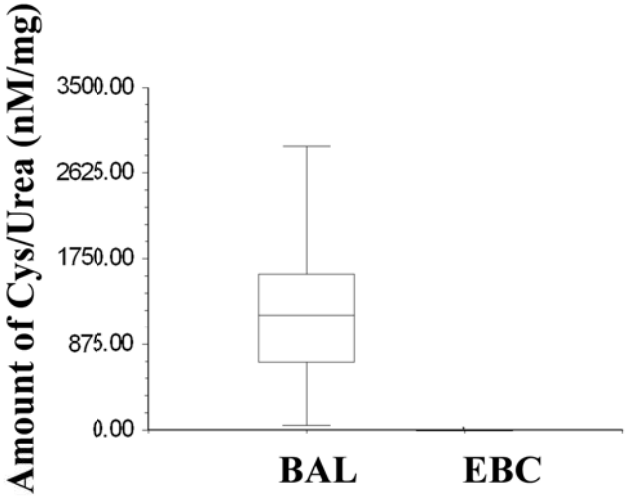

Figure 5b: Box plots of CYS levels measured in BAL fluid and EBC in non-HIV-infected subjects. Median CYS levels in BAL vs. EBC were 1641.37 nM vs.3.49 nM respectively ($p < 0.01$).