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Anne-Genevieve Wheaton Date

Thiol Redox Status in Critically Ill Patients

By

Anne-Genevieve Wheaton
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Nutrition and Health Sciences

_____
Lou Ann S. Brown, Ph.D.
Advisor

_____
David M. Guidot, M.D.
Committee Member

_____
Dean P. Jones, Ph.D.
Committee Member

_____
J. Wylie Nichols, Ph.D.
Committee Member

_____
Thomas R. Ziegler, M.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the Graduate School

_____
Date

Thiol Redox Status in Critically Ill Patients

By

Anne-Genevieve Wheaton
B.S., Georgia Institute of Technology, 1994

Advisor: Lou Ann S. Brown, Ph.D.

An abstract of
a dissertation submitted to the Faculty of the Graduate School of Emory University
in partial fulfillment of the requirements for the degree of
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Abstract

Thiol Redox Status in Critically Ill Patients
By Anne-Genevieve Wheaton

Oxidative stress has been implicated in many pulmonary diseases. Previous studies have focused on the status of the endogenous antioxidant glutathione (GSH), but the status of cysteine (Cys), another important thiol antioxidant present in the lung, has been largely ignored. The bronchoalveolar lavage fluid (BALF) of critically ill patients was more oxidized than controls as evidenced by higher H_2O_2, 4-hydroxynonenal (HNE), and %GSSG (100%*GSSG/GSH+GSSG). The Cys pool was larger in this group but not significantly more oxidized than controls. Higher malondialdehyde (MDA) concentrations and %GSSG in critically ill patients indicated more oxidized plasma, but surprisingly plasma Cys was more reduced in this group. A history of alcohol abuse is a risk factor for the development of and increased mortality from ventilator-associated pneumonia (VAP), a common infection among mechanically-ventilated patients. GSH was depleted to a comparable extent in healthy alcoholics and VAP patients compared to healthy controls. The GSH pools were also more oxidized (%GSSG) in these groups. Cys and CySS (cystine) concentrations were much higher in the VAP groups compared to either healthy group. The total Cys pool (tCys) was larger than the GSH pool (tGSH) in VAP patients compared to healthy subjects. Among VAP patients, a lower tCys/tGSH was associated with survival. Although smoking and chronic alcohol abuse are both oxidative conditions, each is associated with increased risk for different pulmonary disorders. The exhaled breath condensate (EBC), a noninvasive sampling method, of subjects with a history of alcohol abuse and/or smokers was used to monitor the redox status of GSH and Cys. GSH was more oxidized in the EBC of alcoholics, but not different based on smoking status. A history of alcohol abuse was associated with elevated Cys and CySS concentrations in the EBC. A positive smoking history was associated with a 50% decrease in the Cys concentration. Cys was more oxidized by smoking in the control groups and by alcohol in the nonsmoking groups. The ratio of tCys/tGSH was approximately one in nonsmoking controls. This ratio was significantly higher for both alcohol groups and lower for smoking controls, possibly indicative of different forms of oxidative stress.

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ABBREVIATIONS

ALA	Alpha lipoic acid
ARDS	Acute respiratory distress syndrome
$Asc^{\bullet -}$	Dehydroascorbic acid, vitamin C (oxidized)
$AscH^-$	Ascorbate, vitamin C (reduced)
AUDIT	Alcohol Use Disorders Identification Test
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
Cys	Cysteine (reduced)
CySS	Cystine (oxidized)
DHLA	Dihydrolipoic acid
EBC	Exhaled breath condensate
E_h	Redox potential/state
ELF	Epithelial lining fluid
GCL	Cysteine ligase
GGT	γ-Glutamyl transpeptidase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSNO	Nitrosoglutathione
GSSG	Glutathione disulfide (oxidized)
GSS	Glutathione synthetase
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HNE	4-hydroxynonenal
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
iNOS	Inducible NO synthase
LMW	Low molecular weight
LOH	Lipid alcohol
LOOH	Lipid peroxide
MDA	Malondialdehyde
MPO	Myeloperoxidase
MV	Mechanical ventilation
NESARC	National Epidemiologic Survey on Alcohol and Related Conditions
$NO\bullet$	Nitric oxide
NO_2	Nitrogen dioxide
NOX	NAD(P)H-oxidase

ABBREVIATIONS (Continued)

$O_2\bullet^-$	Superoxide anion
$ONOO^-$	Peroxynitrite
ROS	Reactive Oxygen Species
SMAST	Short Michigan Alcohol Screening Test
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactant substances
T-O•	Vitamin E (tocopherol) radical
T-OH	Vitamin E (tocopherol), reduced
VAP	Ventilator-associated pneumonia

CHAPTER 1: INTRODUCTION

1.1 CLINICAL SIGNIFICANCE

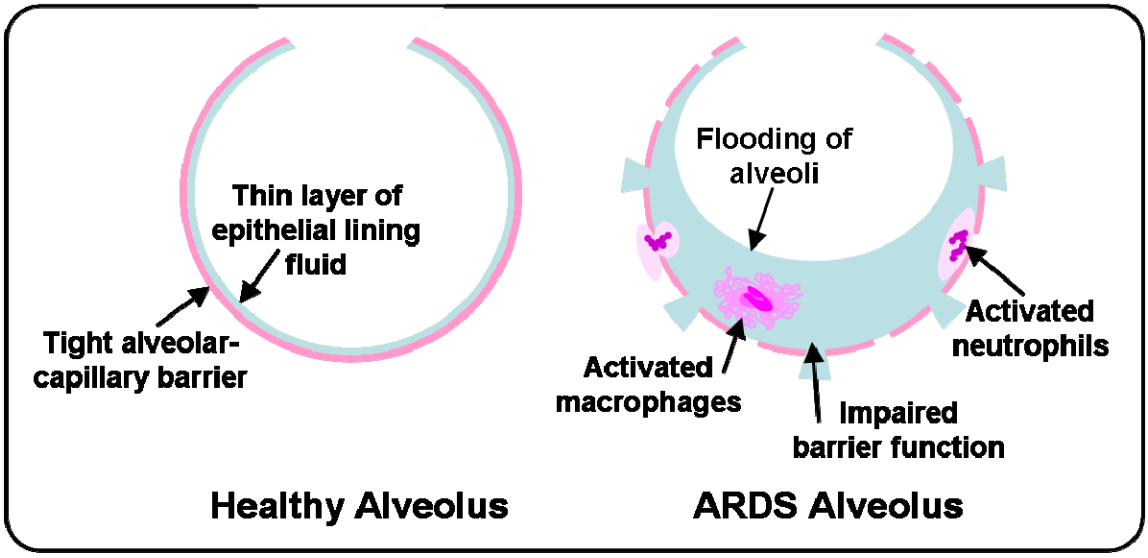
1.1.1 Acute Respiratory Distress Syndrome

The acute respiratory distress syndrome (ARDS) is a form of lung injury characterized by increased permeability of the alveolar-capillary barrier. Alveoli become flooded with neutrophils and other proteinaceous matter (Figure 1-1), as well as the accompanying fluid, resulting in impaired gas exchange, respiratory failure, and in approximately 50% of cases, death. In the United States, more than 100,000 ARDS cases occur each year (1). Even for survivors of ARDS, quality of life is diminished (2).

Several diagnoses are associated with an increased risk of developing ARDS. These at-risk diagnoses include sepsis (serious bacterial infection with fever), trauma, hypertransfusion (resulting from gastrointestinal bleeding), and aspiration of gastric contents (3, 4). Alcohol intoxication is associated with increased incidence of these conditions, while chronic alcohol abuse is associated with poorer prognosis (5-7). In a prospective study of patients with any of these at-risk diagnoses found that patients who also had a history of alcohol abuse were at two times the risk of developing ARDS compared to those with no such alcohol abuse history. For patients with sepsis, a history of alcohol abuse conferred a relative risk of 2.6. Among patients who developed ARDS, nearly two-thirds of patients with a history of alcohol abuse died, compared to just over a third of the patients without an alcohol abuse history (8).

Figure 1-1. Comparison of Healthy and ARDS Alveoli. ARDS is characterized by impaired barrier function, which permits fluid leak into the alveolus and entry of activated neutrophils. As a result of increased fluid, oxygen and carbon dioxide must diffuse across a greater distance and gas-exchange becomes less efficient.

Figure 1-1. Comparison of Healthy and ARDS Alveoli.



1.1.2 VENTILATOR-ASSOCIATED PNEUMONIA

Ventilator-associated pneumonia (VAP) is differentiated from other hospital-acquired pneumonias in that it is a pulmonary infection that occurs after more than 48 hours of mechanical ventilation. Of mechanically ventilated patients, nearly 30% develop VAP (9). In addition to the high incidence of VAP, it is also associated with a high mortality rate, possibly as high as 70% for certain resistant bacteria (10). Although it is difficult to separate the mortality attributable to the VAP itself from that attributable to concomitant conditions, VAP has been associated with a 30% increase in the rate of mortality from underlying illnesses (10).

The primary risk factors for the development of VAP are duration of ventilation and the presence of an underlying illness, especially acute respiratory distress syndrome (ARDS), which often requires mechanical ventilation due to impaired gas exchange. Conditions that impair host defenses, such as sepsis and chronic pulmonary diseases, also predispose patients to develop VAP (11). As for ARDS, patients with a history of alcohol abuse are also at higher risk for VAP and other pulmonary infections (12).

1.1.3 Alcohol Abuse

A significant proportion of the population of the United States abuses alcohol or is alcohol-dependent. According to the 2001-2002 National Epidemiologic Survey on Alcohol and Related Conditions (NESARC), the twelve-month prevalence of alcohol abuse for the adult population was estimated at 4.7% (a population of nearly ten million)

and of alcohol dependence was 3.8% (13). One study estimated the lifetime risk of developing alcohol dependence in the United States at 13% (14).

With respect to chronic alcohol abuse, most public attention is paid to the issues of addiction and liver disease. But alcohol abuse is also associated with an impaired immune system, osteoporosis and bone fractures, as well as various forms of cancer. Although moderate alcohol consumption is linked to decreased risk of several cardiovascular diseases, heavy alcohol use increases the risk of developing cardiomyopathy, hypertension, and arrhythmias (15). A weakened immune system cannot fight off infections, which can lead to sepsis and ARDS.

1.1.4 Smoking

Whereas the association between alcohol abuse and pulmonary disorders may not be common knowledge, the association between cigarette smoking and a variety of pulmonary pathologies is. Cigarette smoking contributes to diseases of the respiratory system, including lung cancer, emphysema, and chronic obstructive pulmonary disease. The health effects of smoking are not limited to the respiratory system, however, and smoking is a factor in cardiovascular disease, pancreatic ulcers, and cancers outside the lung (16). Although the prevalence of smoking in the United States has decreased, it remains high. According to the first edition of The Tobacco Atlas, published by the World Health Organization in 2002, 23.6% of the U.S. population smoked (17). The second edition, published by the American Cancer Society in 2006, the prevalence of smoking was 21.6% (18).

A necessary consideration when conducting clinical or epidemiological research on alcohol abuse or cigarette smoking is the substantial overlap between these two behaviors. Previous studies have found that 80 to 95 percent of alcoholics smoke cigarettes and 70 percent smoke more than one pack a day (19-21).

1.2 OXIDATIVE STRESS

Oxidative stress has been implicated in a large variety of human diseases including cancer, diabetes mellitus, atherosclerosis and other cardiovascular disease, Alzheimer's disease and other neurological disorders, as well as an assortment pulmonary diseases (22-26). A PubMed search for references to "oxidative stress" identifies nearly 10,000 articles, including over 1000 reviews, in 2008 alone. The subject of oxidative stress has also expanded beyond the scientific community. Advertisements for skin creams highlight that their products fight wrinkles by neutralizing free radicals, while cranberry juice producers tout the immune system-strengthening effects of their fruit's antioxidants.

1.2.1 What is Oxidative Stress?

The term "oxidative stress" was coined by Helmut Sies in 1985 (27), when he defined oxidative stress as "a disturbance in the prooxidant-antioxidant balance in favor of the former." Under this definition, pro-oxidants (electron-acceptors) damage proteins, DNA, and lipids when antioxidants (electron-donors) are insufficient to neutralize them. Although this definition is adequate when considering the direct damage caused to cellular components by reactive oxygen species, alterations in redox signaling may occur

when the imbalance is not cytotoxic. In response to the increasing data about redox signaling, a new definition for oxidative stress was proposed by Sies and Jones in 2007 (28) as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage.”

1.2.2 Redox Signaling

Exposure to oxidants can result in altered signaling by several mechanisms. Enzymes with cysteine at their active site may be inactivated by oxidation of the cysteine residue. Some phosphatases, such as protein tyrosine phosphatases, are inactivated in this manner, which results in activation of phosphorylation pathways (29). Certain protein tyrosine kinases, such as protein tyrosine kinases (including the Src family and focal adhesion kinases), are activated by cysteine oxidation, which also results in activation of phosphorylation pathways (30). The formation of disulfide bonds alters the tertiary structure of proteins, which may affect their function and interaction with other proteins. An example of this mechanism is the oxidation-induced formation of disulfide bonds between subunits of the ryanodine receptor (RyR) channel. The formation of these bonds activates the channel, thereby releasing calcium from the sarcoplasmic reticulum in skeletal muscle (31).

1.2.3 Important Pro-Oxidants in the Lung

The lung is constantly exposed to oxidants, some of which are endogenously produced and others of which are inhaled.

1.2.3.1 Molecular Oxygen. The principle purpose of the lung is the exchange of oxygen (O_2) and carbon dioxide (CO_2). Oxygen is essential for aerobic life and required by all cells in the human body, yet it is itself a radical and as such attacks double bonds in other molecules. Its metabolites, including superoxide, the hydroxyl radical, and hydrogen peroxide, are referred to as reactive oxygen species (ROS).

1.2.3.2 Superoxide. The superoxide anion ($O_2\bullet^-$) is formed by the addition of an electron to molecular oxygen. The resulting free radical is highly reactive. Superoxide inactivates enzymes containing iron-sulfur clusters, releasing free iron into cells; initiates lipid peroxidation of membrane lipids; reacts with nitric oxide to form peroxynitrite; and oxidizes thiols with a low pKa. Superoxide does not cross membranes readily, therefore most superoxide remains in the compartment where it was generated. Due to the fact that superoxide production is ubiquitous as well as toxic, most aerobic organisms express superoxide dismutase (SOD), an enzyme that rapidly converts superoxide to hydrogen peroxide (Eqn. 1).

SOD: $$2\ O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

1.2.3.3 Hydrogen Peroxide. Hydrogen peroxide (H_2O_2) is formed primarily through the action of SOD, although peroxisomal oxidases are responsible for producing hydrogen peroxide in peroxisomes. H_2O_2 is a strong oxidant and diffuses easily across membranes, although the diffusion rate is dependent on the concentration gradient across the

membrane. H_2O_2 is reduced enzymatically by glutathione peroxidase (GPx) (Eqn. 2), which requires glutathione as a co-factor, and the peroxisomal enzyme catalase (Eqn. 3).

GPx: $$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG \quad (2)$$

Catalase: $$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (3)$$

1.2.3.4 Peroxynitrite. Peroxynitrite ($ONOO^-$) is formed from the reaction of nitric oxide with superoxide (Eqn. 4). It oxidizes thiols directly, but the products of its decomposition (hydroxyl and NO_2 radicals) will also oxidize thiols. GSH reacts quickly with ONOO-. ONOO- also initiates lipid peroxidation.

$$NO\bullet + O_2\bullet^- \rightarrow ONOO^- \quad (4)$$

1.2.3.5 Hypochlorous Acid. Hypochlorous acid (HOCl) is formed by myeloperoxidase (MPO), an enzyme that catalyzes the reaction of H_2O_2 with the chloride ion (Eqn. 5). MPO is present in activated neutrophils and macrophages. HOCl is a strong oxidant that reacts preferentially with thiols, often irreversibly.

MPO: $$H_2O_2 + 2Cl^- \rightarrow 2HOCl \quad (5)$$

1.2.3.6 Lipid Peroxides. Lipid peroxidation results in the degradation of lipids via a free radical chain reaction. The reaction may be initiated by H_2O_2, the hydroxyl radical, or peroxynitrite, and is propagated by molecular oxygen. Two products of lipid peroxidation

are malondialdehyde (MDA) and 4-hydroxynonenal (HNE), both of which form DNA-adducts.

1.2.4 Antioxidants

Antioxidants present in the lung can be categorized as enzymatic or non-enzymatic.

1.2.4.1 Antioxidant Enzymes. The antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. SOD acts on the superoxide radical to form hydrogen peroxide, which is converted to water and O_2 by either GPx or catalase. GPx requires glutathione (GSH) as an electron donor, which is oxidized to its disulfide (GSSG). Catalase is present primarily in the hydrogen peroxide-rich peroxisomes, but there is evidence of the presence of catalase in epithelial lining fluid also (32, 33).

1.2.4.2 Non-Enzymatic Antioxidants. The non-enzymatic antioxidants in the lung include ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), and uric acid.

1.2.4.3 Glutathione. In addition to the non-enzymatic antioxidants described above, low molecular weight (LMW) thiols, such as glutathione and cysteine, are also non-enzymatic antioxidants with important roles in the lung.

Glutathione is the primary LMW thiol and the most abundant antioxidant in the epithelial lining fluid (ELF) of healthy lungs. The glutathione concentration in the ELF of healthy adults is about 500 μM, nearly as concentrated as it is inside cells (34). When oxidized,

glutathione (GSH) forms a disulfide, referred to as glutathione disulfide or GSSG. Normally, the glutathione present in the ELF is very reduced, with only 3-4 % present as GSSG (34).

Glutathione is a tripeptide composed of the amino acids glutamate, cysteine, and glycine. The thiol group on the cysteine residue is the site of GSH oxidation. GSH is synthesized in two ATP-dependent steps by the actions of the enzymes glutamate cysteine ligase (GCL) and glutathione synthetase (GSS) as shown in Eqs. 6-7. Although GSH can be synthesized in all human cells, most synthesis occurs in the liver.

$$\text{GCL: L-glutamate + L-cysteine + ATP} \rightarrow \text{L-}\gamma\text{-glutamylcysteine + ADP + } P_i \quad (6)$$

$$\text{GSS: L-}\gamma\text{-glutamylcysteine + Glycine + ATP} \rightarrow \text{Glutathione + ADP + } P_i \quad (7)$$

GSH performs as an antioxidant through both direct reaction with its target and as a co-factor for glutathione peroxidase (GPx). **Figure 1-2** demonstrates GSH's role in direct regeneration of Vitamin C [1] and Vitamin E [2], as well as the reduction of protein disulfides [3]. Also illustrated is the regeneration of GSH from GSSG by glutathione reductase (GR) [4] and dihydrolipoic acid (DHLA) [5].

GSH is also a cofactor for GPx. In this role, GSH is also able to reduce hydrogen peroxide, lipid peroxides, and peroxynitrite (**Figure 1-3**: Reactions [1], [2], and [3], respectively). S-glutathionylation, whereby GSH-conjugates are formed through the action of glutathione S-transferase (GST), is another protective mechanism. 4-

Figure 1-2: Non-Enzymatic Functions of GSH and Regeneration of GSH from GSSG. Regeneration of Vitamin C [1]: dehydroascorbic acid ($Asc^{\bullet -}$) is reduced to ascorbate ($AscH^-$). Regeneration of Vitamin E [2]: tocopherol radical (T-O•) is reduced to tocopherol (T-OH). Reduction of disulfide links in proteins [3]: protein disulfide($Pr\text{-}(S)_2$) is reduced to protein (Pr-SH). Reduction of GSSG via glutathione reductase (GR) [4]: GSH is regenerated from GSSG by the oxidation of NADPH to $NADP^+$. GSSG may also be reduced by lipoic acid [5]: GSH is regenerated from GSSG by the oxidation of dihydrolipoic acid (DHLA) to α-lipoic acid (ALA).

Figure 1-2: Non-Enzymatic Functions of GSH and Regeneration of GSH from GSSG.

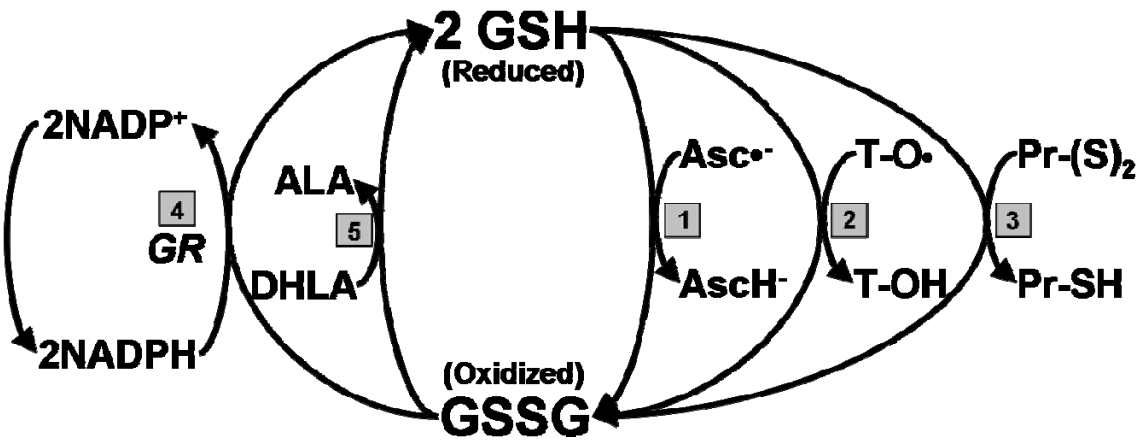
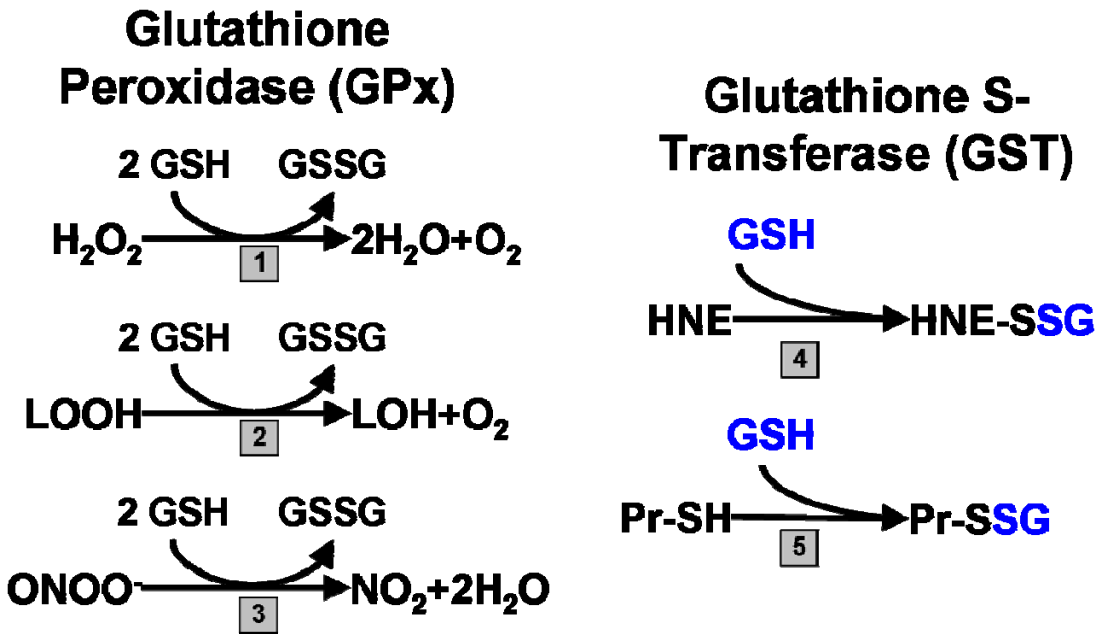


Figure 1-3. Glutathione Peroxidase and Glutathione S-Transferase Enzymes. GSH is a co-factor in glutathione peroxidase (GPx) reactions to reduce peroxides such as hydrogen peroxide [1], lipid peroxides [2], and peroxinitrite [3]. Glutathione S-transferase (GST) forms mixed disulfides between GSH and thiol residues on proteins. Hydroxynonenal (HNE) [4] is a product of lipid peroxidation, but other thiol-containing proteins may also be targeted [5].

Figure 1-3. Glutathione Peroxidase and Glutathione S-Transferase Enzymes



hydroxynonenal (HNE) is a cytotoxic product of lipid peroxidation, but its GSH-conjugate (**Figure 1-3**: Reaction [4]) is less reactive and can be exported from cells. Although glutathionylated proteins are more oxidized than their reduced counterparts, the reaction is reversible and may protect vulnerable thiols from irreversible oxidation (**Figure 1-3**: Reaction [5]).

In the bronchoalveolar lavage fluid of ARDS patients, one study found that GSSG was increased, without a difference in GSH (35). GSSG was measured using an enzymatic assay that is not as sensitive as the HPLC protocol used in the studies included in the following chapters. Another study found that total GSH was significantly decreased in ARDS patients, but the researchers were unable to accurately measure GSSG and were therefore unable to assess the redox state (36). GSH concentrations in the epithelial lining fluids of cystic fibrosis patients, as well as patients with idiopathic pulmonary fibrosis, have been found to be lower than for healthy subjects (37).

Various clinical and animal studies have demonstrated decreased GSH concentrations in the liver. In a study of patients with mild to moderate alcoholic hepatitis, researchers found very low plasma GSH concentrations, as well as low levels of hepatic GSH, methionine, and cysteine in these patients (38). Expression of hepatic GCL (catalytic subunit) and GSS, the enzymes responsible for GSH synthesis, was also diminished in these patients. Animal studies into the effect of chronic alcohol feeding have found decreased liver GSH, even in cases where GSH-synthetic enzymes were upregulated,

evidence that increased GSH consumption and efflux, but not decreased synthesis, are responsible for lower hepatic GSH levels (39, 40).

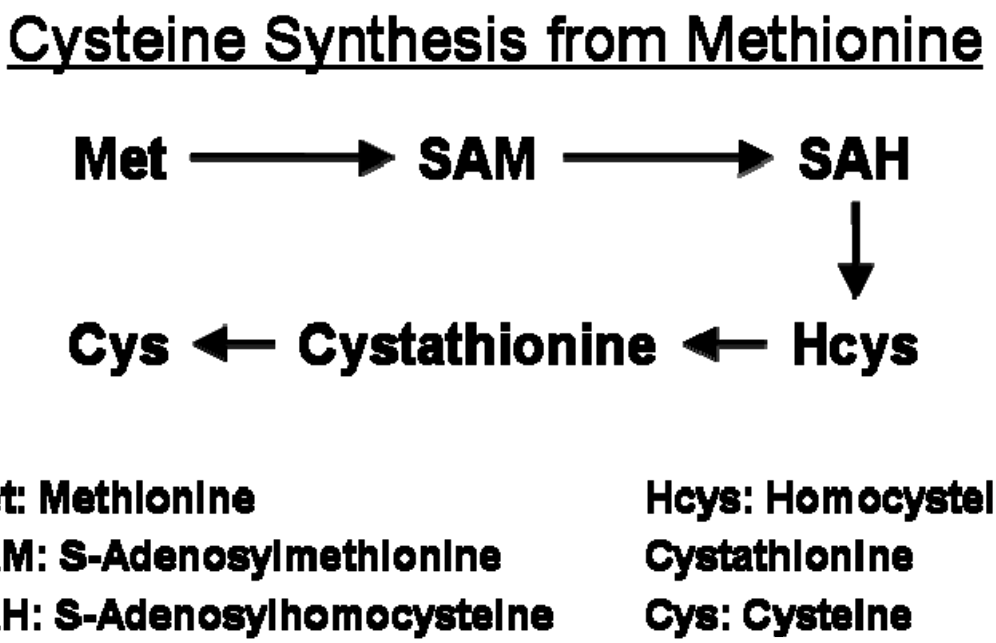
The discovery that alcohol abuse independently increases the risk for developing ARDS and evidence of depletion of pulmonary GSH in ARDS patients led to the investigation of pulmonary GSH with chronic alcohol abuse. Reduced GSH in the epithelial lining fluid of subjects with a history of alcohol abuse was greatly decreased to less than a fourth of that in subjects with no history of alcohol abuse (41). This depletion of the reduced GSH was not accompanied by a decrease in GSSG. As a result, the redox state of the GSH/GSSG redox couple was 40 mV more oxidized in the alcohol abusers.

Investigations into levels of pulmonary GSH in smokers have provided different results, with some studies showing an increase (34, 41) and others a significant decrease (42) in epithelial lining fluid GSH. These differences may be due partly to the different populations studied in terms of age and smoking history. The concentration of reduced GSH in the plasma of smokers was found to be significantly lower than in non-smokers, with an 11 mV oxidation of the GSH/GSSG redox state (43).

1.2.4.4 Cysteine. Cysteine (Cys) is a nonessential amino acid. Cells are capable of cysteine synthesis (**Figure 1-4**), but under conditions of methionine deficiency endogenously formed cysteine may be inadequate to perform all its functions. Cysteine is important for protein synthesis. In fact, very few proteins have no Cys residues. Disulfide

Figure 1-4. Cysteine Biosynthesis. Cysteine is not considered an essential amino acid because it can be synthesized from methionine.

Figure 1-4. Cysteine Biosynthesis.



bonds within proteins play an important role in protein folding and stability. Cysteine metabolism may occur through various routes with products including GSH and taurine, a bile acid precursor. Cys is also the principle low molecular weight extracellular thiol and its redox state is the primary determinant of extracellular redox.

The disulfide (oxidized) form of cysteine is cystine (CySS), which is reduced back to cysteine by cystine reductase (Eqn. 8). L-Cystine is not highly soluble in aqueous solution, which in some situations may cause the formation of cystine kidney stones.

Cystine Reductase: $CySS + NADH + H^+ \rightarrow 2\ Cys + NAD^+$ (8)

Recently, the extracellular cysteine redox state has been shown to affect the function in cells in culture. Ramirez et al found that lung fibroblast proliferation and matrix expression was altered by changing the Cys/CySS redox potential of the media (44). Extracellular Cys/CySS redox state has also been shown to affect oxidant-induced apoptosis in human retinal pigment epithelial cells (45) and proliferation of Caco-2 cells (46), independent of GSH status.

Hepatic levels of cysteine have been shown to be decreased in patients with mild to moderate alcoholic hepatitis, as has expression of methionine adenosyltransferase (MAT) and cystathionine β-synthase (CBS), both involved in the synthesis of cysteine from methionine (38).

Jones et. al. measured Cys concentrations and calculated the Cys/CySS redox state in the plasma of smokers and found that plasma concentrations of both Cys and CySS were lower in the plasma of smokers compared to non-smokers (43). The Cys/CySS redox state was also more oxidized (12 mV) in smokers.

It is interesting to note that the reaction of Cys with acetaldehyde, the primary metabolite of ethanol and a component of cigarette smoke, forms a stable, non-toxic product (2-methylthiazolidine-4-carboxylic acid). This has led to the development of chewing gums and lozenges containing L-cysteine to bind acetaldehyde in the saliva (47, 48) and to further research into the protective effects of L-cysteine supplementation against alcohol-derived acetaldehyde (49, 50).

1.2.5 Sources of Oxidants in the Lung

1.2.5.1 Cellular Respiration. Normal cell function results in the production of reactive oxygen species. The mitochondrial electron transfer chain couples the transfer of electrons from NADH or succinate to O_2 with the transfer of H^+ ions across the inner mitochondrial membrane via a series of redox reactions. The electrochemical potential gradient developed by the transmembrane transfer of H^+ drives ATP synthase, the enzyme responsible for synthesis of adenosine triphosphate (ATP), the primary energy source in living organisms. Although the electron transport chain is highly efficient, there is some leak of electrons to O_2, which results in the production of superoxide radicals ($O\cdot_2^-$). The enzyme SOD converts superoxide to hydrogen peroxide (H_2O_2) as shown in

Eqn. 1. Superoxide has low membrane permeability so that superoxide arising from the mitochondrial electron transfer chain remains within the mitochondria.

1.2.5.2 The Respiratory Burst. Phagocytic cells, such as neutrophils and macrophages, play a role in the innate immune response. The appropriate pro-inflammatory stimulus triggers the respiratory burst in these cells. The respiratory burst is a process by which NAD(P)H-oxidases (NOX) in the plasma membrane generate superoxide. Superoxide and secondary ROS such as H_2O_2 act as bactericides (51). In the presence of certain transition metals, H_2O_2 can undergo the Fenton reaction and be converted to the hydroxyl radical, which can initiate the process of lipid peroxidation. Two products of lipid peroxidation, malondialdehyde and hydroxynonenal, can form DNA-adducts. In activated neutrophils and macrophages, the enzyme myeloperoxidase (MPO) produces the cytotoxic hypochlorous acid (HOCl) from H_2O_2 and the chloride anion. HOCl reacts with DNA, fatty acids, cholesterol, and proteins. Activated neutrophils also produce nitric oxide (NO) from the oxidation of L-arginine by inducible NO synthase (iNOS). Nitric oxide may then react with superoxide to form peroxynitrite, a strong oxidizing agent.

1.2.5.3 Cigarette Smoke. One puff of cigarette smoke contains more than 10^{14} carbon and oxygen centered radicals (52). These radicals react readily with the nitric oxide also present at high concentrations to form alkyl peroxynitrites and peroxynitrate esters. The tar component in cigarette smoke also contains a semiquinone radical that is membrane permeable and that can react with oxygen to form superoxide (53).

1.2.5.4 Chronic Alcohol Abuse.

Many of the detrimental health effects of alcohol abuse are believed to occur through ethanol's primary metabolite acetaldehyde, but the reactions that metabolize ethanol to acetaldehyde are themselves a source of oxidants. Ethanol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH, Eqn. 9) or the inducible enzyme Cyp2E1. Both reactions are somewhat leaky and may produce ROS such as superoxide. The metabolism of acetaldehyde to acetate may also produce ROS. One of the acetaldehyde metabolizing enzymes is aldehyde dehydrogenase (ALDH). The reactions catalyzed by ADH and ALDH generate NADH, whose reoxidation in mitochondria is an additional potential source of ROS (54). Acetaldehyde also induces lipid peroxidation (55).

ADH: $\text{Ethanol} + \text{NAD}^+ \rightarrow \text{Acetaldehyde} + \text{NADH} + \text{H}^+$ (9)

Acetaldehyde binds to proteins and depletes the antioxidant pool by binding to GSH and Cys (56). In a rat model, acetaldehyde was shown to alter the activity of enzymes involved in GSH synthesis and GSH redox, increasing glutathione S-transferase and glutathione peroxidase activity, while decreasing glutathione reductase and γ-glutamylcysteine synthetase (the rate-limiting enzyme in GSH synthesis) activity (57).

In addition to these effects of ethanol and acetaldehyde, alcohol abuse is usually accompanied by malnutrition, partly due to substitution of nutrients by alcohol, but also to altered nutrient absorption. Alcoholics may be deficient in selenium (a co-factor for GPx), ascorbate (Vitamin C) or Vitamin E (58, 59).

1.2.5.5 Mechanical Ventilation.

Mechanical ventilation (MV) is commonly used to treat patients with acute lung injury, including acute respiratory distress syndrome (ARDS). Although MV may improve oxygenation of the blood, it is itself associated with increased ROS. Various aspects of MV may contribute to this increase in pro-oxidants. First, the patient is exposed to concentrations of molecular oxygen than are present normally. Next, the stretching of the alveoli may result in cell damage, decreased barrier integrity, and the influx of ROS-generating inflammatory cells. Finally, an in vitro study investigating the association between cyclic stretch and the generation of ROS found that cyclic strain of endothelial cells increased the activity of an endothelial NAD(P)H-oxidase (NOX), which generates superoxide (60).

1.3 THE PULMONARY MILIEU

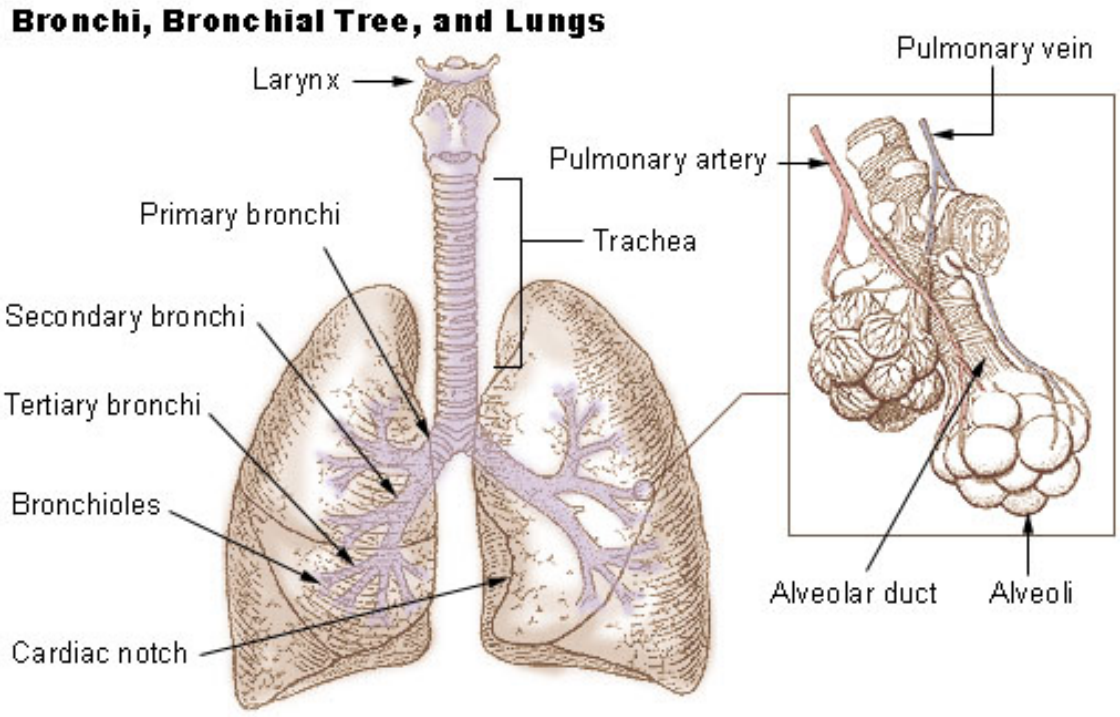
The lower respiratory system is composed of the bronchi, bronchioles, alveolar ducts, and alveoli **(Figure 1-5)** and can be divided into the conducting zone and the gas exchange zone.

1.3.1 The "Conducting Zone"

The bronchi and bronchioles act as passageways for air into and out of the lungs and may be referred to as the "conducting zone". The anatomy of the conducting passageways

Figure 1-5. Anatomy of the Respiratory System. (Source: http://training.seer.cancer.gov. National Cancer Institute)

Figure 1-5. Anatomy of the Respiratory System.



gradually changes as the branching progresses from the proximal bronchi to bronchioles. The walls of the bronchi contain a large amount of hyaline cartilage and are lined with a mucous membrane composed of ciliated pseudostratified columnar epithelium. As the passageway divides, it loses cartilage and gains smooth muscle, while the epithelium shifts to simple cuboidal epithelium and then to simple squamous epithelium.

1.3.2 Gas Exchange Zone

Further branching results in alveolar ducts with alveoli at their termini. The alveolar ducts and alveoli are sites for gas exchange, where oxygen is transported to the circulation and carbon dioxide is released from the bloodstream. The squamous epithelium of the alveolar ducts and alveoli provide a short path for gas exchange. In order for oxygen to reach the red blood cells in the bloodstream, it must cross through the surfactant-containing epithelium lining fluid, the epithelial lining itself, a matrix-containing interstitial space, the endothelial capillary wall, before it reaches its destination. The epithelium lining the alveolus is composed of alveolar type I cells, which cover most of the alveolar surface area, and alveolar type II cells, which produce surfactant.

1.3.3 Pulmonary Defenses

As a result of performing the invaluable function of respiration, the entire respiratory system is exposed to the environment, including toxins and microbes, and has developed extensive protective mechanisms. The cilia lining the bronchi move mucus containing inhaled particles and microbes towards the larynx in a process called mucociliary clearance. Macrophages present in the alveolar space protect against threats that are not

cleared by the cilia. The epithelial lining itself forms a tight barrier between the environment and the circulation. Another important source of protection is the epithelial lining fluid that contains antioxidants and various compounds such as interferon, in addition to surfactant. When any of these protective mechanisms are impaired, the results may include cellular damage, less efficient gas exchange, or infection.

1.4 SAMPLING METHODOLOGIES FOR ASSESSING OXIDATIVE STRESS

Oxidative stress can be assessed in a variety of fluid samples. Bronchoalveolar lavage is used to sample the epithelial lining fluid of the lungs. Plasma provides an indication of systemic oxidative stress. In recent years, a non-invasive method to sample the pulmonary airspace referred to as exhaled breath condensate has been used. Each method has advantages and disadvantages that are important to consider when assessing oxidative stress.

1.4.1 Bronchoalveolar Lavage

The epithelial lining fluid of the lungs is most commonly sampled by performing a bronchoalveolar lavage (BAL). In this procedure, a bronchoscope is inserted into the lungs, saline is instilled, and the fluid is recovered for analysis. The bronchoalveolar lavage fluid (BALF) contains cells, pathogens, as well as epithelial lining fluid. Clinically, the cells may be separated from the fluid in order to perform a differential cell count. If a bacterial infection is suspected, a sample of the lavage fluid may be cultured to confirm an infection and identify the pathogen. The acellular portion can be analyzed for the

presence of a multitude of compounds such as proteins, fatty acids, markers of oxidative stress, and low molecular weight antioxidants.

The BAL procedure has several limitations. First of all, it is invasive. The subject must be sedated and is at risk for complications such as bronchospasm, distal airway collapse, transient infiltrates, and a transient decrease in lung function or PaO2 (Thoracic.org). In patients with already compromised respiratory status, the transient decrease in lung function may demand the use of mechanical ventilation. These risks limit the utility of the procedure in certain patients.

Another limitation of the BAL as a method to sample the epithelial lining fluid (ELF) is the dilution of the ELF with the instillate. Various methods have been proposed to correct for this dilution, all of which have their own limitations. One method uses urea concentrations based on the premise that urea should be in equilibrium across the alveolar-capillary barrier (61). If this is the case, $[urea]_{plasma} = [urea]_{epithelial\ lining\ fluid}$ and the urea dilution factor may be calculated as $[urea]_{plasma}/[urea]_{lavage}$. There are a couple of possible problems with this method. First, the instillate dilutes the epithelial lining fluid, which causes increased influx of urea in an attempt to return to equilibrium. The longer the lavage procedure takes, the greater the amount of extra urea in the lavage fluid (62). This would result in an underestimate of the dilution of the epithelial lining fluid. Disruption of the epithelial cells during the lavage procedure could also cause a rise in urea levels. Another approach is to normalize to the amount of total protein or a specific large protein (such as albumin) that should not readily cross the alveolar-capillary barrier.

As for the urea method, these lavage protein concentrations could be altered by disruption of the barrier during the lavage procedure or by increased permeability due to a condition such as ARDS (63).

Another consideration for the BAL procedure for research purposes is the difficulty in determining the contribution of the alveolar vs. bronchial portions of the lung to the sample. There have been some significant differences in antioxidant status between that determined in BALF and in samples collected from induced sputum or nasal lavage (64, 65). Because of these findings and the heterogeneity of the lung with respect to cell types, structure, and exposure to oxidants, it is certainly plausible that oxidative status could vary along the airways. One technique that has been proposed to address this issue is the fractional processing of sequential bronchoalveolar lavage in which the first aliquot of recovered lavage fluid is processed separately from the remaining aliquots, which are pooled. In a study performed by Rennard et al, analysis of the first aliquot revealed a higher content of ciliated epithelial cells, such as those that predominate in the bronchi, indicating a sampling of the bronchial airways (66).

1.4.2 Plasma

Plasma samples are often used to assess systemic oxidative stress. Venous blood samples are drawn and cells. Cells are separated from plasma by simply centrifuging the blood. The blood cells collect at the bottom of the tube, leaving the plasma to be pipetted to a fresh container.

Although this procedure is not nearly as invasive as the BAL and much easier to perform, it has its own limitations. First of all, even a low rate of hemolysis could increase GSH estimates since the concentration of GSH is many times higher in erythrocytes than in plasma. Hemolysis can be minimized by avoiding the use of evacuated tubes (such as Vacutainer tubes) for blood draws. GSH in plasma is also easily oxidized or degraded so that proper processing of samples to minimize oxidation and inhibit degradation via γ-glutamyl transpeptidase is necessary (67).

More importantly for pulmonary research, the plasma does not reflect the antioxidant status of the lung. In a study evaluating the impact of a history of alcohol abuse on GSH redox in the lung, plasma redox state was unaltered in contrast to the 40 mV oxidation found of the BALF (41).

1.4.3 Exhaled Breath Condensate

Another sampling procedure for oxidative stress analysis is exhaled breath condensate (EBC). This method works on the principal that moisture in the breath will condense on a cold surface, along with any proteins or other substances in the microdroplets. The protocol is non-invasive and requires only that the subject breathe into a specially-designed tube for several minutes. Many compounds have been measured in the resulting condensate, including molecules as small as nitric oxide and as large as surfactant proteins.

As with the other sampling methods, EBC also has limitations. Most compounds in the EBC are present in very low concentrations, sometimes below the detection limit of assays used with other fluids. This problem may demand the concentration of samples prior to making any measurements. Another concern with respect to EBC samples is the possible contribution of compounds in the oral or nasal cavities. The contribution from nasal cavities can be reduced by performing oral inhalation and exhalation rather than nasal inhalation with oral exhalation (68). Contribution from saliva is minimized by the incorporation of a saliva trap in the EBC collection apparatus. Salivary amylase activity in the EBC sample can also provide an estimate of salivary contribution.

1.5 THE STUDIES

1.5.1 Altered Pulmonary and Systemic Thiols in Critically Ill Patients (Chapter 3)

1.5.1.1 Background. Oxidative stress has been implicated in many pulmonary diseases. Previous studies have found an oxidation of the endogenous antioxidant glutathione **(GSH)** in the lungs of patients with acute respiratory distress syndrome (ARDS), but the status of cysteine **(Cys)**, another important thiol antioxidant present in the lung, has been largely ignored.

1.5.1.2 Methods. Bronchoalveolar lavage fluid **(BALF)** and plasma were collected from healthy controls **(controls)** and from critically ill, mechanically ventilated patients **(patients)**. Reduced and oxidized moieties of GSH and Cys were measured in BALF and

plasma using HPLC. Other markers of oxidative stress were also measured in the BALF: 4-hydroxynonenal **(HNE)**, hydrogen peroxide **(H_2O_2)**, and malondialdehyde **(MDA)**. Plasma MDA was also measured.

1.5.1.3 Results. In the BALF of patients, H_2O_2 and HNE were elevated; the GSH pool was depleted and oxidized; and Cys and cystine (CySS) concentrations were higher than for controls. In the plasma of patients, MDA concentrations were significantly elevated; GSSG, total GSH (tGSH), and %GSSG were increased; concentrations of Cys and tCys were increased; but cysteine was more reduced (lower %CySS).

1.5.1.4 Conclusion. The BALF of critically ill patients was more oxidized than controls as evidenced by higher H_2O_2, HNE, and %GSSG. The Cys pool was larger in this group but the %CySS was not significantly higher than controls. Higher plasma MDA concentrations and %GSSG in patients indicated a more oxidized state, but surprisingly plasma Cys was significantly more reduced in this group.

1.5.2 Thiols in the Bronchoalveolar Lavage Fluid of Ventilator-Associated Pneumonia Patients with and without Alcohol Abuse (Chapter 4)

1.5.2.1 Background. Ventilator-associated pneumonia **(VAP)** is a common infection among mechanically-ventilated patients. A history of alcohol abuse is a risk factor for the development of VAP and increased mortality. Oxidative stress may provide a mechanism for the increased risk seen with alcohol abuse, specifically through the alteration of

glutathione **(GSH)** and cysteine **(Cys)**, low molecular weight thiols and important pulmonary antioxidants.

1.5.2.2 Methods. Bronchoalveolar lavage fluid **(BALF)** was collected from healthy controls and VAP patients, with and without a history of alcohol abuse. Reduced and oxidized moieties of glutathione **(GSH** and **GSSG)** and cysteine **(Cys** and **CySS)** were measured using HPLC. Other markers of oxidative stress were also measured: 4-hydroxynonenal **(HNE)**, hydrogen peroxide **(H_2O_2)**, and malondialdehyde **(MDA)**.

1.5.2.3 Results. H_2O_2 and HNE concentrations were higher in the BALF of VAP patients compared to the healthy subjects. GSH was depleted to a comparable extent in healthy alcoholics and VAP patients compared to healthy controls. The GSH pools were also more oxidized (%GSSG) in these groups. Cys and CySS concentrations were much higher in VAP controls and VAP alcoholics compared to either healthy group. The total Cys pool **(tCys)** was larger than the GSH pool **(tGSH)** in VAP patients compared to healthy subjects. Among VAP patients, a lower tCys/tGSH was associated with survival. **1.5.2.4 Conclusions.** These results support the idea that chronic alcohol abuse depletes the antioxidant potential of the lung, leaving it vulnerable to attack by oxidants resulting from the initiation and resolution of infection. The tCys/tGSH ratio may provide a prognostic indicator in VAP.

1.5.3 Divergent Effects of Alcohol and Smoking on Thiols in Exhaled Breath Condensate (Chapter 5)

1.5.3.1 Background. Although smoking and chronic alcohol abuse are both oxidative conditions, each is associated with increased risk for different pulmonary disorders. We demonstrated in previous studies of otherwise healthy alcoholics that the exhaled breath condensate **(EBC)**, a noninvasive sampling method, could be used to monitor pulmonary oxidative stress of the glutathione redox pair. In the current study, the EBC of subjects with a history of alcohol abuse and/or smokers was used to monitor the redox balance of the other key thiol pair, cysteine **(Cys)** and cystine **(CySS)**.

1.5.3.2 Methods. EBC was collected from healthy subjects with and without a history of alcohol abuse. GSH, GSSG, Cys and CySS were measured by HPLC.

1.5.3.3 Results. GSSG and **%GSSG** (100%*GSSG/GSH+GSSG) were higher in the EBC of alcoholics, but not different based on smoking status. A history of alcohol abuse was associated with elevated Cys and CySS concentrations in the EBC. A positive smoking history was associated with a 50% decrease in the Cys concentration. The **%CySS** (100%*CySS/Cys+CySS) was increased by smoking in the control groups and by alcohol in the nonsmoking groups. The ratio of total Cys to total GSH **(tCys/tGSH)** was approximately 1 in nonsmoking controls. This ratio was significantly higher for both alcohol groups and lower for smoking controls.

1.5.3.4 Conclusions. Although both smoking and alcohol abuse were associated with a more oxidized Cys pool (%CySS), the concentrations of tCys were much higher in alcohol abusers and lower in smokers.

CHAPTER 2: METHODS

2.1 SUBJECT RECRUITMENT

Subjects were recruited from three populations in Atlanta, Georgia. Healthy controls (no history of alcohol abuse) were recruited from the community around Atlanta. Healthy alcohol abusers were recruited from the drug and alcohol dependency unit at Grady Memorial Hospital or the alcohol detoxification unit at the VA Medical Center. Critically ill patients were recruited from the ICU at Grady Memorial.

2.1.1 Healthy Subjects

Exclusion criteria from either healthy group included a medical history of lung diseases determined either by radiography or spirometry; liver dysfunction including cirrhosis or hepatitis, aspartate aminotransferase greater than 100 U/L, total bilirubin exceeding 2.2 mg/dL, a serum albumin level less than 3.5 g/dL or any abnormal hepatic imaging; kidney disease or abnormal serum creatinine levels; gastrointestinal disorders or bleeding; cardiac diseases; diabetes mellitus; positive for HIV infection; pregnancy; any concurrent illicit drug use or recent acetaminophen use. Subjects with concomitant illicit drug use by prior history or urine drug screen were also excluded.

2.1.2 Alcohol Abuse Status

The Short Michigan Alcohol Screening Test (SMAST) and Alcohol Use Disorder Identification Test (AUDIT) were used to determine subjects' alcohol abuse status. (Refer to section 2.2 for descriptions of these screening tools.) Subjects who had actively

consumed alcohol within 7 days of recruitment and with a SMAST score greater than 3 and an AUDIT score greater than 8 were classified as having a history of alcohol abuse. Classification as having a negative history of alcohol abuse required a SMAST score of zero or AUDIT score less than 8.

2.2 SCREENING TESTS FOR ALCOHOL ABUSE

For the purposes of categorizing the subjects in the following studies as alcohol abusers, one of two screening questionnaires was used: the Short Michigan Alcohol Screening Test (SMAST) and the Alcohol Use Disorders Identification Test (AUDIT). For our studies, subjects with a score greater than 3 on the SMAST or greater than 8 on the AUDIT were classified as having a positive history of alcohol abuse.

2.2.1 Short Michigan Alcohol Screening Test

The SMAST is a series of 13 questions that are answered by a simple "Yes" or "No" that has been in use for over 30 years to screen for alcoholism (**Table 2-1**). The SMAST is easy to administer and can be completed in less than ten minutes. A result of more than three positive responses identifies alcohol dependence with high sensitivity (more than 0.7) and high specificity (more than 0.8) (69). Due to the nature of the questions, the questionnaire is useful for the identification of subjects with a history of chronic alcohol abuse. The SMAST does not provide information about quantities or patterns of alcohol consumption.

Table 2-1. The Short Michigan Alcohol Screening Test. Thirteen "Yes" or "No" questions used to identify alcohol dependent subjects. More than three positive responses indicate alcohol dependence. The SMAST does not provide information about quantities or patterns of alcohol consumption.

Short Michigan Alcohol Screening Test	
1	Do you feel that you are a normal drinker? (by normal we mean do you drink less than or as much as most other people.)
2	Does your wife, husband, a parent, or other near relative ever worry or complain about your drinking?
3	Do you ever feel guilty about your drinking?
4	Do friends or relatives think you are a normal drinker?
5	Are you able to stop drinking when you want to?
6	Have you ever attended a meeting of Alcoholics Anonymous (AA)?
7	Has your drinking ever created problems between you and your wife, husband, a parent or other near relative?
8	Have you ever gotten into trouble at work because of your drinking?
9	Have you ever neglected your obligations, your family, or your work for two or more days in a row because you were drinking?
10	Have you ever gone to anyone for help about your drinking?
11	Have you ever been in a hospital because of drinking?
12	Have you ever been arrested for drunken driving, driving while intoxicated, or driving under the influence of alcoholic beverages?
13	Have you ever been arrested, even for a few hours, because of other drunken behaviors?

2.2.2 Alcohol Use Disorders Identification Test

The AUDIT is a series of ten questions, including three that address quantity and frequency of alcohol consumption (70). In contrast to the SMAST, for which answers are limited to "Yes" or "No", the AUDIT provides several possible answers for each question. Each item is scored from 0 to 4 points, depending on the answer chosen. A total score of 0 to 40 points is possible, with a score greater than 8 suggesting harmful alcohol consumption patterns. Whereas the SMAST focuses on long-term alcohol abuse, the AUDIT gives more weight to the consequences of alcohol abuse over the previous year.

2.3 SAMPLE COLLECTION

For the studies in Chapters 3 and 4, bronchoalveolar lavage (BAL) samples were obtained to assess the thiol status of the pulmonary epithelial lining fluid. Plasma samples were collected to assess systemic thiol status. For the study in Chapter 5, exhaled breath condensate samples were used.

2.3.1 Bronchoalveolar Lavage

Bronchoscopy was performed according to standard procedures. Subjects fasted overnight and all samples were collected within the same three hours of the day. Subjects who were smokers were asked to abstain from smoking for the six hours prior to their visit. After sedation with short-acting benzodiazepine, a flexible fiberoptic bronchoscope (model BF-1T20D; Olympus America, Inc., Melville, NY) was inserted transnasally into a subsegmental bronchus of the right middle lobe. 50 mL of sterile saline were instilled and immediately aspirated into suction traps under continuous low-pressure suction. The

Table 2-2. The Alcohol Use Disorders Identification Test. Each item is scored from 0 to 4 points, depending on the answer chosen. A total score of 0 to 40 points is possible, with a score greater than 8 suggesting harmful alcohol consumption patterns. Whereas the SMAST focuses on long-term alcohol abuse, the AUDIT gives more weight to the consequences of alcohol abuse over the previous year.

The Alcohol Use Disorders Identification Test						
		0	1	2	3	4
1	How often do you have a drink containing alcohol?	Never	Monthly or less	2-4 times a month	2-3 times a week	4 or more times a week
2	How many drinks containing alcohol do you have on a typical day when you are drinking?	1 or 2	3 or 4	5 or 6	7 to 9	10 or more
3	How often do you have six or more drinks on one occasion?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
4	How often during the last year have you found that you were not able to stop drinking once you had started?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
5	How often during the last year have you failed to do what was normally expected from you because of drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
6	How often during the last year have you been unable to remember what happened the night before because you had been drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
7	How often during the last year have you needed an alcoholic drink first thing in the morning to get yourself going after a night of heavy drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
8	How often during the last year have you had a feeling of guilt or remorse after drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
9	Have you or someone else been injured as a result of your drinking?	No		Yes, but not in the last year		Yes, during the last year
10	Has a relative, friend, doctor, or another health professional expressed concern about your drinking or suggested you cut down?	No		Yes, but not in the last year		Yes, during the last year

instillation/aspiration procedure was repeated for two additional 50 mL aliquots for a total of 150 mL. The collected fluid was filtered through coarse gauze and centrifuged at 750 x g for 10 minutes in order to remove the cellular components.

2.3.2 Plasma

A 10 mL blood specimen was collected from a peripheral vein within 10 minutes of the bronchoscopy. A portion of the plasma was preserved for HPLC analysis (using the same preservation solution as for BALF) and stored at -70°C.

2.3.3 Exhaled Breath Condensate

EBC was collected with an R-tube (Respiratory Research, Charlottesville, VA) that included a sterile polypropylene collection tube, a mouthpiece, and a saliva trap. A chilled (-70 °C) outer aluminum sleeve placed over the collection tube kept it cooled during the collection period allowing breath to condense on the tube wall. Subjects were asked to breathe tidally for 10 minutes. Subjects did not wear a noseclip during collection and were instructed to swallow any fluid that built up in the mouth to minimize the introduction of saliva into the collection tube. The one-way valve trap on the tube also served as a barrier against salivary contamination.

2.4 MEASUREMENT OF THIOLS

Thiols were measured using high performance liquid chromatography (HPLC).

2.4.1 Sample Preservation

After each sample was collected, a portion was transferred to a solution that maintained the redox state of the thiols (GSH preservation solution).

2.4.1.1 Bronchoalveolar Lavage Fluid and Plasma. 500 µl of the BALF or plasma was added directly to 500 µl of a GSH preservation solution (a 5% perchloric acid (PCA) solution containing 6.7 µM iodoacetic acid (IAA), 0.1 M boric acid, and an internal standard of 5 µM γ-glutamyl-glutamate (γ-Glu-Glu)) and stored at -70°C.

2.4.1.2 Exhaled Breath Condensate. Since thiol concentrations in EBC are much less than in either BALF or plasma, the EBC samples were preserved in a modification of the preservation solution used for those fluids. Immediately after collection, a 240 µl EBC aliquot was treated with 40 µl of 7x GSH preservation solution (35% PCA, 43.9 mM IAA, and 0.7 M boric acid). An internal standard γ-Glu-Glu (final 5 nM) was added with the preservation fluid. Preserved samples were stored at -70^{o}C

2.4.2 Derivatization of Samples

Prior to derivitization, acidified samples were thawed on ice.

BALF and plasma samples: Thawed samples were centrifuged at 3500 x g for 5 minutes, after which 300 µl of the top clear solution was transferred to a clean tube. Sample pH was adjusted to pH 9.0 ± 0.2 with approximately 200 µl of a 1M KOH solution saturated with potassium tetraborate.

EBC samples: The entire sample was adjusted to pH 9.0 ± 0.2 with approximately 70 µl of a 3M KOH solution saturated with potassium tetraborate.

After pH adjustment, all samples were left to stand at room temperature for 20 minutes. Samples were then dansylated with 300 µl of dansyl chloride (Sigma) dissolved in acetone (20 mg/ml). Samples were vortexed thoroughly, then incubated in the dark for 24 hours. The addition of 500 µl chloroform terminated the reactions. After centrifugation at 10,000 x g for 5 minutes, a portion of the clear aqueous layer was transferred to vials in preparation for HPLC analysis.

2.4.3 High Performance Liquid Chromatography

Derivatized samples were injected by the autosampler (plasma: 25 µl, BALF: 50 µl, EBC: 100 µl). The run method followed a previously established method (71). Separation of compounds was made with a 10-µm Ultrasil amino column. The mobile solvent was 80% (v/v) HPLC grade methanol in water and the salt solvent was 0.8 M sodium acetate in acetic acid and methanol. Fluorescence detection was recorded by two detectors (Waters 474 from Waters Corp. (Milford, MA) and Gilson Model 121 from Gilson Inc. (Middleton, WI)). Quantitation of GSH, GSSG, Cys, and CySS concentrations was obtained by integration relative to the internal standard γ-Glu-Glu.

2.5 MEASUREMENT OF OXIDANTS

2.5.1 Hydrogen Peroxide

The concentration of H_2O_2 was determined in all samples by the Amplex Red Assay from Molecular Probes (Portland, OR). In the presence of horseradish peroxidase (HRP), the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in a 1:1 stoichiometry to produce a red-fluorescent product.

2.5.2 4-Hydroxynonenal (HNE)

HNE concentrations were measured using a quantitative indirect enzyme immunoassay. In a 96-well plate, standards and samples of BALF or diluted plasma (1:50 in PBS) were incubated at 4°C overnight in 0.05% carbonate buffer. Blocking buffer (0.1% nonfat dry milk in PBS) was added and kept at room temperature for 1 h. After rinsing wells in PBS, the primary antibody (anti-HNE polyclonal from Alexis Biochemicals (San Diego, CA)) at a 1:5000 dilution was added and allowed to incubate overnight at 4°C. Then, unbound antibody was removed by washing and the wells were treated with a secondary antibody conjugated to streptavidin–peroxidase for 1 h. Wells were washed and OPD Sigma-Fast substrate was added. After 20 minutes protected from light, absorbance was determined at a wavelength of 450 nm.

2.5.3 Malondialdehyde (MDA)

MDA concentrations were also measured using a quantitative indirect enzyme immunoassay. In a 96-well plate, standards and samples of BALF or diluted plasma (1:50

in PBS) were incubated at 4°C overnight in 0.05% carbonate buffer. Blocking buffer (0.1% nonfat dry milk in PBS) was added and kept at room temperature for 1 h. After rinsing wells in PBS, the primary antibody (anti-MDA polyclonal from Academy Biomedical (Houston, TX)) at a dilution of 1:3000 was added and allowed to incubate overnight at 4°C. After unbound antibody was removed by washing, the wells were treated with a secondary antibody conjugated to streptavidin–peroxidase for 1 h. Wells were washed and OPD Sigma-Fast substrate was added. After 20 minutes protected from light, absorbance was determined at a wavelength of 450 nm.

CHAPTER 3: ALTERED PULMONARY AND SYSTEMIC THIOLS IN CRITICALLY ILL PATIENTS

3.1 BACKGROUND

Oxidative stress has been implicated in many pulmonary diseases, including ventilator associated pneumonia (VAP) and acute respiratory distress syndrome (ARDS) (72, 73), two conditions common in mechanically ventilated patients in the intensive care unit. In VAP patients, increased alveolar and plasma TBARS (thiobarbituric acid reactant substances), markers of lipid peroxidation, have been observed (72). In addition, these patients had decreased erythrocyte and alveolar glutathione peroxidase activity. Increased lipid peroxidation, as indicated by TBARS, has also been seen in the plasma of ARDS patients (74). In the bronchoalveolar lavage fluid (BALF) of ARDS patients, one study found that the oxidized moiety of glutathione (GSSG) was increased, without a difference in the reduced form (GSH) (35), while another study found that total GSH (tGSH) was significantly decreased in ARDS patients (36). The net result in both studies was an increase in the ratio of oxidized to reduced glutathione.

Mechanical ventilation (MV) is commonly used to treat patients with acute lung injury, including acute respiratory distress syndrome (ARDS). Although MV may improve oxygenation of the blood, it is itself associated with increased reactive oxygen species (ROS). Various aspects of MV may contribute to this increase in ROS. First, the patient is exposed to concentrations of molecular oxygen that are higher than are present normally. Next, the stretching of the alveoli may result in cell damage, decreased barrier

integrity, and the influx of ROS-generating inflammatory cells. Finally, cyclic strain of endothelial cells appears to increase the activity of an endothelial NAD(P)H-oxidase (NOX), which generates superoxide (60).

In order to defend against all these oxidants, the epithelial lining fluid (ELF) of the lungs is equipped with an arsenal of antioxidants that includes antioxidant enzymes, vitamins, and low molecular weight thiols. The primary antioxidant in the ELF of healthy lungs is the low molecular weight thiol glutathione; but cysteine is also significant, as an antioxidant, as a component of proteins and GSH, and for the maintenance of cell homeostasis (75). The objective of our study was to assess the pulmonary and systemic redox status of both GSH and Cys in critically ill, mechanically ventilated patients.

3.2 METHODS

3.2.1 Subject Recruitment

All research protocols concerning subjects have been fully approved by the Institutional Review Board at Emory University in accordance with guidelines provided by the National Institutes of Health. Subjects were recruited from patients admitted to the ICU at Grady Memorial Hospital in Atlanta, Georgia. All patients were mechanically ventilated, with a diagnosis of ARDS and/or VAP. All participants or their surrogate provided informed consent. Healthy controls were also recruited. Subjects were excluded from the control group if they had any prior history of cardiac, liver, kidney or lung disease by prior history or medical testing, diabetes mellitus, human immunodeficiency virus

infection, or gastrointestinal bleeding. Since alcoholics are at higher risk for VAP and ARDS, even without trauma (12, 76), subjects with a history of chronic alcohol abuse (a SMAST score >3) were excluded to eliminate the possible confounding effects of alcohol abuse. Subjects with concomitant illicit drug use by prior history or urine drug screen were also excluded.

3.2.2 Sample Collection

Bronchoscopy was performed according to standard procedures as discussed previously (77). A bronchoscope (model BF-1T20D; Olympus America, Inc., Melville, NY) was inserted transnasally into a subsegmental bronchus of the right middle lobe. Three 50-ml aliquots of sterile saline were injected and immediately aspirated into suction traps under continuous low-pressure suction. The fluid was filtered through coarse gauze and centrifuged at 750 x g for 10 minutes in order to remove the cellular components. For thiol measurements, 500 µl of the resulting fluid was added directly to 500 µl of the preservation fluid (a 5% perchloric acid solution containing 6.7 µM iodoacetic acid, 0.1 M boric acid, and an internal standard of 5 µM γ-glutamyl-glutamate (γ-Glu-Glu)) and stored at -70°C. A 10 ml blood specimen was collected within 10 minutes of the bronchoscopy. A portion of the plasma was also preserved for HPLC analysis and stored at -70°C.

3.2.3 Markers of Oxidative Stress

Hydrogen peroxide (H_2O_2) is a radical oxygen species (ROS) that is a byproduct of mitochondrial respiration and a secondary product of superoxide generated by NADPH-

oxidases. The concentration of H_2O_2 in the BALF was determined using the Amplex Red Assay from Molecular Probes (Portland, OR). 4-Hydroxynonenal (HNE) is a product of lipid peroxidation and forms protein adducts that can affect many cellular processes. Like HNE, malondialdehyde (MDA) is an aldehyde end-product of lipid peroxidation and forms protein adducts. It also forms mutagenic adducts with DNA. HNE and MDA concentrations were measured using a quantitative indirect enzyme immunoassay. In a 96-well plate, standards and samples of BALF or diluted plasma (1:50 in PBS) were incubated at 4°C overnight in 0.05% carbonate buffer. Blocking buffer (0.1% nonfat dry milk in PBS) was added and kept at room temperature for 1 h. After rinsing wells in PBS, the appropriate primary antibody (anti-MDA polyclonal (1:3000) from Academy Biomedical (Houston, TX) or anti-HNE polyclonal (1:5000) from Alexis Biochemicals (San Diego, CA)) was added and allowed to incubate overnight at 4°C. After unbound antibody was removed by washing, the wells were treated with a secondary antibody conjugated to streptavidin–peroxidase for 1 h. Wells were washed and OPD Sigma-Fast substrate was added. After 20 minutes protected from light, absorbance was determined at a wavelength of 450 nm.

3.2.4 HPLC Measurements of Thiols

After protein removal, preserved samples were derivatized with dansyl chloride and separated by HPLC on a 10-µm Ultrasil amino column (Waters 2690; Waters Corp. Milford, MA). Fluorescence detection was performed by two detectors (Waters 474 and Gilson Model 121; Gilson, Inc., Middleton, WI]). The concentrations of GSH, GSSG,

Cys, and CySS were calculated by quantifying the integrated areas relative to that of γ-Glu-Glu.

3.2.5 Statistical Analysis

SPSS software (version 15.0; SPSS Inc., Chicago, IL) was used for all analyses. Results were reported as median [25%, 75%]. Group median comparisons were made using the nonparametric Mann-Whitney test. Relations between variables were evaluated using the nonparametric Spearman's correlation coefficient. Statistical significance was obtained with a p-value less than 0.05.

3.3 RESULTS

3.3.1 Subject Demographics

Subject demographics are displayed in **Table 3-1**. The critically ill patients were several years older than the healthy subjects (47 ± 19 years vs. 40 ± 8 years). The proportion of males was not significantly different (56% of healthy controls vs. 71% of critically ill patients). The proportion of smokers was also not significantly different between groups (53% of healthy controls vs. 52% of critically ill patients).

3.3.2 BALF H_2O_2 and HNE were Higher in the Critically Ill Patients

Two markers of oxidative stress were higher in the BALF of the critically ill patients. H_2O_2 (**Figure 3-1A**) for the patients was more than double that for the controls (2.8 μM [1.8, 4.0] vs. 1.0 μM [0.4, 1.2]), as was the concentration of hydroxynonenal (HNE)

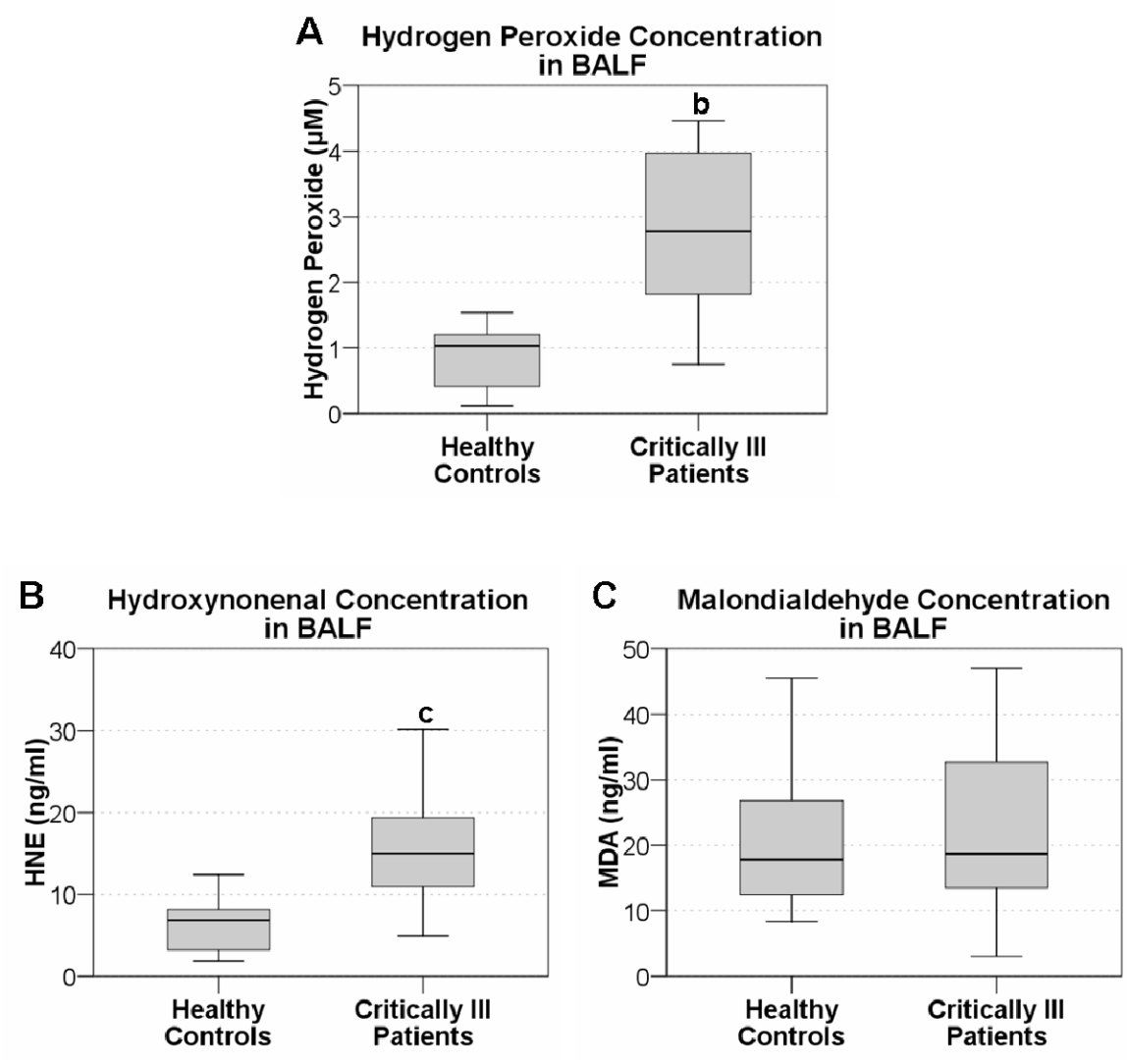
Table 3-1: Subject Demographics. * indicates a statistically significant difference compared to Healthy Controls.

	Healthy Controls	Critically Ill Patients
N	61	32
Mean Age (SD)	40 (8)	49 (19) *
% Male	56%	72%
% Smoker	53%	50%

*** p<0.05 compared to Healthy Controls**

Figure 3-1. Oxidative Stress Markers in the BALF. The bronchoalveolar lavage fluid was collected from healthy subjects and critically ill patients. Various markers of oxidative stress were measured. H_2O_2 concentrations **(A)** and HNE concentrations **(B)** were found to be significantly higher in the BALF of critically ill patients. MDA concentrations **(C)** were not different. Data were expressed as boxplots. [b] $p<0.01$ compared to healthy controls. [c] $p<0.001$ compared to healthy controls.

Figure 3-1. Oxidative Stress Markers in the BALF.



(**Figure 3-1B**) (15.0 ng/ml [10.9, 19.4] vs. 6.8 ng/ml [3.2, 8.1]. Levels of malondialdehyde (MDA) **(Figure 3-1C)** did not differ in the BALF of the two groups.

3.3.3 Lower GSH Concentrations and Higher %GSSG in Patient BALF

In BALF, critically ill patients had significantly lower GSH concentrations compared to healthy controls (0.5 μM [0.2, 3.9] vs. 10.0 μM [5.3, 25.7]) (**Figure 3-2A**), but concentrations of GSSG were not significantly altered in the critically ill patients (**Figure 3-2B**). Median GSSG concentrations were less than 0.5μM for both groups. The total GSH pool (tGSH) in the BALF primarily reflected changes in GSH, resulting in significantly lower concentrations for the patients (1.5 μM [0.4, 5.0]) compared to controls (11.8 μM [5.6, 27.3]). Oxidative stress was assessed by calculating the proportion of the GSH pool present as the oxidized moiety GSSG (%GSSG = GSSG/GSH+GSSG). Since GSH levels were lower in the critically ill patients compared to the healthy controls, the %GSSG for the patients was 33.2% [17.3, 58.4], compared to only 3.5% [1.7, 7.1] for controls (**Figure 3-2C**).

3.3.4 Increased BALF Cys and CySS Concentrations in the Critically Ill

BALF Cys and CySS concentrations were both significantly higher in the patients compared to controls (**Figure 3-3A, B**). Cys concentrations were 0.3 μM [0.2, 0.4] for healthy controls, but 0.9 μM [0.3, 40.6] for the patients. The corresponding CySS concentrations were 0.1 μM [0.0, 0.3] and 1.7 μM [0.3, 19.3] for controls and patients, respectively. As a consequence, the total Cys concentrations (tCys) were higher in the

Figure 3-2. Glutathione in the BALF. Using γ-glutamyl glutamate as an internal standard, the GSH **(A)** and GSSG **(B)** concentrations in the BALF were determined by HPLC. The percentage of the total glutathione present as the oxidized moiety was determined (%GSSG = [GSSG]/([GSH]+[GSSG]) x 100%) **(C)**. Sample size: Healthy Controls (30), Critically Ill Patients (30). ^{c} $p<0.001$ compared to healthy controls.

Figure 3-2. Glutathione in the BALF.

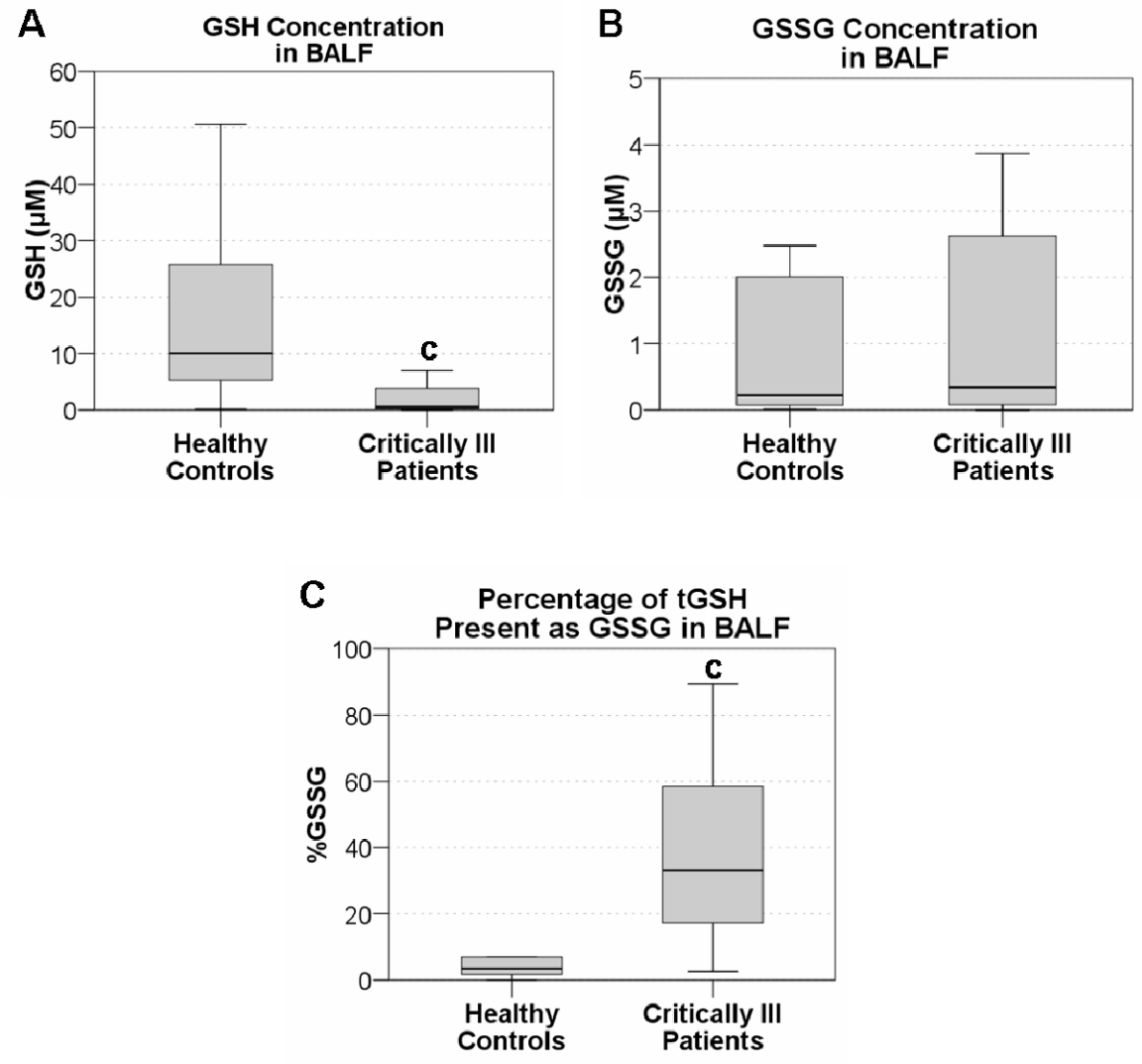
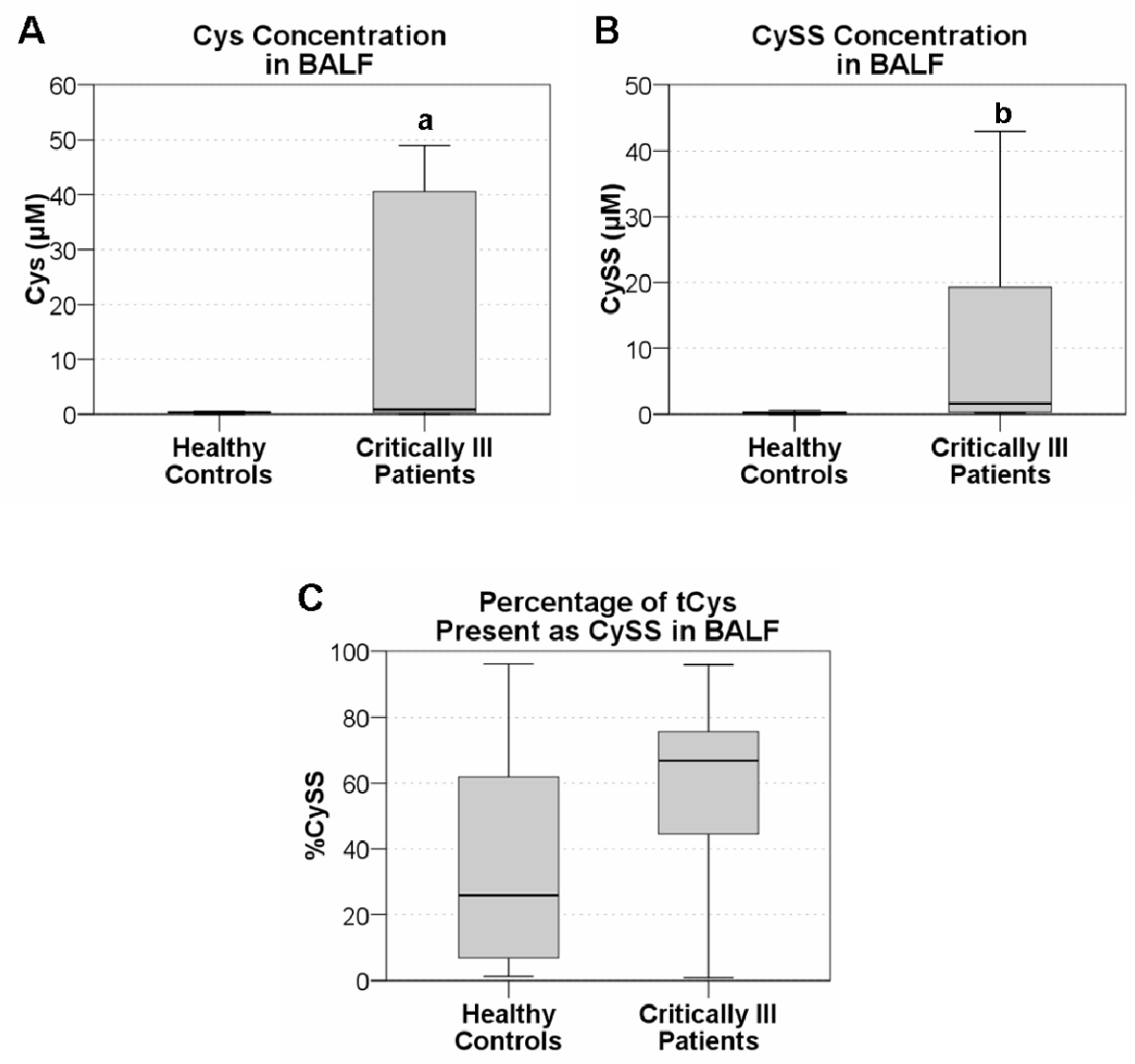


Figure 3-3. Cysteine in the BALF. The Cys **(A)** and CySS **(B)** concentrations in the bronchoalveolar lavage fluid were determined by HPLC. The percentage of the total cysteine present as the oxidized moiety in the BALF of healthy subjects and critically ill patients was determined (%CySS = [CySS]/([Cys]+[CySS]) x 100%) **(C)**. Sample size: Healthy Controls (10), Critically Ill Patients (18). [a] $p<0.05$ compared to healthy controls. [b] $p<0.01$ compared to healthy controls.

Figure 3-3. Cysteine in the BALF.



patient population. The large difference in %CySS (26% [7, 62] for controls vs. 67% [45, 76] for patients) was not statistically significant (p=0.08) (**Figure 3-3C**).

3.3.5 Associations between BALF Glutathione and Cysteine

In the BALF, there were moderate correlations between GSH and Cys concentrations (ρ=0.541, p<0.01, N=26) and tGSH and tCys (ρ=0.572, p<0.01), but the correlation between GSSG and CySS (ρ=0.643, p<0.01) was somewhat stronger. The correlation between %GSSG and %CySS was not significant (ρ=0.354, p<0.08). The ratio of tCys/tGSH was significantly higher for the patients (4.8 [2.6, 8.9]) than the controls (0.3 [0.1, 0.4]) (p<0.01) (**Figure 3-4**).

3.3.6 Correlations Between Thiols and Oxidative Stress Markers in the BALF

HNE concentrations correlated with Cys (ρ=0.624, p<0.01), CySS (ρ=0.591, p<0.05), and tCys (ρ=0.661, p<0.01). HNE did not correlate with any measures of GSH. Neither H_2O_2 nor MDA concentrations correlated with any measures of GSH or Cys in the BALF.

3.3.7 Lipid Peroxidation in Plasma of Patients

MDA concentrations were more than 10 times higher in the plasma of the critically ill patients (5400 ng/ml [2100, 8400] vs. 390 ng/ml [280, 470]) (**Figure 3-5**).

Figure 3-4. Ratio of tCys/tGSH in BALF. The ratio of tCys ([Cys]+[CySS]) to tGSH ([GSH]+[GSSG]) in the BALF was calculated. Sample size: Healthy Controls (9), Critically Ill Patients (17). [b] $p<0.01$ compared to healthy controls.

Figure 3-4. Ratio of tCys/tGSH in BALF.

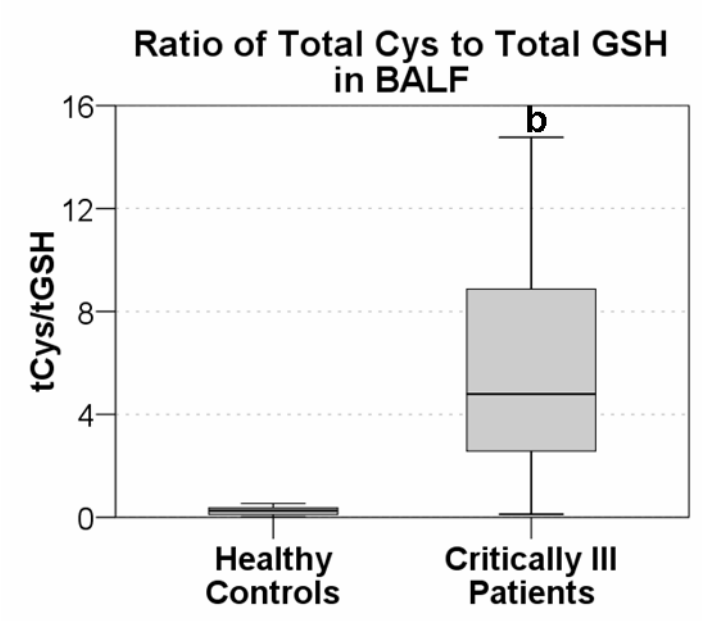
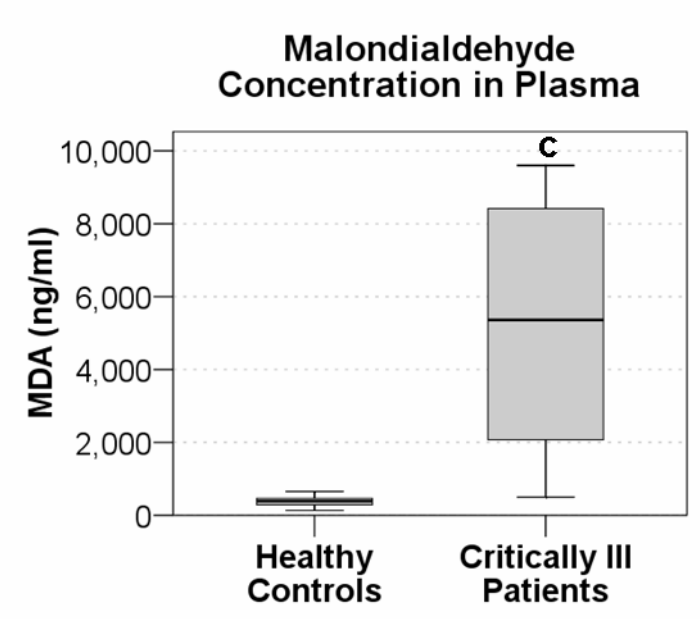


Figure 3-5. Plasma Malondialdehyde (MDA). MDA, a marker of oxidative stress we measured in the plasma, was significantly higher in the plasma of critically ill patients. [c] $p<0.001$ compared to healthy controls.

Figure 3-5. Plasma Malondialdehyde (MDA).



3.3.8 Increased Plasma GSSG and tGSH Concentrations, and %GSSG in Critically Ill.

Plasma GSH and GSSG concentrations were greater in critically ill patients compared to healthy controls (**Figure 3-6A, B**), although the increase in GSH from 4.1μM [2.2, 7.2] for controls to 11.3 μM [2.6, 24.4] for the patients was not significant (p=0.08), but the increases in GSSG (0.2 μM [0.2, 0.3] vs. 1.6 μM [0.4, 4.1]) and tGSH (4.3 μM [2.4, 7.4] vs. 13.5 μM [3.6, 29.4]) were both significant. Plasma %GSSG was also greater in the patients (21.1% [8.1, 30.6]) compared to controls (5.9% [4.0, 8.8]) (**Figure 3-6C**).

3.3.9 Increased Plasma Cys and tCys, but Lower %CySS in Critically Ill

Plasma Cys and tCys concentrations were also higher in the critically ill patients, although the difference was not as dramatic as in the BALF (**Figure 3-7A**). Plasma Cys was 8.4 μM [4.7, 10.8] in control subjects vs. 36.8 μM [28.3, 47.4] in patients. The difference in CySS was not statistically significant (**Figure 3-7B**). CySS concentrations were 82.1 μM [64.7, 100.3] for controls and 109.1 μM [59.1, 140.6] for patients. The percent oxidized (%CySS) was significantly decreased in critically ill patients (**Figure 3-7C**). The difference in %CySS was nearly 20% (90% [88, 93] for controls vs. 71% [60, 76] for patients).

3.3.10 Associations between Plasma Glutathione and Cysteine

In the plasma, there were no significant correlations between measures of GSH and Cys. In contrast to the larger tCys/tGSH ratio in the BALF, the tCys/tGSH ratio in the plasma

Figure 3-6. Plasma Glutathione. GSH **(A)** and GSSG **(B)** concentrations in plasma were determined by HPLC. The percentage of the total glutathione present in the plasma as the oxidized moiety (% GSSG) was determined **(C)**. Sample size: Healthy Controls (41), Critically Ill Patients (16). ^{c} $p<0.001$ compared to healthy controls.

Figure 3-6. Plasma Glutathione.

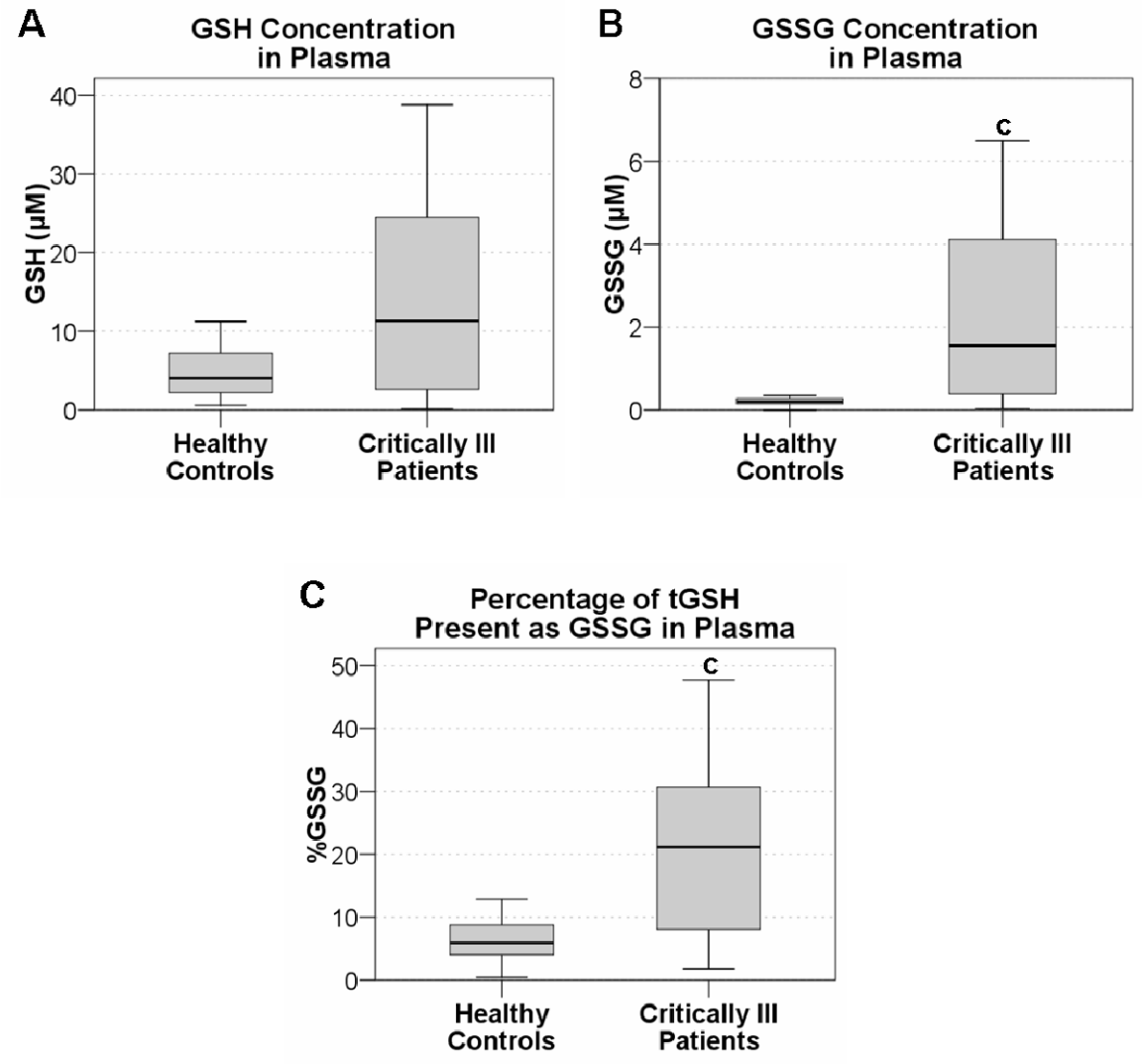
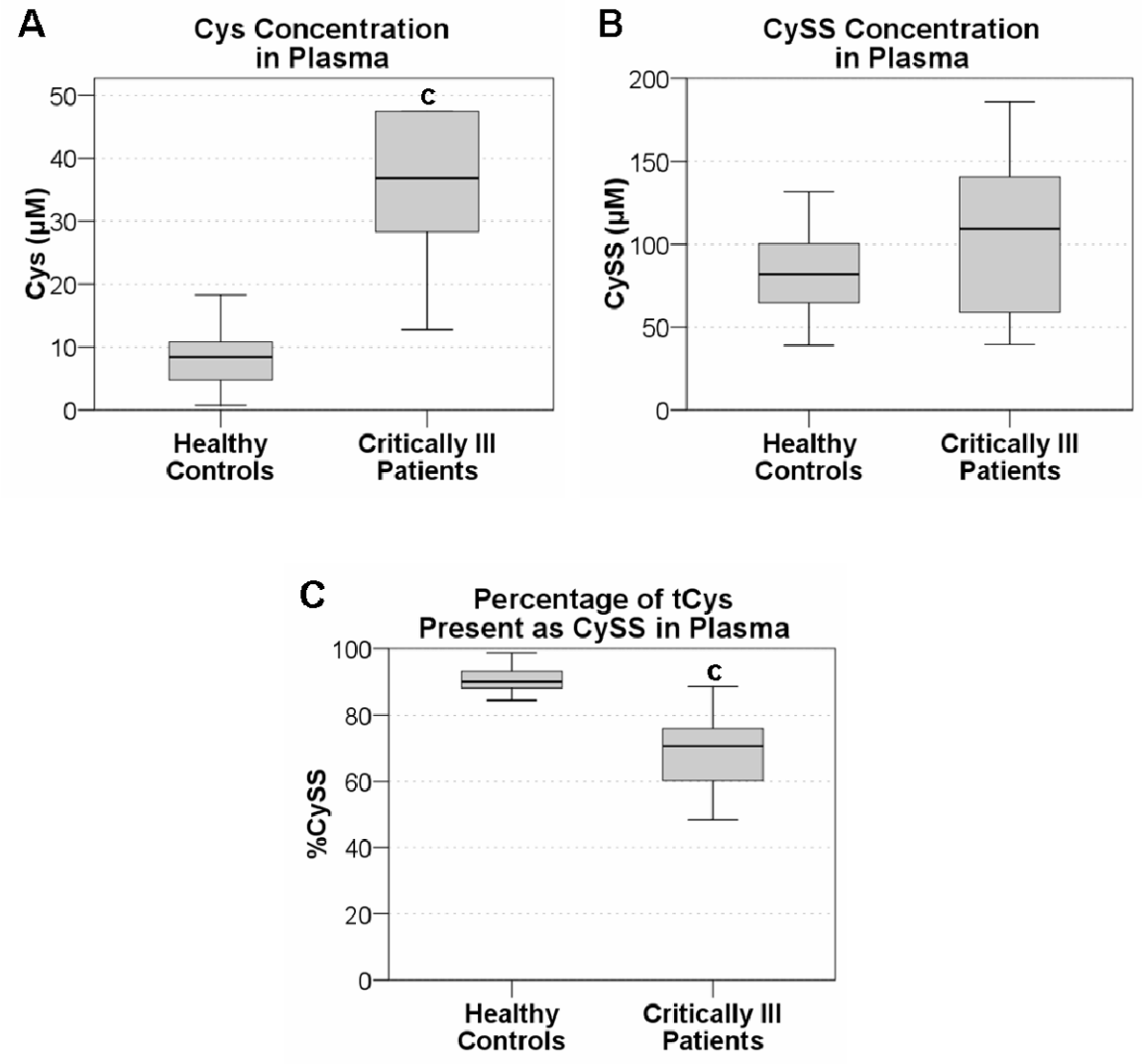


Figure 3-7. Plasma Cysteine. Plasma Cys **(A)** and CySS **(B)** concentrations in plasma were determined by HPLC. The percentage of the total cysteine present in the plasma as the oxidized moiety (% CySS) was determined **(C)**. Sample size: Healthy Controls (36), Critically Ill Patients (10). ^{c} $p<0.001$ compared to healthy controls.

Figure 3-7. Plasma Cysteine.



of the critically ill patients was significantly smaller (33 [9, 42] for controls vs. 7 [4, 10] for patients) (**Figure 3-8**).

3.3.11 Correlations between Thiols and Oxidative Stress Markers in Plasma

In plasma, MDA correlated with Cys (ρ=0.649, $p<0.01$, N=18) and GSSG (ρ=0.508, $p<0.01$, N=27) concentrations. MDA also correlated with %GSSG (ρ=0.461, $p<0.05$, N=27) and inversely with %CySS (ρ=-0.668, $p<0.01$, N=18).

3.3.12 Correlations of Thiols in BALF and Plasma

BALF vs. plasma glutathione: BALF GSSG correlated with plasma GSSG (ρ=0.553, $p<0.01$, N=30). The moderate correlation for GSH was not significant (ρ=0.342, $p=0.06$), but that for tGSH was (ρ=0.489, $p<0.01$). The correlation for %GSSG between the two compartments was moderate, but significant (ρ=0.386, $p<0.05$).

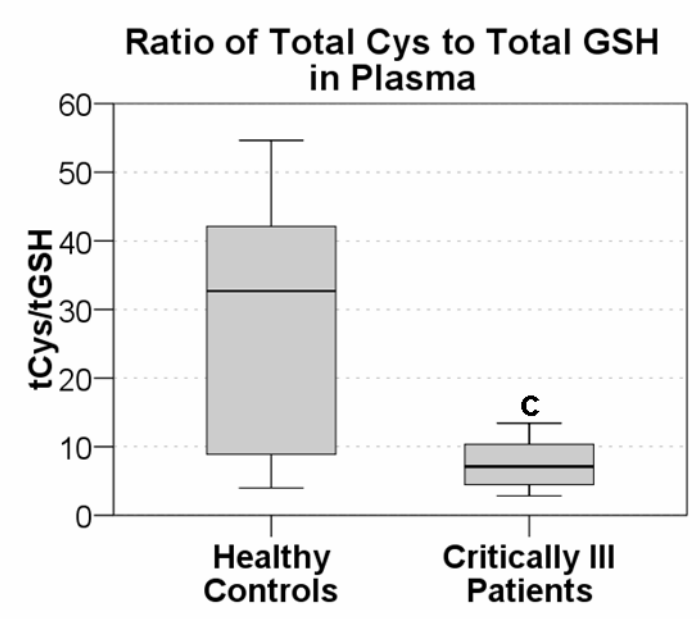
BALF vs. plasma cysteine: We found a strong positive association between the BALF and plasma measures for Cys (ρ=0.698, $p<0.01$, N=17) and tCys (ρ=0.623, $p<0.01$). Neither CySS nor %CySS correlated between the BALF and plasma compartments.

3.4 DISCUSSION

Ventilator associated pneumonia (VAP) and acute respiratory distress syndrome (ARDS) are common in mechanically ventilated patients in the intensive care unit. Among mechanically ventilated patients, nearly 30% develop VAP (9), a pulmonary infection that occurs after more than 48 hours of mechanical ventilation. In addition to the high

Figure 3-8. Ratio of tCys/tGSH in Plasma. The ratio of tCys to tGSH in the plasma was calculated. Sample size: Healthy Controls (32), Critically Ill Patients (10). [c] $p<0.001$ compared to healthy controls.

Figure 3-8. Ratio of tCys/tGSH in Plasma.



incidence of VAP, it is also associated with a high mortality rate, possibly as high as 70% for certain resistant bacteria (10). Although it is difficult to separate the mortality attributable to the VAP itself from that attributable to concomitant conditions, VAP has been associated with a 30% increase in the rate of mortality from underlying illnesses (10). The acute respiratory distress syndrome (ARDS) is a form of lung injury characterized by increased permeability of the alveolar-capillary barrier. Alveoli become flooded with neutrophils and other proteinaceous matter, as well as the accompanying fluid, resulting in impaired gas exchange, respiratory failure, and in approximately 50% of cases, death. In the United States, more than 100,000 ARDS cases occur each year (1). ARDS is associated with a high mortality rate, but even for survivors of ARDS, quality of life is diminished (1, 2).

Oxidative stress has been implicated in many pulmonary diseases, including VAP and ARDS, which are both associated with activation of immune cells and a subsequent increase in release of reactive oxygen species (72, 73). In VAP patients, increased alveolar and plasma lipid peroxidation have been observed (72). In addition, these patients had decreased erythrocyte and alveolar glutathione peroxidase activity. Increased lipid peroxidation, as indicated by TBARS, has also been seen in the plasma of ARDS patients (74). In the bronchoalveolar lavage fluid (BALF) of ARDS patients, one study found that the oxidized moiety of glutathione (GSSG) was increased, without a difference in the reduced form (GSH) (35), while another study found that total GSH (tGSH) was significantly decreased in ARDS patients (36). The net result in both studies was an increase in the ratio of oxidized to reduced glutathione.

We found higher concentrations of H_2O_2 and hydroxynonenal (HNE) in the BALF of critically ill patients. These results agreed with the findings of Duflo et al demonstrating greater oxidation in the BALF of VAP patients (72). Increased H_2O_2 has also been found in the exhaled breath of ARDS patients (78, 79).

In order to prevent collateral oxidative damage, the lung is equipped with an arsenal of antioxidants. The primary extracellular antioxidant in the healthy lung is glutathione. We found that GSH concentrations in the BALF of critically ill patients were about 5% of those in healthy controls. Although BALF GSSG concentrations were similar in the two groups, the lower GSH resulted in a much higher proportion of the total GSH pool present as the oxidized moiety GSSG (%GSSG) in the critically ill patients. This suggested increased oxidative stress and impaired antioxidant defenses in the BALF of the critically ill patients, which agrees with previous findings in ARDS patients (35, 36).

Another low molecular weight thiol normally present in the lung is cysteine. This amino acid is a component of proteins and GSH, but it also acts as an antioxidant. In addition, extracellular cysteine redox is important for cellular homeostasis (44, 45, 80). In the epithelial lining fluid of healthy individuals, the concentration of cysteine is about a tenth of the GSH concentration (81, 82). This apparent predominance of GSH in the epithelial lining may explain why studies of pulmonary antioxidant capacity have focused on GSH and neglected Cys. Our results indicate that Cys status is significantly altered in the BALF of critically ill patients. We found that not only were Cys and CySS concentrations

higher in the critically ill patients compared to the healthy subjects, the cysteine pool (tCys) was larger than the glutathione pool (tGSH). When the two groups of subjects were compared, the patients had a higher %CySS in the BALF, although the difference was not significant.

In the BALF, the reduced moiety GSH was decreased in the VAP patients but the Cys moiety was increased. The source of the cysteine in the BALF of the critically ill patients is not clear, but the high levels found could be due to increased leak across the alveolar-capillary barrier or to increased γ-glutamyl-transpeptidase (GGT) activity. Whereas GSH normally predominates in the epithelial lining fluid, cysteine (mostly as CySS) is the primary low molecular weight thiol in plasma. Impaired barrier function, such as accompanies mechanical ventilation and more specifically ARDS, would therefore shift the balance in the airspace towards cysteine. γ-glutamyl-transpeptidase (GGT) is an enzyme that initiates the liberation of cysteine from glutathione and has been used as an indicator of oxidative stress. GGT also functions to increase intracellular GSH synthesis by the transport of cystine across cell membranes (83). The inflammatory cytokine IL-1 has been shown to increase levels of GGT in lung tissue (84). One study also found increased GGT activity in the BALF of ARDS patients (85). Increased GGT activity could explain both the decrease in GSH and the increase in Cys we found in the critically ill patients. The large difference in Cys oxidation (%CySS) was not significant due to the wide range of values for both groups.

In the plasma of the critically ill patients, we observed higher MDA concentrations. These results were in agreement with previous studies of ARDS and VAP patients (72, 86). As in the BALF, plasma %GSSG was significantly greater in the critically ill patients. In contrast to our findings in the BALF where the increase in %GSSG was driven by a decrease in GSH, in plasma the increase in %GSSG was driven by an increase in GSSG. The overall increase in tGSH may be partly due to GSH release from damaged cells such as erythrocytes, since intracellular concentrations are very high (on the order of 1 mM) compared to plasma concentrations. A similar elevation of plasma tGSH was found in a study of ICU patients with multiple organ failure (87).

Plasma Cys, but not CySS, was higher in the critically ill patients. The study by Wernerman et al (87) that found elevated plasma tGSH in ICU patients also found elevated plasma tCys, although they did not assess Cys and CySS separately. In the BALF, the increase in both Cys and CySS led to an increase in %CySS that was not significant. In contrast, plasma Cys was more reduced in the critically ill groups as reflected by a lower %CySS. Unlike in the BALF, the difference was significant. As proposed for the BALF, alterations in GGT activity could contribute to increased Cys in plasma. In a mouse model of multiple organ dysfunction syndrome (MODS), mechanical ventilation alone increased plasma GGT, with a further increase when mechanical ventilation was accompanied by endotoxin administration (88).

Another possible source for increased plasma Cys is muscle wasting. Malnutrition and inactivity, common among critically ill patients, lead to a loss of lean muscle mass. Muscle is broken down to free amino acids for protein synthesis elsewhere.

One of the reasons our analysis included both BALF and plasma samples was to determine if the plasma could be used to assess pulmonary redox status. BALF GSSG correlated with plasma GSSG ($\rho=0.553$, $p<0.01$). The correlation for %GSSG between the two compartments was more moderate, but significant ($\rho=0.386$, $p<0.05$). For Cys, we found a strong positive association between the BALF and plasma measures for Cys ($\rho=0.698$, $p<0.01$, $N=17$) and tCys ($\rho=0.623$, $p<0.01$), but %CySS did not correlate between the BALF and plasma compartments.

In summary, the BALF of critically ill patients was under greater oxidative stress than healthy controls as evidenced by higher H_2O_2 and HNE. A higher %GSSG in the BALF of these patients provide further evidence of greater oxidative stress. The Cys pool (tCys) was larger in the BALF of the critically ill patients but the %CySS was not significantly different from controls. Higher plasma MDA concentrations and %GSSG in critically ill subjects indicated greater oxidative stress, but surprisingly plasma Cys was significantly more reduced in this group. Although the BALF and plasma of the critically ill patients were more oxidized, the effect was not consistently reflected by the oxidative status of the two principal thiol antioxidants and suggested that these two thiol pools were regulated independently of each other.

CHAPTER 4: THIOLS IN THE BRONCHOALVEOLAR LAVAGE FLUID OF VENTILATOR-ASSOCIATED PNEUMONIA PATIENTS WITH AND WITHOUT ALCOHOL ABUSE

4.1 BACKGROUND

Nearly 30% of mechanically ventilated patients develop ventilator-associated pneumonia (VAP) (9). VAP is differentiated from other hospital-acquired pneumonias in that it is a pulmonary infection that occurs after more than 48 hours of mechanical ventilation. In addition to the high incidence of VAP, it is also associated with a high mortality rate, possibly as high as 70% for certain resistant bacteria (10). Although it is difficult to separate the mortality attributable to the VAP itself from that attributable to concomitant conditions, VAP has been associated with a 30% increase in the rate of mortality from underlying illnesses (10).

The primary risk factors for the development of VAP are duration of ventilation and the presence of an underlying illness, especially acute respiratory distress syndrome (ARDS). Conditions that impair host defenses, such as sepsis and chronic pulmonary diseases, also predispose patients to develop VAP (11). In addition, patients with a history of alcohol abuse are also at higher risk for VAP (12). Although patients with a history of alcohol abuse have been shown to be at increased risk for VAP and other pulmonary infections, the mechanisms by which this increased risk may be conferred have not been extensively investigated.

Oxidative stress has been implicated in many pulmonary diseases, including ARDS and COPD (73, 89), which are themselves risk factors for VAP. While studies into the possible association between oxidative stress and VAP have been limited, evidence in support of an association between oxidative stress and VAP was published by Duflo et al, who found alteration of certain markers of oxidative stress in VAP patients (72). They found that alveolar and plasma TBARS (thiobarbituric acid reactant substances) are increased in VAP patients. They also observed decreased erythrocyte and alveolar glutathione peroxidase activity in this group.

We have previously demonstrated that pulmonary levels of glutathione (GSH), the primary endogenous antioxidant in the fluid lining the alveolar epithelium, were depleted in healthy subjects with a history of alcohol abuse (41). In addition, the redox potential of GSH (E_h) was also more oxidized in this population by 40 mV. We have hypothesized that this oxidative stress may increase the risk of VAP. Although GSH is the primary antioxidant in the ELF of healthy lungs is, cysteine, another low molecular weight thiol, is also significant, as an antioxidant, as a component of proteins and GSH, and for the maintenance of cell homeostasis (75).

In the study presented here, we assessed pulmonary GSH and Cys status to determine if it was altered with VAP in subjects with a history of alcohol abuse. We also evaluated the association of the thiol measures with outcomes for the VAP patients.

4.2 METHODS

4.2.1 Subject Recruitment

All research protocols concerning subjects were fully approved by the Institutional Review Board at Emory University in accordance with guidelines provided by the National Institutes of Health. Subjects were recruited into one of four groups based on presence of VAP and alcohol abuse status: VAP Alcoholics, VAP Controls, Healthy Alcoholics, or Healthy Controls. References to "alcoholics" in our study indicate a positive history of alcohol abuse as explained below.

VAP subjects were recruited from patients admitted to the ICU at Grady Memorial Hospital in Atlanta, Georgia, within 72 hours of VAP diagnosis. All participants or their surrogate provided informed consent and provided information concerning alcohol abuse status as determined by either the Short Michigan Alcohol Screening Test (SMAST) questionnaire (69) or the AUDIT test. Subjects with a SMAST score greater than 3 or an AUDIT score of greater than 8 were assigned to the VAP Alcoholic group. Subjects with a SMAST score of zero were included in the VAP Control group.

Healthy Alcoholics were recruited as previously reported (90). Briefly, subjects with a history of chronic alcohol abuse were recruited from the drug and alcohol dependency unit at Grady Memorial Hospital and from the alcohol detoxification unit at the VA Medical Center. Recruitment eligibility was determined by a SMAST score greater than 3

or an AUDIT score greater than 8 and alcohol consumption within 7 days of recruitment, although intoxicated subjects were excluded.

Healthy Controls without a history of chronic alcohol abuse who had a SMAST score of 0 were also recruited. Subjects were excluded from the "Healthy" groups if they had any prior history of cardiac, liver, kidney or lung disease by prior history or medical testing, diabetes mellitus, human immunodeficiency virus infection, or gastrointestinal bleeding. Subjects with concomitant illicit drug use by prior history or urine drug screen were not eligible. Subjects who had recently taken acetaminophen, which is known to alter GSH status, were also excluded.

4.2.2 Sample Collection

Bronchoscopy was performed according to standard procedures as discussed previously (77). A bronchoscope (model BF-1T20D; Olympus America, Inc., Melville, NY) was inserted transnasally into a subsegmental bronchus of the right middle lobe. Three 50-mL aliquots of sterile saline were injected and immediately aspirated into suction traps under continuous low-pressure suction. The fluid was filtered through coarse gauze and centrifuged at 750 x g for 10 minutes in order to remove the cellular components. For GSH measurements, 500 µl of the resulting fluid was added directly to 500 µl of the preservation fluid (a 5% perchloric acid solution containing 6.7 µM iodoacetic acid, 0.1 M boric acid, and an internal standard of 5 µM γ-glutamyl-glutamate (γ-Glu-Glu)) and stored at -70°C. A 10 mL blood specimen was collected within 10 minutes of the bronchoscopy. A portion of the plasma was also preserved for HPLC analysis and stored

at stored at -70°C. A fraction of the BALF and plasma were also preserved for urea analysis.

4.2.3 Markers of Oxidative Stress

Hydrogen peroxide (H_2O_2) is a radical oxygen species (ROS) that is a byproduct of mitochondrial respiration and a secondary product of superoxide generated by NADPH-oxidases. The concentration of H_2O_2 in the BALF was determined using the Amplex Red Assay from Molecular Probes (Portland, OR). 4-Hydroxynonenal (HNE) is a product of lipid peroxidation and forms protein adducts that can affect many cellular processes. Like HNE, malondialdehyde (MDA) is an aldehyde end-product of lipid peroxidation and forms protein and DNA adducts. HNE and MDA concentrations were measured using a quantitative indirect enzyme immunoassay. In a 96-well plate, standards and samples of BALF or diluted plasma (1:50 in PBS) were incubated at 4°C overnight in 0.05% carbonate buffer. Blocking buffer (0.1% nonfat dry milk in PBS) was added and kept at room temperature for 1 h. After rinsing wells in PBS, the appropriate primary antibody (anti-MDA polyclonal (1:3000) from Academy Biomedical (Houston, TX) or anti-HNE polyclonal (1:5000) from Alexis Biochemicals (San Diego, CA)) was added and allowed to incubate overnight at 4°C. After unbound antibody was removed by washing, the wells were treated with a secondary antibody conjugated to streptavidin–peroxidase for 1 h. Wells were washed and OPD Sigma-Fast substrate was added. After 20 minutes protected from light, absorbance was determined at a wavelength of 450 nm.

4.2.4 HPLC Measurements of Thiols

After protein removal, preserved samples were derivatized with dansyl chloride and separated by HPLC on a 10-μm Ultrasil amino column (Waters 2690; Waters Corp. Milford, MA). Fluorescence detection was performed by two detectors (Waters 474 and Gilson Model 121; Gilson, Inc., Middleton, WI]). The concentrations of GSH and GSSG were calculated by quantifying the integrated areas relative to that of γ-Glu-Glu.

4.2.5 Statistical Analysis

SPSS software (version 15.0; SPSS Inc., Chicago, IL) was used for all analyses. Results were reported as median [25%, 75%] and group median comparisons were made using the nonparametric Mann-Whitney test. Relations between variables were evaluated using the nonparametric Spearman's correlation coefficient. Statistical significance was obtained with a p-value less than 0.05.

4.3 RESULTS

4.3.1 Subject Demographics

Subject demographics are displayed in **Table 4-1**. The subjects in the Healthy Control group were significantly younger than subjects in the other three groups. All groups were predominantly male, with no significant difference between groups. All groups were also predominantly smokers, with no significant difference between groups.

Table 4-1. Subject Demographics

	Healthy Controls	Healthy Alcoholics	VAP Controls	VAP Alcoholics
N	30	44	23	13
Mean Age (SD)	41 (7)	47 (5) *	55 (20) *	52 (7) *
% Male	74%	93%	71%	77%
% Smoker	50%	77%	57%	85%

* p≤0.05 compared to Healthy Control

4.3.2 Oxidative Stress Markers were increased in the BALF of VAP Patients

We measured the concentrations of the reactive oxygen species hydrogen peroxide (H_2O_2), as well as two products of lipid peroxidation, 4-hydroxynonenal (HNE) and malondialdehyde (MDA). H_2O_2 (**Figure 4-1A**) for the VAP control group was more than double that for the healthy groups (2.8 μM [1.8, 4.0] vs. 0.9 μM [0.4, 1.2] for Healthy Controls and 0.5 μM [0.2, 1.3] for Healthy Alcoholics). Due to the wide range of values, the VAP alcoholic group was not significantly different from either of the healthy groups or the VAP control group. The concentration of HNE (**Figure 4-1B**) was significantly higher in the VAP groups (15.0 ng/ml [10.9, 19.4] for VAP Controls and 14.3 ng/ml [12.5, 18.6] for VAP Alcoholics) compared to either healthy group (6.9 ng/ml [3.3, 8.1] for Healthy Controls and 9.9 ng/ml [5.9, 15.3] for Healthy Alcoholics). There was no significant difference in MDA concentration between groups (**Figure 4-1C**).

4.3.3 GSH Concentrations in the BALF of Healthy Alcoholics were Comparable to VAP Patients

The concentration of BALF GSH **(Figure 4-2A)** was significantly lower in the otherwise Healthy Alcoholics (2.2 μM [1.0, 3.9]) and in both populations of VAP patients (VAP Controls: 1.5 μM [0.5, 6.0] and VAP Alcoholics: 2.8 μM [1.3, 7.8]), when compared to the Healthy Controls (10.6 μM [5.5, 25.7]). There was no statistical difference between the Healthy Alcoholics and either VAP group. BALF GSSG concentrations were similar in all groups **(Figure 4-2B)**, with medians varying from 0.3 μM in Healthy Controls to 1.0 μM in VAP Alcoholics. Total GSH concentrations (tGSH) were significantly lower in

Figure 4-1. Concentrations of Oxidative Stress Markers: H_2O_2 (A), HNE (B), and MDA (C). Oxidative stress markers were measured in the BALF. Sample sizes: Healthy Controls (14), Healthy Alcoholics (24), VAP Controls (18), VAP Alcoholics (13). Data were expressed as boxplots. [a] $p<0.05$ compared to healthy controls. [b] $p<0.05$ compared to healthy alcoholics.

Figure 4-1. Concentrations of Oxidative Stress Markers: H_2O_2 (A), HNE (B), and MDA (C).

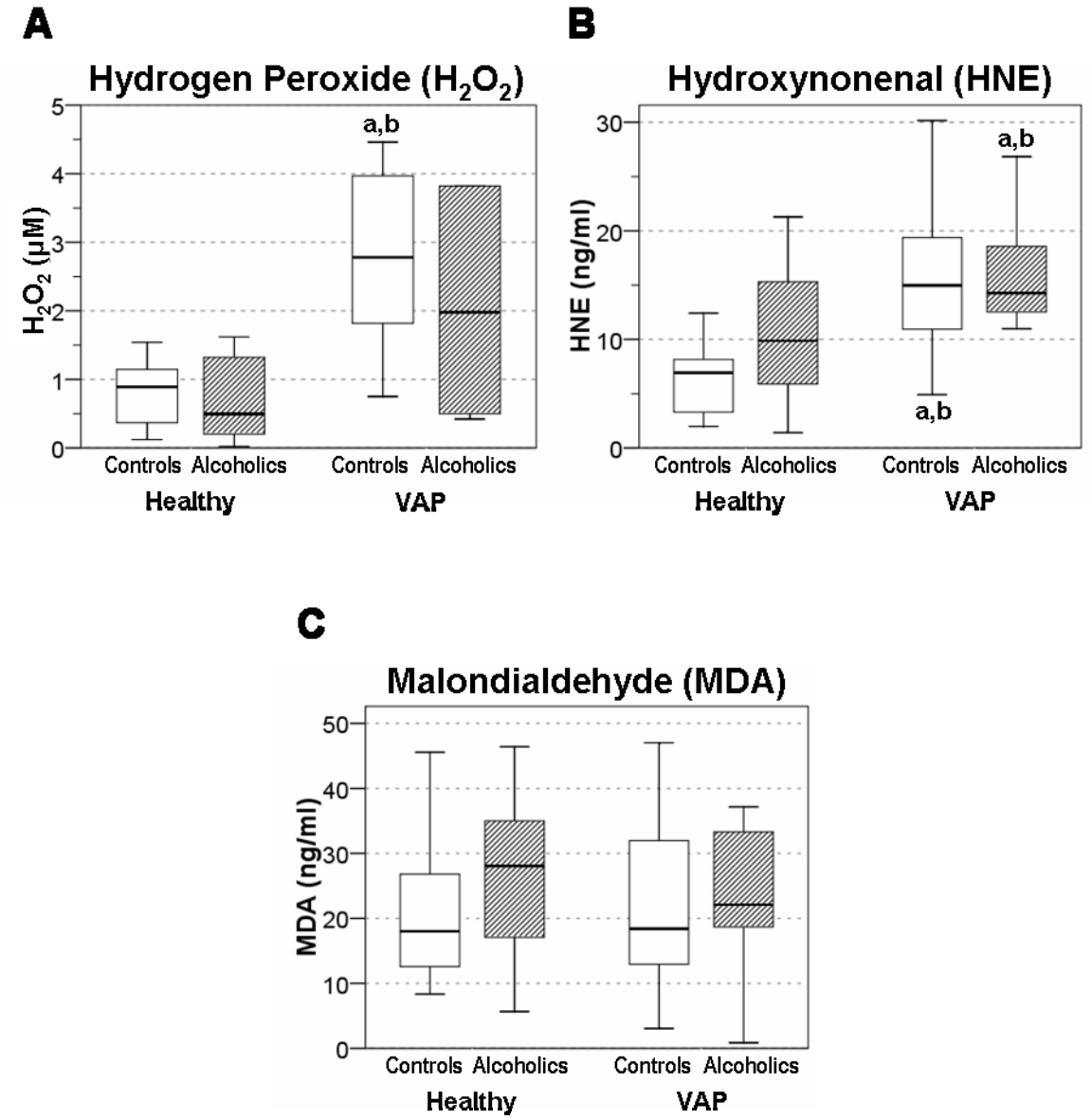
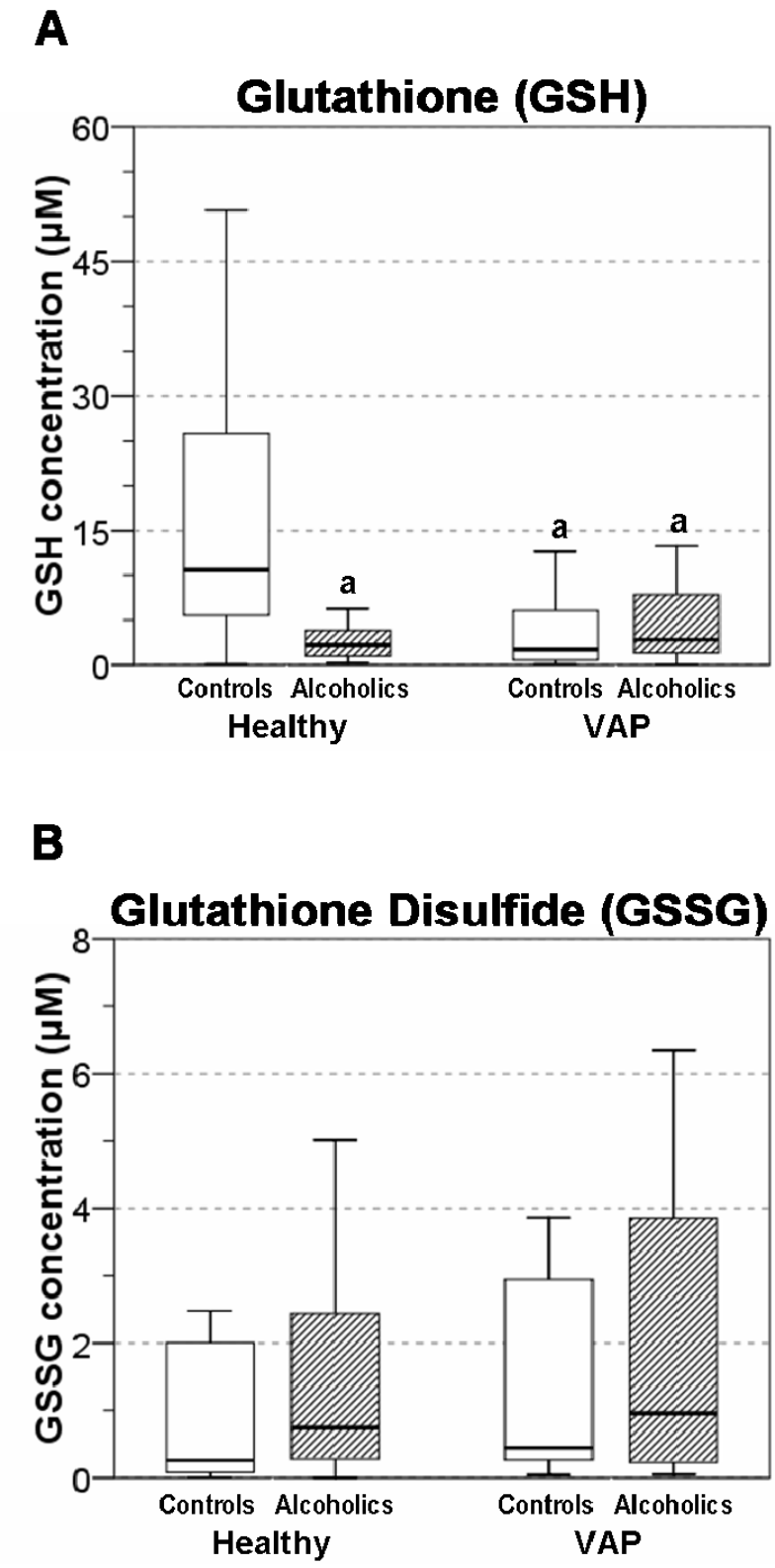


Figure 4-2. Concentrations of GSH and GSSG. Using γ-glutamyl glutamate as an internal standard, the GSH and GSSG concentrations in the BALF were determined by HPLC. Sample sizes: Healthy Controls (29), Healthy Alcoholics (42), VAP Controls (22), VAP Alcoholics (13). [a] $p<0.05$ compared to healthy controls.

Figure 4-2. Concentrations of GSH and GSSG.



the Healthy Alcoholics (3.6 μM [1.7, 5.7]) and VAP Controls (2.3 μM [0.6, 13.1]) with respect to Healthy Controls (12.4 μM [5.7, 27.3]). In the VAP Alcoholics, the total GSH pool (6.3 μM [2.2, 13.4]) was not significantly different from the Healthy Alcoholics or VAP Controls.

4.3.4 BALF GSH was More Oxidized in Healthy Alcoholics and VAP Patients

Whereas the percentage of GSH present as the oxidized moiety, %GSSG (100% x GSSG/tGSH), **(Figure 4-3)** was only 3.6% [1.7, 7.1] in the BALF of Healthy Controls, %GSSG was approximately ten times higher in all the other groups: Healthy Alcoholics (31.3% [11.7, 50.2]), VAP Controls (33.0% [17.3, 58.4]), and VAP Alcoholics (41.6% [11.8, 45.9]). %GSSG in Healthy Alcoholics was not different from the VAP groups.

4.3.5 Cys Concentrations in the BALF of VAP Patients were Higher Than in Healthy Subjects

The concentration of Cys **(Figure 4-4A)** was significantly higher in both populations of VAP patients (VAP Controls: 4.7 μM [0.4, 48.8] and VAP Alcoholics: 12.7 μM [2.1, 26.3]) when compared to the Healthy Controls (0.3 μM [0.2, 0.4]) and Healthy Alcoholics (0.3 μM [0.1, 0.5]). CySS concentrations **(Figure 4-4B)** were also significantly higher in the VAP patients (VAP Controls: 19.1 μM [1.8, 39.4] and VAP Alcoholics: 26.9 μM [2.4, 39.2]) compared to either healthy group (Healthy Controls (0.1 μM [0.0, 0.2]) and Healthy Alcoholics (0.4 μM [0.2, 0.6]). In addition, the CySS in Healthy Alcoholics was significantly higher than in Healthy Controls. Total Cys

Figure 4-3. Oxidation of GSH. Relative oxidation of GSH is expressed as %GSSG (100%x[GSSG]/[tGSH]). Sample sizes: Healthy Controls (29), Healthy Alcoholics (42), VAP Controls (22), VAP Alcoholics (13). [a] $p<0.05$ compared to healthy controls.

Figure 4-3. Oxidation of GSH.

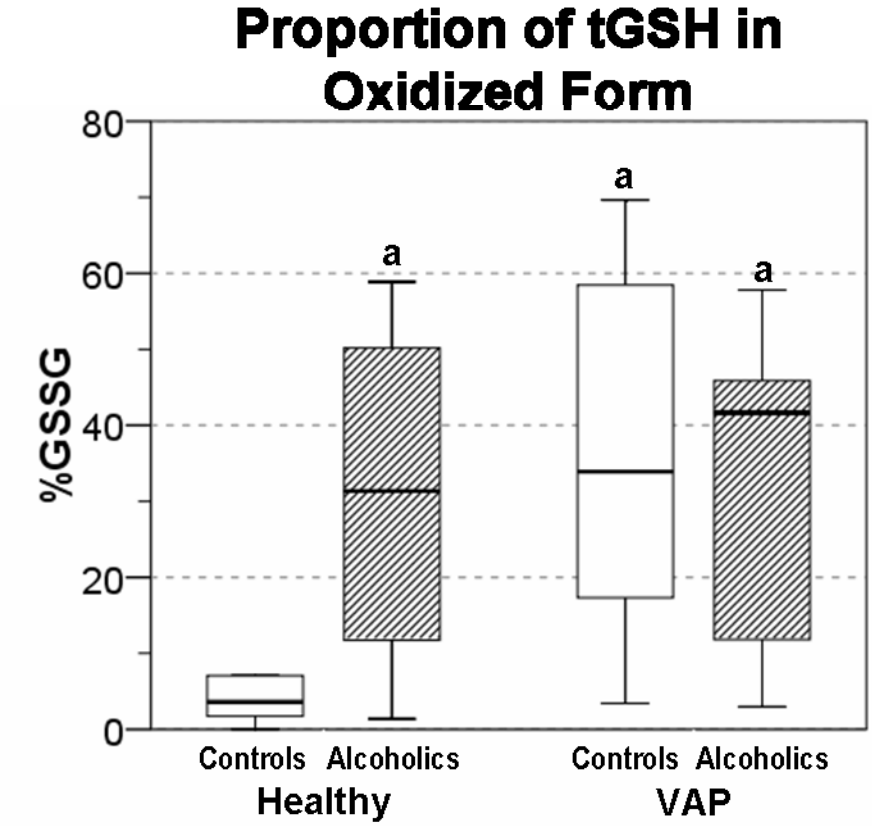
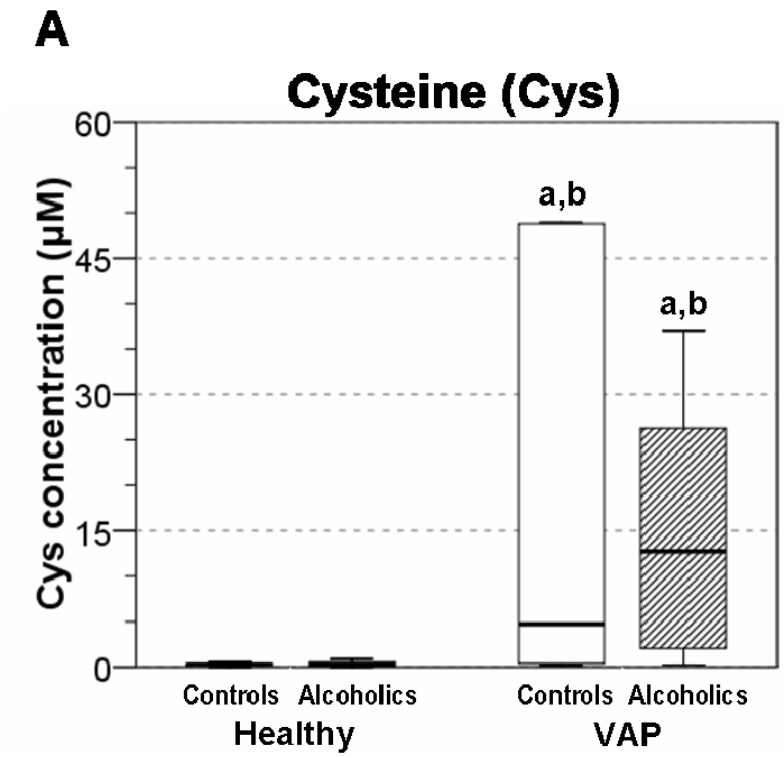
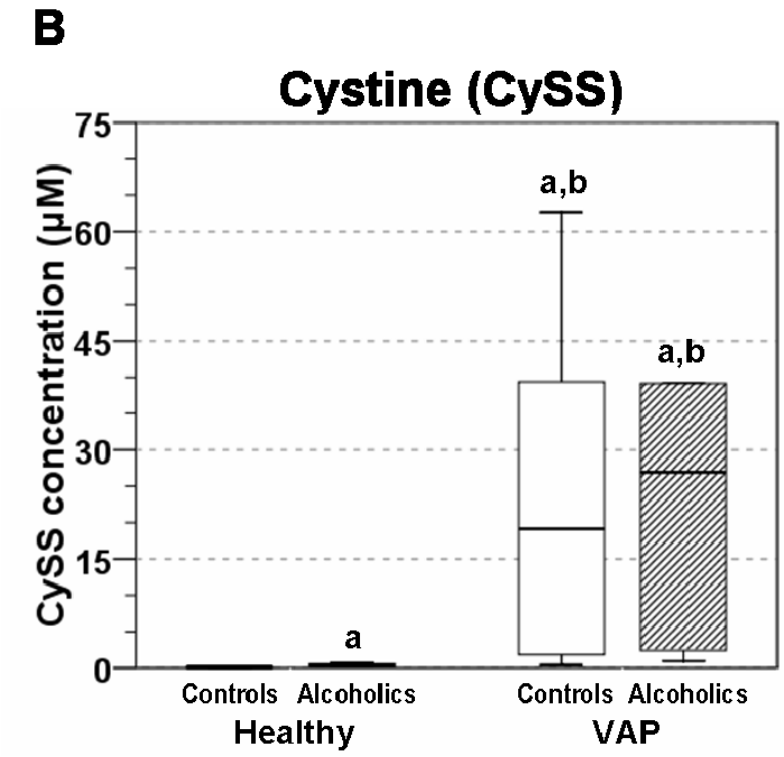


Figure 4-4. Concentrations of Cys and CySS. Using γ-glutamyl glutamate as an internal standard, the Cys and CySS concentrations in the BALF were determined by HPLC. Sample sizes: Healthy Controls (9), Healthy Alcoholics (22), VAP Controls (10), VAP Alcoholics (6). [a] $p<0.05$ compared to healthy controls. [b] $p<0.05$ compared to healthy alcoholics.

Figure 4-4. Concentrations of Cys and CySS.





concentrations (tCys) followed the pattern seen in CySS. tCys was several times higher in the VAP patients (VAP Controls: 24.7 μM [2.2, 88.3] and VAP Alcoholics: 40.0 μM [4.5, 75.6]) than in the Healthy Controls (0.4 μM [0.3, 0.6]) and Healthy Alcoholics (0.8 μM [0.4, 1.0]). tCys for Healthy Alcoholics was double that for Healthy Controls.

4.3.6 BALF Cys was Oxidized in Healthy Alcoholics

The percentage of Cys present as the oxidized moiety, %CySS (100% x CySS/tCys), **(Figure 4-5)** was more than doubled in Healthy Alcoholics compared to Healthy Controls (65% [48, 70] vs. 24% [7, 57]. %CySS for the VAP groups (VAP Controls 62% [51, 76] and VAP Alcoholics 72% [54, 81] were not significantly different from Healthy Controls, although they were also not significantly different from Healthy Alcoholics.

4.3.7 The Cys Pool (tCys) in VAP Patients was Larger than the GSH Pool (tGSH)

The relative sizes of the two thiol pools were assessed by calculating the ratio tCys/tGSH (**Figure 4-6**). For the healthy groups, tGSH was larger than tCys (tCys/tGSH for Healthy Controls was 0.2 [0.1, 0.4] and for Healthy Alcoholics was 0.1 [0.1, 0.2]). For the VAP groups, the inverse was true (VAP Controls 2.6 [0.2, 6.1] and VAP Alcoholics 3.2 [0.7, 6.0].

4.3.8 Survival Corresponded with a Lower tCys/tGSH Ratio

We evaluated the association between several outcomes and our thiols measures. The size of the GSH pool (**Figure 4-7A**) was the same in VAP patients who survived their hospital stay and those who did not survive (4.7 μM [0.7, 13.6] vs. 2.5 μM [1.4, 13.4],

Figure 4-5. Oxidation of Cys. Relative oxidation of Cys is expressed as %CySS (100%x[CySS]/[tCys]). Sample sizes: Healthy Controls (9), Healthy Alcoholics (22), VAP Controls (10), VAP Alcoholics (6). [a] $p<0.05$ compared to healthy controls. [b] $p<0.05$ compared to healthy alcoholics.

Figure 4-5. Oxidation of Cys.

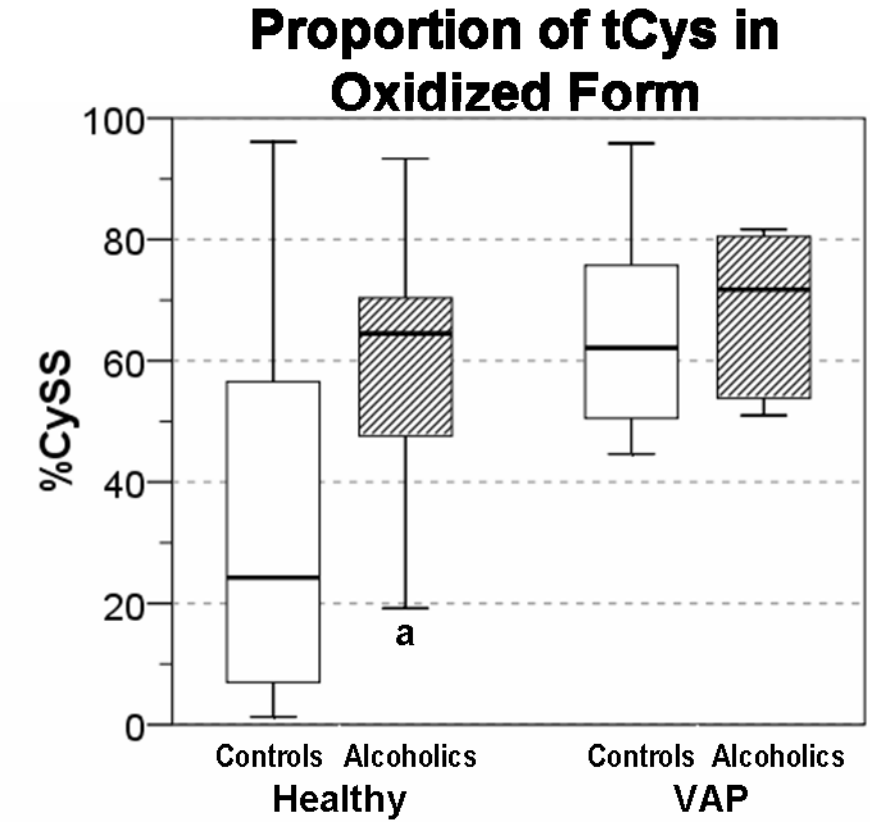


Figure 4-6. Ratio of tCys/tGSH. Sample sizes: Healthy Controls (8) Healthy Alcoholics (20), VAP Controls (9), VAP Alcoholics (6). [a] $p<0.05$ compared to healthy controls. [b] $p<0.05$ compared to healthy alcoholics.

Figure 4-6. Ratio of tCys/tGSH.

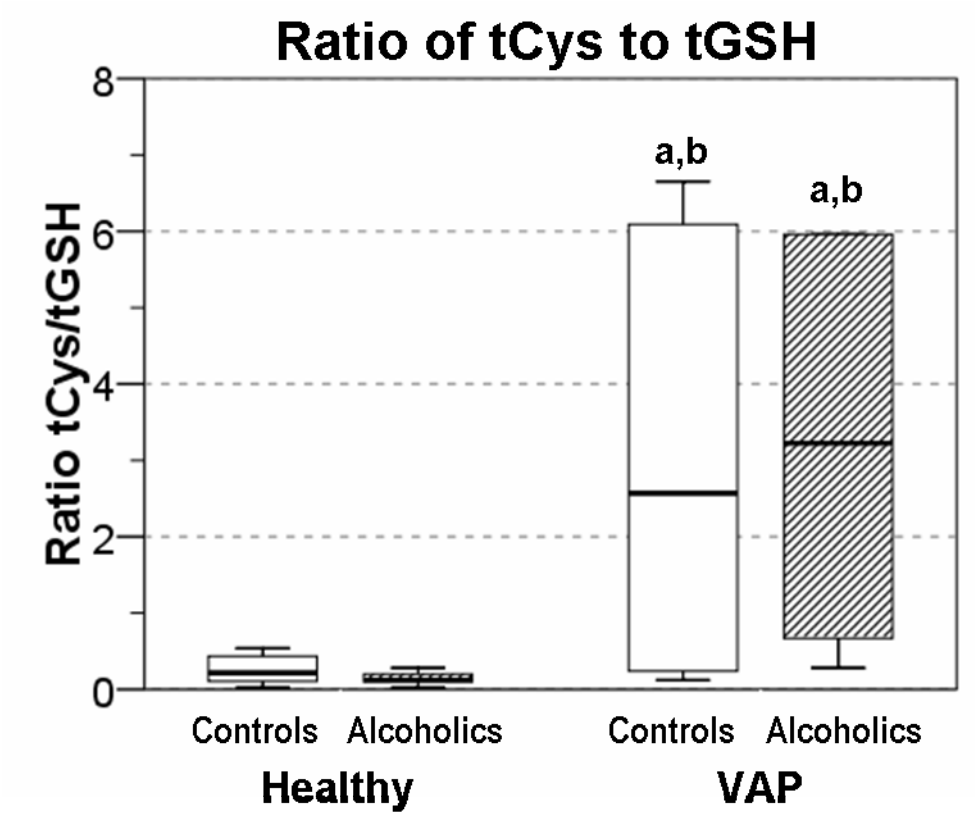
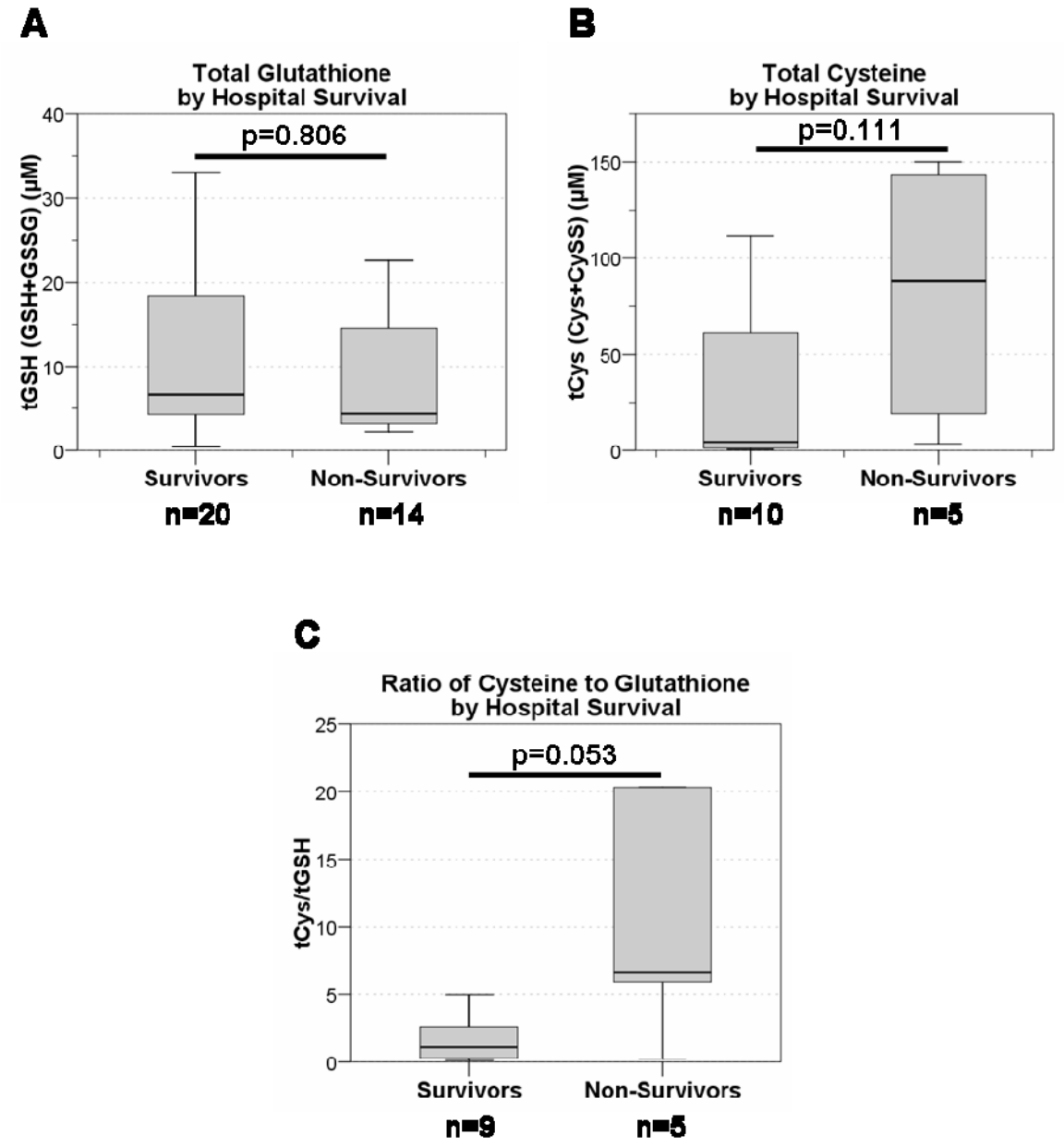


Figure 4-7. Hospital Survival and Thiol Status: tGSH (A), tCys (B), and tCys/tGSH (C). Thiol concentrations for all VAP patients, regardless of alcohol abuse history, were compared for patients who survived their hospital stay vs. those who did not.

Figure 4-7. Hospital Survival and Thiol Status: tGSH (A), tCys (B), and tCys/tGSH (C).



p=0.806). The Cys pool (**Figure 4-7B**) was smaller in survivors, but not significantly (12.3 μM [1.2, 60.9] vs. 88.3 μM [19.0, 143.6], p=0.111). The ranges for the tCys/tGSH ratio (**Figure 4-7C**) were tighter than those for tCys (1.1 [0.3, 2.6] vs. 6.7 [6.0, 20.3]). As a result, the p-value for the difference between the survivors and non-survivors was 0.053.

4.4 DISCUSSION

Ventilator-associated pneumonia (VAP) is a common pulmonary infection in the ICU with a significant impact on mortality in affected patients (9). A hyper-inflammatory response to surgery, trauma, or other severe illness requiring special care in the ICU increases the release of pro-inflammatory cytokines and activation of neutrophils and macrophages (91). Exposure to infectious agents triggers an immune response, which also entails neutrophil and macrophage activation, as well as feedback into the inflammatory cascade. All of this activity increases the generation of reactive oxidant species. Reactive oxygen species (ROS) play an essential role in immunity, but no longer limit their attack on the foreign invaders if they overwhelm the body's antioxidant defenses. The consequences of this imbalance between ROS and antioxidants are tissue injury and a decreased ability to prevent infection or recover from it. While studies into the possible association between oxidative stress and VAP have been limited, an increase in oxidative stress (as measured by a TBARS assay) and a decrease in the antioxidant activity of glutathione peroxidase in the lungs and circulation of VAP patients has been reported (72).

Patients with a history of alcohol abuse are at an increased risk for developing VAP (12). Previously, we found that healthy subjects with a history of alcohol abuse have depleted pulmonary GSH pools and that the GSH redox potential (E_h) is more oxidized when compared to subjects with no history of alcohol abuse (41). In the current study, we compared oxidative stress markers and the thiol status of healthy subjects with a history of alcohol abuse with VAP patients, with or without a history of alcohol abuse.

The previous study by Deflo et al suggested an association between oxidative stress and VAP (72). We also found an increase in the oxidative stress markers H_2O_2 and HNE in the VAP patients. In addition, the BALF from VAP patients had a significant decrease in GSH and tGSH when compared to healthy controls. Although there was no significant increase in the GSSG pool, the decreases in GSH resulted in a significant increase in the percentage of tGSH present in the oxidized moiety (%GSSG). These results indicate a depletion of the primary antioxidant in the epithelial lining fluid. In contrast, both moieties of cysteine (Cys and CySS) were increased by more than ten-fold compared to healthy controls in the VAP patients. Whereas GSH is considered to be the primary antioxidant in the epithelial lining fluid, greatly overshadowing Cys, we found that the depletion of GSH and increase in Cys in the VAP patients resulted in a Cys pool that was in fact larger than the GSH pool.

The above findings can be readily explained by the activation of immune cells and by impairment of the alveolar-epithelial barrier, although increased γ-glutamyl transpeptidase (GGT) and NAD(P)H-oxidase (NOX) activities are also possible

contributors. In response to bacteria in the airspace, immune cells are activated, which results in a release of ROS and pro-inflammatory cytokines. GSH serves to neutralize H_2O_2 and other peroxides as a cofactor for glutathione peroxidase, as well as to reduce the oxidized forms of the antioxidants Vitamin C, Vitamin E, and lipoic acid. During these reactions, GSH becomes oxidized and forms the glutathione disulfide (GSSG). An excess of oxidants would result in a shift towards a more oxidized GSH pool (higher %GSSG). GSH also forms mixed disulfides with free Cys and Cys residues in proteins, which would decrease the amount of free GSH.

Impaired function of the alveolar-capillary barrier could also explain our findings. The filling of the alveoli with fluid is characteristic of pneumonia, but the stretching associated with mechanical ventilation may also damage cells and cause leak across the normally tight alveolar-capillary barrier. A compromised barrier permits pro-inflammatory cells to enter the airspace, which would go on to contribute to the oxidant load. The composition of the fluid is also important. The infiltrate originates in the plasma, where Cys and CySS are the primary thiols and GSH and GSSG are present at much lower concentrations than in the epithelial lining fluid. The introduction of this fluid would therefore dilute the GSH in the epithelial lining fluid and cause a rise in Cys concentrations, although this dilution effect would not explain the increase in %GSSG that is unaffected by dilution.

The enzyme γ-glutamyl transpeptidase (GGT) initiates the release of cysteine from glutathione and has been used as an indicator of oxidative stress. GGT also functions to

increase intracellular GSH synthesis by the transport of cystine across cell membranes (83). The inflammatory cytokine IL-1 has been shown to increase levels of GGT in lung tissue (84). Increased GGT activity could explain at least a portion of the alteration in thiol concentrations. Mechanical ventilation alone increased plasma GGT, with a further increase when mechanical ventilation was accompanied by endotoxin administration, in a mouse model of multiple organ dysfunction syndrome (MODS) (88).

There is another possible source for increased ROS in mechanically ventilated patients. NAD(P)H-oxidase (NOX) generates superoxide and is a principal source of ROS from activated immune cells. But, other isoforms of the protein exist in other cells. One study has demonstrated that cyclic strain of endothelial cells appears to increase the activity of an endothelial NAD(P)H-oxidase (NOX) (60).

Similar to our previously published studies (41), we found that healthy alcoholics had a greatly depleted BALF GSH pool, with a significant increase in GSH oxidation when compared to healthy controls. When we compared the otherwise healthy alcoholics to the VAP patients with or without a history of alcohol abuse, there was no significant difference in GSH concentrations or %GSSG. This suggested that the oxidative stress as measured by oxidation of GSH in the BALF of otherwise healthy alcoholics was equivalent to that observed in patients with VAP. The fact that the effects of alcohol abuse and VAP were not additive with respect to GSH concentrations and %GSSG is not that surprising given the 80% decrease in the GSH pool with either exposure alone. It is possible that further decreases are not compatible with life.

In contrast to the comparable GSH status in healthy alcoholics and VAP patients, H_2O_2, HNE, Cys, and CySS concentrations were not similar in the healthy alcoholics and VAP patients. Rather, these measures were higher in both VAP groups compared to either healthy group. Therefore, whereas GSH status may reflect oxidant stress in the epithelial lining fluid, the increased Cys and CySS concentrations may be better indicators of tissue injury.

In order to evaluate whether Cys status correlated with severity of VAP, we compared the thiol status of VAP patients who survived their hospital stay and those who did not. We found no difference between these two groups for any of the GSH measures. The Cys concentration appeared to be greater in non-survivors, but the concentration ranges were too large for the difference to be statistically significant. Calculating the ratio of tCys/tGSH provided much tighter ranges for both groups, with a lower ratio for the survivors. The difference was still not significant ($p=0.053$), but these results in spite of the small sample sizes, indicate the possibility of using this ratio as a prognostic indicator. More studies, with larger sample sizes, will need to be conducted to validate its use.

In summary, otherwise healthy subjects with a history of alcohol abuse expressed similar decreases in GSH concentration and oxidation of GSH. This suggested that the oxidative stress in the alveolar space of otherwise healthy alcoholic subjects was equivalent to that observed in VAP patients in the ICU. This oxidative stress at baseline may provide a mechanism by which a history of alcohol abuse increases the risk of VAP and ARDS.

Cys and CySS concentrations were raised in VAP patients compared to either healthy group, which suggested that increased Cys and CySS may be markers of tissue injury. Finally, further research should be pursued into the value of a high ratio of tCys/tGSH in the BALF as a prognostic indicator in VAP patients.

CHAPTER 5: DIVERGENT EFFECTS OF ALCOHOL AND SMOKING ON THIOLS IN EXHALED BREATH CONDENSATE

5.1 BACKGROUND

Although smoking and chronic alcohol abuse are both oxidative conditions, they are each associated with an increased risk for different pulmonary disorders. Cigarette smoking increases the risk for the development of lung cancer, chronic obstructive pulmonary disease (COPD), emphysema, and interstitial lung diseases (89, 92, 93); whereas chronic alcohol abuse is a major risk factor for acute respiratory distress syndrome (ARDS) and pulmonary infections (8, 77).

The prevalence of smoking in the United States has decreased, but remains high. According to the second edition of The Tobacco Atlas, published by the American Cancer Society in 2006, the prevalence of smoking among adults in the United States was 21.6% (18). A significant proportion of the population of the United States abuses alcohol or is alcohol-dependent. According to the 2001-2002 National Epidemiologic Survey on Alcohol and Related Conditions (NESARC), the twelve-month prevalence of alcohol abuse for the adult population was estimated at 4.7% (a population of nearly ten million) and of alcohol dependence was 3.8% (13). A necessary consideration when conducting clinical or epidemiological research on alcohol abuse or cigarette smoking is the substantial overlap between these two behaviors. Previous studies have found that 80 to 95 percent of alcoholics smoke cigarettes and 70 percent smoke more than one pack a day (19-21).

Alcohol-induced oxidative stress appears to play a key role in increasing susceptibility of alcoholic patients to pulmonary dysfunction. In previous clinical studies, we found that a history of alcohol abuse in otherwise healthy individuals was linked to a significant decrease in the important antioxidant glutathione (GSH) in the bronchoalveolar lavage (41). There was a corresponding increase in the percentage of the total glutathione pool present as the oxidized moiety (%GSSG). These effects were independent of smoking history. Investigations into levels of pulmonary GSH in smokers by other groups have provided variable results, with some studies showing an increase (34, 41) and others a significant decrease (42) in epithelial lining fluid GSH associated with smoking. These differences may be due partly to the different populations studied in terms of age and smoking history.

The bronchoalveolar lavage (BAL) procedure is quite invasive, requiring sedation of the subject. Complications such as bronchial collapse are not common, but are potential risks for patients undergoing BAL. Other effects, such as excess fluid in the airspace, are relatively common, but normally resolve on their own. As a result, the suitability of BAL for serial monitoring of the epithelial lining fluid is limited. The use of exhaled breath condensate (EBC) as a proxy for the epithelial lining fluid of the lung has been proposed. This procedure is non-invasive, relatively inexpensive, and simple to perform. Various markers of oxidative stress have been measured in EBC, including isoprostane, H_2O_2, and myeloperoxidase (94). Brown et al demonstrated that the GSSG concentration and the %GSSG were significantly increased in the EBC of otherwise healthy subjects with a

history of alcohol abuse (hereafter referred to as "alcoholics") (95). This alcohol-induced oxidative stress was further verified by increased hydrogen peroxide in the EBC of otherwise healthy alcoholics. The study did not separate subjects on the basis of smoking status and did not assess the status of another important thiol pair.

Although GSH is a very important thiol antioxidant in the lung, it is not the only one. Cysteine (Cys) is not only an amino acid component of GSH, but it also has antioxidant properties in its own right. In plasma, the cysteine/cystine (Cys/CySS) thiol couple is present at much higher concentrations than GSH/GSSG. The extracellular redox state of Cys/CySS is important for cell homeostasis and alters cell signaling involved in proliferation and apoptosis (75). In addition, Cys binds to acetaldehyde, an oxidant that is present in cigarette smoke and a metabolite of ethanol, to form the non-toxic 2-methylthiazolidine-4-carboxylic acid (47). However, the impact of pulmonary oxidant stress on this critical thiol pair has received little attention. Given the significant oxidation of the GSH/GSSG pair in the lavage fluid and the EBC of otherwise healthy alcoholics, we analyzed the impact on the Cys/CySS redox pair in the EBC of alcoholic subjects with or without a smoking history. We also assessed the association between the two thiol pools and the impact of smoking status on the GSH/GSSG thiol pair.

5.2 METHODS

5.2.1 Subjects

The EBC was collected from 17 otherwise healthy alcoholic subjects whose positive alcohol abuse status was determined by a score greater than 3 on the Short Michigan Alcohol Screening Test (SMAST) questionnaire (69) or a score of greater than 8 on the AUDIT test (70). The otherwise healthy alcoholics had consumed alcohol within the previous 7 days, but were excluded if they were intoxicated at the time of sample collection. Smokers had smoked within the previous 24 hours. None of the subjects were positive for the exclusion criteria previously established, including a prior history of cardiac disease, liver dysfunction, kidney disease, diabetes mellitus, lung disease, human immunodeficiency virus infection, gastrointestinal bleeding, pregnancy or concomitant illicit drug use (90). Subjects who had recently taken acetaminophen were also excluded. For comparison, 15 healthy subjects without a history of alcohol abuse, verified by a SMAST score of zero, with similar age and smoking history as the alcohol abusers were recruited. All recruitment, processing, and analysis were in accordance with requirements set by the Investigation Review Board of Emory University.

5.2.2 Sample Collection

The EBC was collected from subjects who breathed tidally for 10 minutes using an R-tube (Respiratory Research, Charlottesville, VA). All parts of the R-tube including a clear polypropylene body, mouthpiece, saliva trap, and tube caps were sterile. The polypropylene tube was kept cooled with an outer aluminum sleeve chilled at -70 ^{0}C

during the collection period so that breath condensed on the inner wall of the R-tube. Subjects did not wear a noseclip during collection. To minimize contamination of EBC samples with saliva, subjects were instructed to swallow any fluid that accumulated in their mouths. The one-way valve trap on the R-tube also served as a barrier against salivary contamination.

5.2.3 HPLC Measurement of Thiols

Immediately after collection, a 300 μl EBC aliquot was treated with a preservation solution that contained perchloric acid (5% final), iodoacetic acid (13.4 mM final), boric acid (0.1M final), and an internal standard γ-Glu-Glu (5 nM final). Preserved samples were stored at -70°C until analysis. The acidified samples were thawed on ice and then centrifuged for 5 minutes (13,000Xg at 4°C) to remove precipitated protein. Samples were derivatized with dansyl chloride as described previously (95). The dansylated derivatives were separated on a 10 μm Ultrasil amino column (Waters Corp., Milford, MA) by HPLC. The mobile solvent was 80% HPLC-grade methanol in water and the salt solvent was 0.8 M sodium acetate in acetic acid and methanol. An autosampler module (Waters 2690) was used to inject 100 μl of the aqueous layer of the derivatized sample. The concentrations of the respective derivatives were calculated in reference to the area of the internal standard.

5.2.4 Statistical Analysis

Group medians were compared using the non-parametric Mann-Whitney U test. Data are presented as median [25%, 75%].

5.3 RESULTS

5.3.1 Subject demographics

General demographics data for the subjects in the study are presented in **Table 5-1**. Although there appeared to be a large difference in the proportion of males in the nonsmoking alcoholics (40%) vs. smoking alcoholics (100%), this difference was not statistically significant. We also found no significant difference in age between any of the groups.

5.3.2 Alcohol Abuse was Associated with a Decrease in GSH and an Increase in GSSG

There was no difference in GSH concentrations between the four groups (**Figure 5-1A**), but when subjects were grouped solely by alcohol history (all controls vs. all alcohol abusers), the GSH concentrations for the alcoholics (5.7 nM [3.1, 13.7]) was significantly lower than for controls (10.9 nM [8.9, 14.2]). GSSG concentrations (**Figure 5-1B**) were significantly higher for both alcoholic groups (nonsmokers: 4.5 nM [1.9, 6.6] and smokers: 5.7 nM [0.4, 10.2]) when compared to the nonsmoking controls (0.2 nM [0.2, 0.5]).

5.3.3 Alcohol Abuse was Associated with an Oxidation of GSSG

The GSH pool was significantly more oxidized (expressed as %GSSG, **Figure 5-2**) in the alcoholic groups (nonsmokers: 36.7% [21.1, 42.6] and smokers: 30.3% [7.2, 61.1])

Table 5-1: Subject Demographics.

	Nonsmoking Control Subjects	Smoking Control Subjects	Alcoholic Nonsmokers	Alcoholic Smokers
Number	9	6	5	12
Male, n (%)	5 (56)	4 (67)	2 (40)	12 (100)
Age, yr (SD)	43 (7)	44 (6)	44 (9)	46 (6)

compared to their respective controls (nonsmokers: 2.6% [1.6, 4.8] and smokers: 3.7% [3.1, 4.4]).

5.3.4 Alcohol Abuse was Associated with an Increase in Cys and Smoking with a Decrease in Cys

When compared to controls, the Cys concentration in the EBC of otherwise healthy alcoholics was increased 10-fold, with controls at 7.3 nM [4.5, 15.6] vs 70.2 nM [18.7, 110.5] for alcoholics (**Figure 5-3A**). This increase in the Cys concentration in subjects that chronically abused alcohol was significant regardless of their smoking history. In contrast to alcohol abuse, a positive history for smoking was associated with an approximately 50% decrease in the Cys concentration. Within the control groups, the Cys concentration was 13.3 nM [11.1, 16.9] for nonsmokers and 5.6 nM [4.4, 6.8] for smokers. For subjects who chronically abused alcohol, the Cys concentrations were 110.5 nM [109.5, 145.5] for nonsmokers and 27.6 nM [11.8, 76.4] for smokers.

5.3.5 Alcohol Abuse was Associated with an Increase in CySS

For the oxidized thiol, the concentration of CySS was increased approximately 9-fold in all alcoholic subjects, with 0.9 nM [0.6, 1.2] for controls vs. 7.7 nM [3.7, 51.9] for otherwise healthy alcoholics (**Figure 5-3B**). When compared to the control groups, this increase in CySS in the alcoholic subjects was independent of smoking status. However, CySS was generally lower in smokers, although the difference was not statistically significant. As for Cys and CySS, the total Cys pool (tCys) was increased 10-fold in alcoholic subjects compared to control subjects. Although smokers trended towards a

Figure 5-1. Concentrations of GSH (A) and GSSG (B). The EBC was collected from controls (with and without a smoking history) and otherwise healthy alcoholics (with and without a smoking history). Using γ-glutamyl glutamate as an internal standard, the GSH and GSSG concentrations in the EBC were determined by HPLC. Data were expressed as boxplots. [a] $p<0.05$ compared to nonsmoking controls.

Figure 5-1. Concentrations of GSH (A) and GSSG (B).

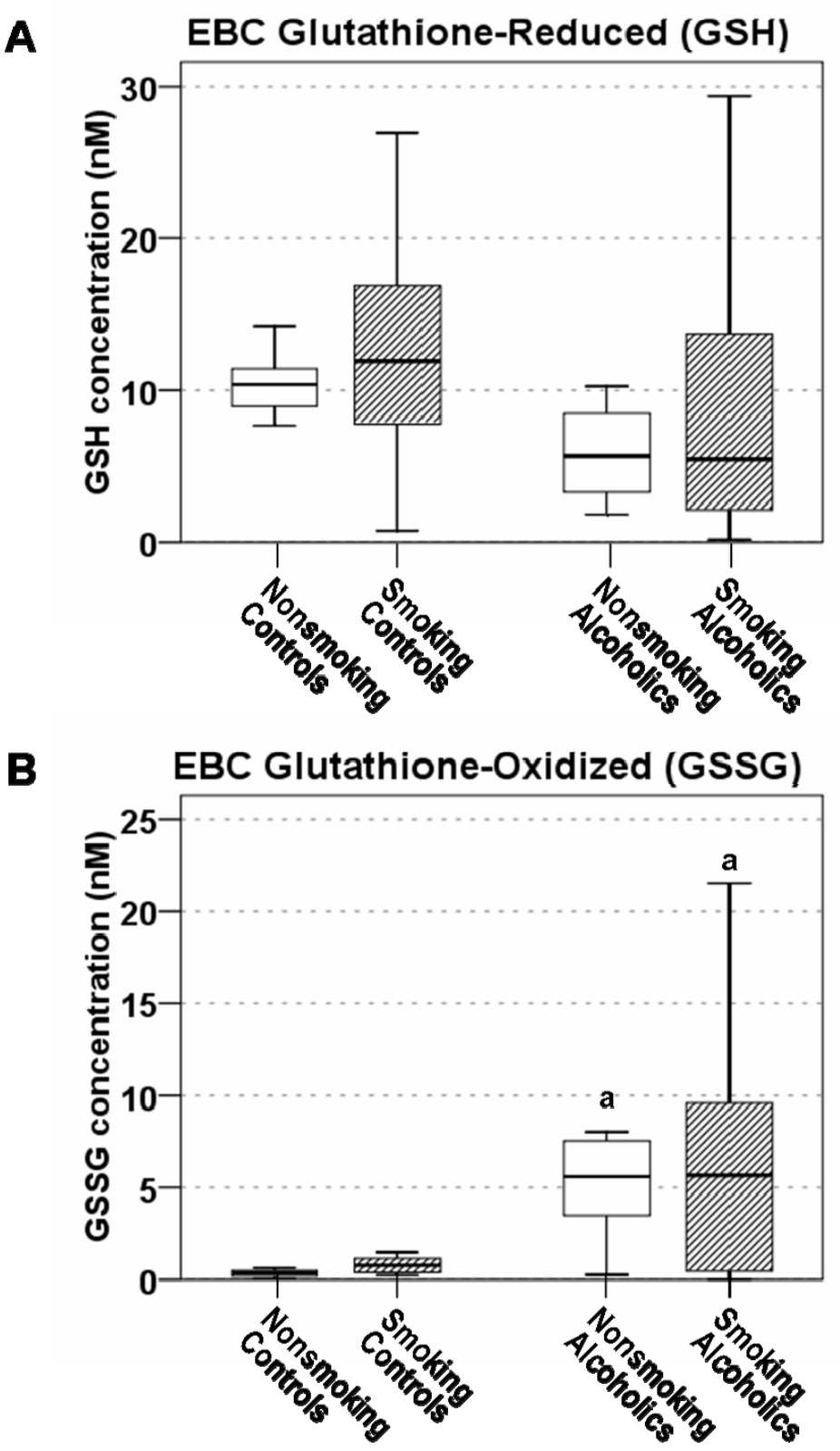
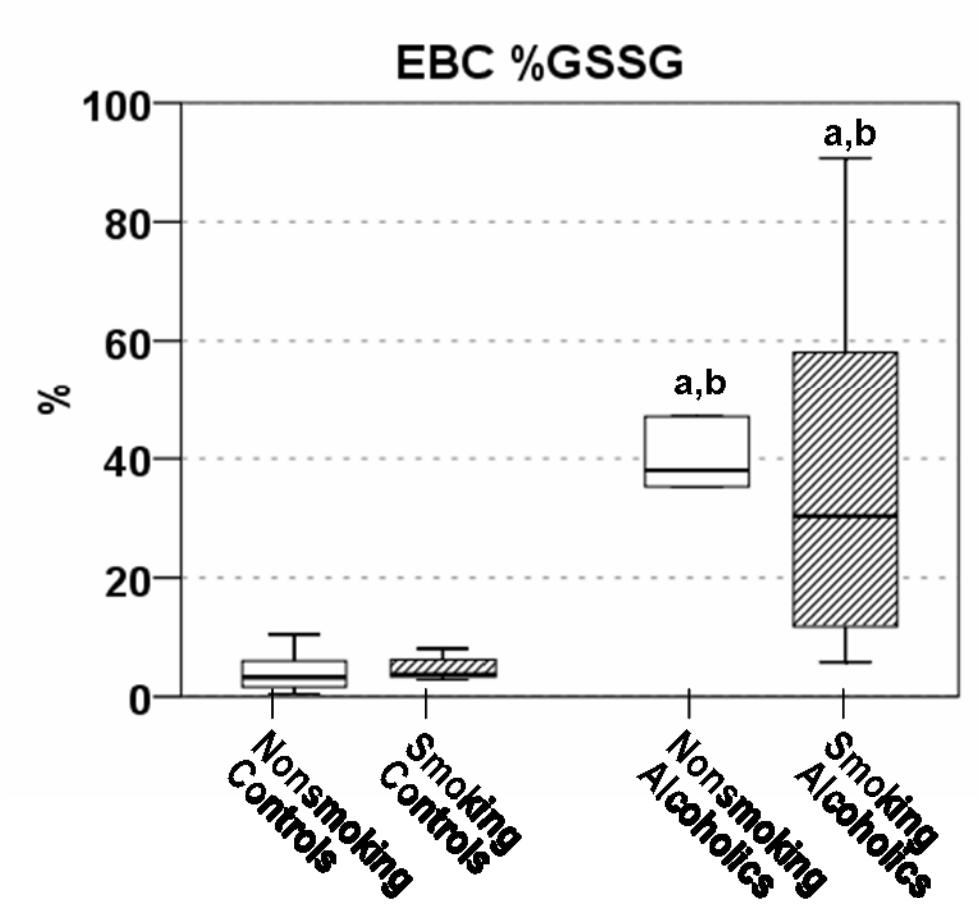


Figure 5-2. Glutathione Oxidation. The percentage of the total glutathione present as the oxidized moiety was determined for the EBC of chronic alcoholics and controls (%GSSG = [GSSG]/([GSH]+[GSSG]) x 100%). [a] $p<0.05$ compared to nonsmoking controls. [b] $p<0.05$ compared to smoking controls.

Figure 5-2. Glutathione Oxidation.



smaller tCys pool than nonsmokers, the difference was not significant for either control subjects or alcoholic subjects.

5.3.6 Cys was More Oxidized in Alcoholics and Smokers

Given the concern of dilution by the breath condensate, we first calculated the simple ratio of the oxidized moiety (CySS) to tCys, referred to as % CySS. For the control groups, the % CySS was increased approximately 2-fold for subjects that had a smoking history (**Figure 5-4**). When nonsmoking controls were compared to nonsmoking alcoholics, there was a 4-fold increase in the % CySS. For the smoking alcoholic subjects, there was a large spread of values for % CySS that was not statistically different from any group.

5.3.7 The Ratio of Total Cysteine to Total Glutathione was Increased with Alcohol Abuse, but Decreased with Smoking

Glutathione and cysteine are not completely independent of each other. Cysteine is a component of glutathione, whereas glutathione acts as an extracellular store for cysteine. Therefore we assessed the ratio of these thiols in the EBC. The ratio of tCys to tGSH in the EBC of nonsmoking controls was 1.1 [0.9, 2.1] (**Figure 5-5**). This ratio was significantly lower for smoking controls (0.4 [0.3, 0.6]). The ratio was significantly higher in the alcohol groups with respect to their respective controls (nonsmokers: 15.1 [12.9, 20.0] and smokers: 2.8 [1.0, 6.8]).

Figure 5-3. Concentrations of Cys (A) and CySS (B). The Cys and CySS concentrations in the EBC were determined by HPLC. [a] $p<0.05$ compared to nonsmoking controls. [b] $p<0.05$ compared to smoking controls. [c] $p<0.05$ compared to nonsmoking alcoholics.

Figure 5-3. Concentrations of Cys (A) and CySS (B).

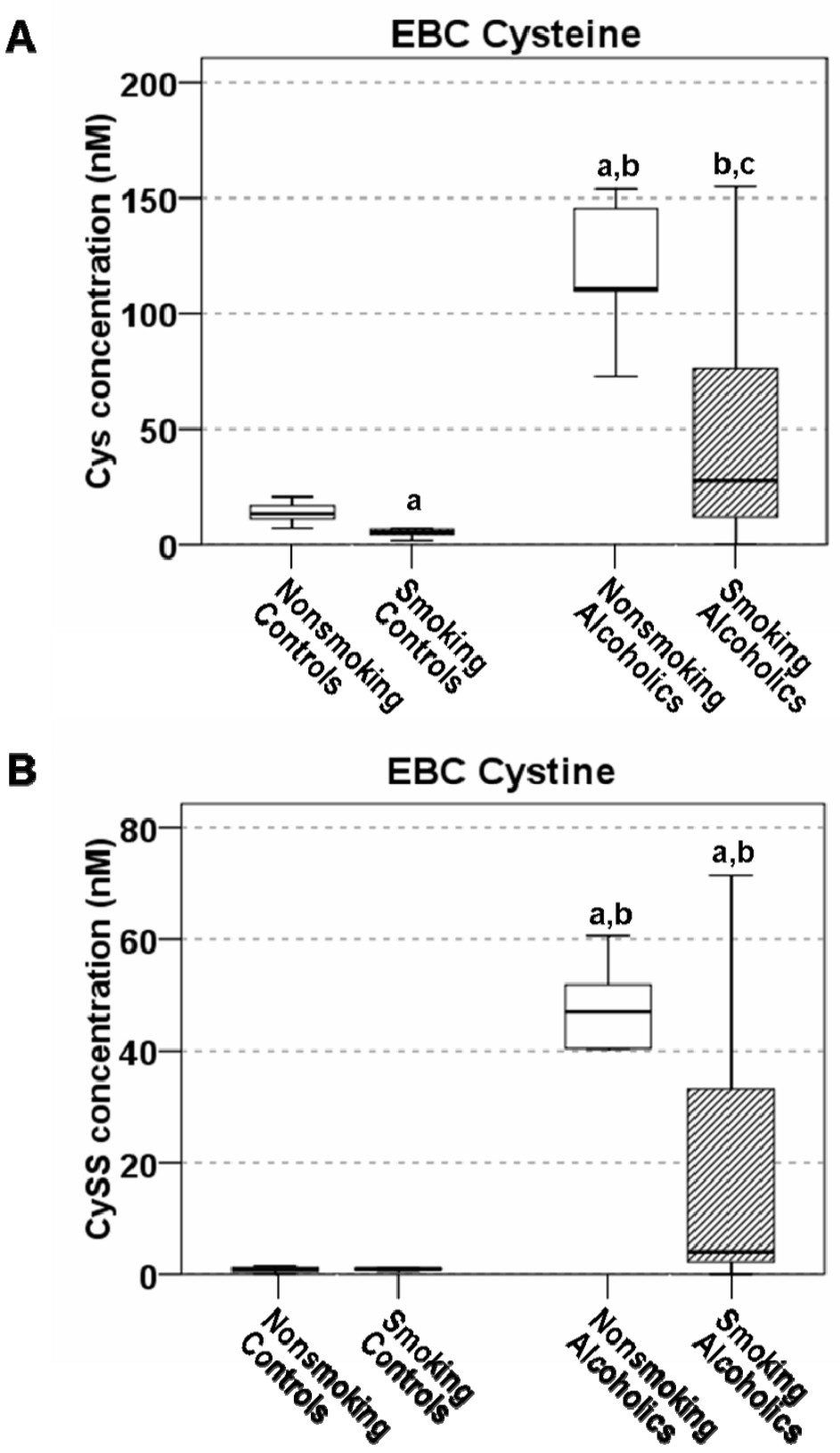


Figure 5-4. Cysteine Oxidation. The percentage of the total cysteine present as the oxidized moiety was determined for the EBC of chronic alcoholics and controls (%CySS = [CySS]/([Cys]+[CySS]) x 100%). [a] $p<0.05$ compared to nonsmoking controls.

Figure 5-4. Cysteine Oxidation.

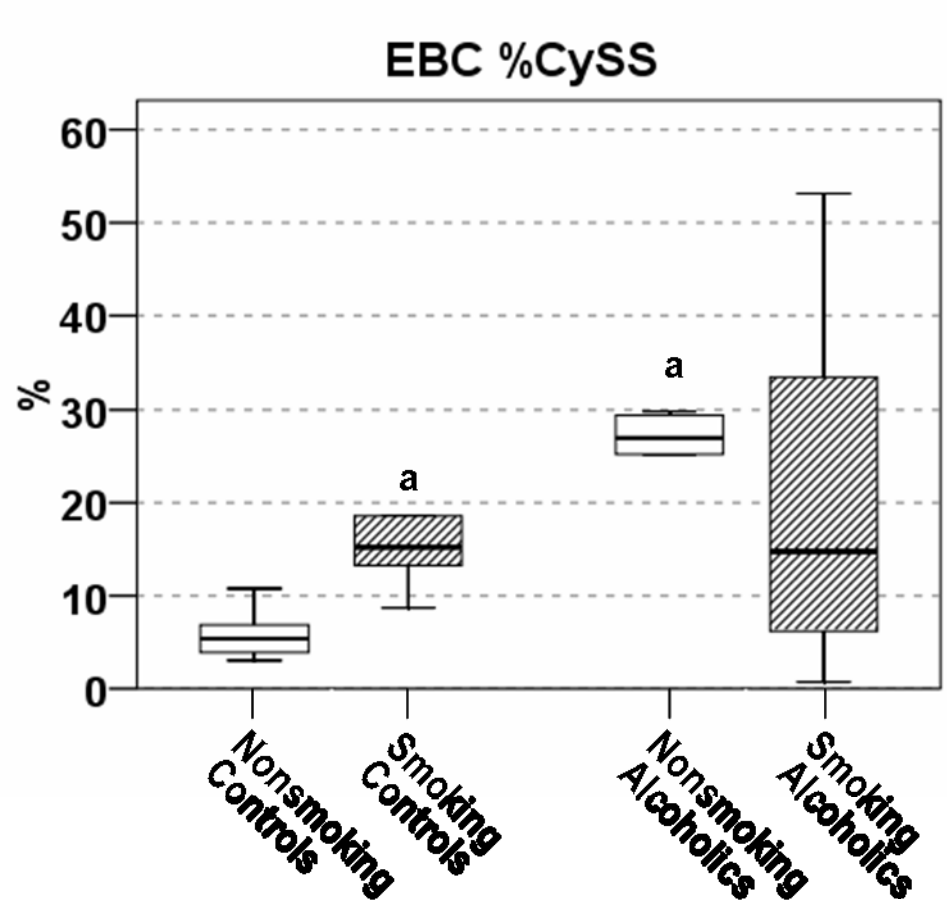
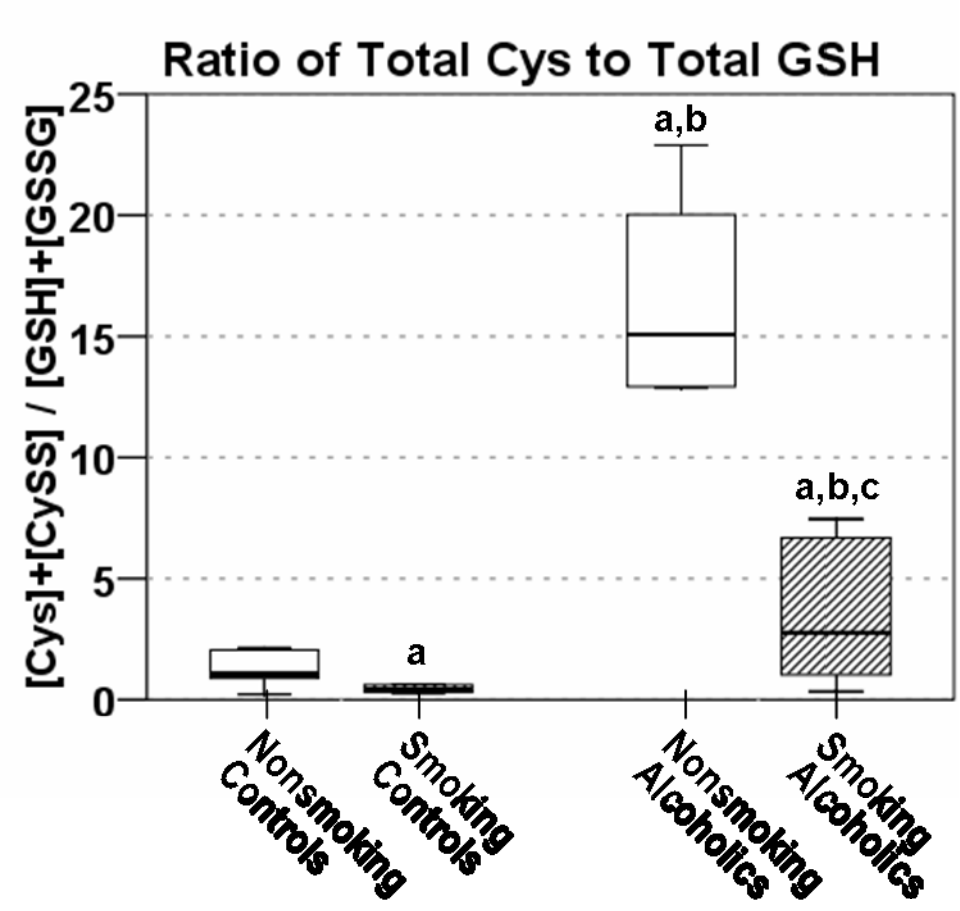


Figure 5-5. Ratio of Total Cysteine to Total Glutathione Concentrations. The ratio of tCys to tGSH was calculated. [a] $p<0.05$ compared to nonsmoking controls. [b] $p<0.05$ compared to smoking controls. [c] $p<0.05$ compared to nonsmoking alcoholics.

Figure 5-5. Ratio of Total Cysteine to Total Glutathione Concentrations.



5.4 DISCUSSION

Although smoking and alcohol both generate oxidative stress, they are each associated with an increased risk for different pulmonary disorders. Cigarette smoking increases the risk for the development of lung cancer, chronic obstructive pulmonary disease (COPD), emphysema, and interstitial lung diseases (89, 92, 93); whereas chronic alcohol abuse is a major risk factor for acute respiratory distress syndrome (ARDS) and pulmonary infections (8, 77). This suggests that different mechanisms may be associated with the response to these exposures.

A history of alcohol abuse in otherwise healthy individuals has been linked to an 80% decrease in GSH in the alveolar epithelial lining fluid, with a corresponding 5-fold increase in the %GSSG and oxidation of the GSH redox potential by 50 mV (41). In plasma, alcohol abuse was not associated with significant changes in the GSH and GSSG concentrations, but the GSH redox potential was more oxidized, although only among smokers. This suggested that plasma was not an appropriate sampling site to monitor the pulmonary oxidative stress associated with a history of alcohol abuse. However, our group observed that alterations in the GSH/GSSG couple in the EBC of otherwise healthy alcoholics were similar to those observed in the lavage fluid (41, 95). In the EBC, a history of alcohol abuse was associated with significant decreases in GSH and increases in GSSG and the percentage of the total pool present in the oxidized moiety (%GSSG) (95). This shift in the GSH/GSSG redox potential of the EBC to a more oxidized state was associated with increased hydrogen peroxide. Thus, these studies suggested that a history of alcohol abuse was associated with oxidative stress of the GSH/GSSG redox

pair throughout the respiratory tree and the GSH/GSSG redox potential of the EBC was representative of that in the alveolar space.

Given this shift towards a more oxidized state for the GSH/GSSG thiol pair in the EBC of otherwise healthy alcoholics, we expected similar results for the other thiol pair Cys/CySS. What we observed is that a history of alcohol abuse was associated with a significant increase in the concentration of both Cys and CySS in the EBC, regardless of smoking history. When the %CySS was used as an indicator of oxidative stress, the significant increase in the nonsmoking subjects suggested that alcohol abuse was associated with oxidative stress.

Our previous study of glutathione status in the EBC of chronic alcohol abusers did not separate subjects based on smoking status. In another study however, our group found that smoking was associated with a five-fold increase in GSSG and a significant increase in %GSSG in the epithelial lining fluid (41). In the study presented here, we found no significant association between smoking status and any of our glutathione measures. In contrast, subjects that smoked presented with a significant decrease in the Cys pool compared to nonsmokers, regardless of alcohol abuse history. Although the CySS pool in smoking alcoholics was lower than for nonsmoking alcoholics, this difference was not significant ($p=0.09$). The CySS pool in nonsmoking controls was very low and was not significantly different from smoking controls. As a consequence of the smoking-associated decreases in Cys and CySS, the tCys pool was lower in smoking subjects, for both control ($p=0.01$) and alcoholic ($p=0.06$) subjects. Although the CySS concentration

in the EBC of controls was not statistically altered by smoking, the decreases in Cys resulted in a greater percentage of the total pool present as the oxidized moiety CySS. There was no statistical difference in cysteine oxidation, expressed as % CySS, associated with smoking among alcoholic subjects.

There is growing evidence that the redox potentials of GSH and Cys are independently regulated (80, 96), but concentrations of these thiols are inherently linked. Cysteine is one of the three amino acids that compose glutathione. One of the functions of glutathione is to provide a source of cysteine for protein synthesis. Therefore, we assessed the ratio of the concentrations of the two thiol pools (tCys/tGSH). We found that this ratio for nonsmoking controls was 1.1 [0.9, 2.1], much greater than the corresponding ratio in the epithelial lining fluid (approximately 0.1), where GSH is by far the predominant thiol in healthy adults (81, 82). This increase in the ratio may be due to the different volatilities and/or solubilities of the compounds in the microdroplets of BAL fluid captured and present in the EBC, but this issue is outside the scope of the present study. Studies to address the association between thiol measures in BAL fluid and EBC are being designed.

In the EBC, we found that a history of alcohol abuse was associated with a significantly higher tCys/tGSH ratio, while a positive smoking history was associated with a significantly lower ratio. Since tGSH was similar in the four groups we investigated, the ratios were dependent on tCys. Increased tCys, such as what we saw with alcohol abuse, may be due to impaired function of the alveolar-capillary barrier. Cysteine is the predominant low molecular weight thiol in plasma. Leak into the airspace would

therefore result in a relative increase in cysteine concentrations in the epithelial lining fluid. Another possible mechanism for increased tCys is increased activity of γ-glutamyl transpeptidase (GGT), an enzyme that initiates the process of liberating cysteine from glutathione. Chronic alcohol abuse has been shown to be associated with both increased alveolar-capillary barrier permeability (97) and increased GGT (98, 99). Decreased tCys may be attributed to increased cysteine utilization. Plasma tCys has been found to be lower in smokers (43), in spite of increased serum GGT activity (100).

One factor that may play an important role is the route of entry of the offending oxidants. Since cigarette smoke is inhaled, the first line of defense in the lungs is the epithelial lining, with the cilia of the upper airways and the antioxidant-containing epithelial lining fluid. Alcohol, on the other hand, is transported by the circulation. It therefore must pass through the capillary endothelium, the interstitium, and the epithelium before encountering the epithelial lining fluid.

A limitation regarding subject demographics is the large difference in the proportion of males in the two alcoholic groups. Whereas the nonsmoking group is 40% male, the smoking group is 100% male. Due to the small number in the nonsmoking group, the difference in proportions was not found to be significant, but the possible impact should be addressed. The only statistically significant difference between these two groups was in the Cys concentration, where the smoking alcoholics had significantly lower concentrations than nonsmoking alcoholics. A smoking-associated decrease was also found in the two control groups, which had comparable proportions of males. This

finding supports that the smoking-associated differences are not actually due to the possible influence of gender on Cys. Although the effect of smoking on plasma CySS and tCys has been found to depend on gender, nothing is known about the effect of gender on thiols in the lung. In the present study, we had insufficient numbers to further stratify by gender.

In summary, these studies suggested that in contrast to the alveolar lining fluid, the concentrations of GSH and Cys were approximately equal in the EBC of nonsmoking controls. A history of alcohol abuse was associated with increased GSSG and %GSSG, which suggested alcohol-induced oxidative stress. In addition, a history of alcohol abuse was associated with increased Cys and CySS concentrations and an increase in oxidation of the Cys/CySS thiol pair as indicated by an increased %CySS. In contrast to alcohol abuse, smoking status was not associated with any alteration of GSH/GSSG status. A smoking-associated oxidation of Cys/CySS (increased %CySS) among subjects with no history of alcohol abuse accompanied a decrease in Cys concentrations. These observations suggest that GSH may be a better discriminator of alcohol-induced oxidative stress, but that Cys may be more sensitive to smoking-induced oxidative stress. If this observation is validated in larger studies, it will indicate that Cys may be the best thiol for monitoring smoking-induced oxidative stress and conditions associated with smoking (such as COPD), while GSH may be preferred for alcohol-induced oxidative stress.

CHAPTER 6: DISCUSSION

6.1 ALTERED THIOL STATUS IN CRITICALLY ILL PATIENTS

The BALF of critically ill patients was significantly oxidized compared to healthy controls, as evidenced by increased H_2O_2 and HNE concentrations, as well as increased %GSSG. These results agreed with previous studies in ARDS patients (35, 36). The status of Cys in critically ill patients had not been previously studied. In these patients, we found increased concentrations of Cys and CySS, without a significant change in %CySS. Plasma concentrations of MDA were dramatically increased in the critically ill patients compared to the healthy controls. The patients also had higher concentrations of GSSG and higher %GSSG in plasma. As in the BALF, plasma Cys and CySS were also increased in the patients, but surprisingly %CySS was significantly lower in the patients.

6.1.1 Possible Mechanisms for Alteration of Thiol Status

A smaller GSH pool was responsible for the more oxidized state of the GSH in the BALF of critically ill patients. This decline in the GSH pool may have been a result of reduced GSH synthesis, either in the liver or in the pulmonary epithelium. Another explanation is the formation of mixed disulfides with other low molecular weight thiols, such as free cysteine, or with proteins. GSH may also react with nitric oxide to form nitrosoglutathione (GSNO). These mechanisms may be combined with impaired GSH efflux from epithelial cells.

Different mechanisms appear to be involved in plasma GSH status. The reduced GSH concentration was unaltered in critically ill patients, so the increase in %GSSG was attributable to increased GSSG concentrations. The concentration of tGSH in plasma is low compared to that of Cys, making it more sensitive to the efflux of intracellular GSH, which could come from damaged cells from tissues such as the liver or the vasculature itself. Impaired barrier function could also result in GSH from the epithelial lining fluid crossing into the circulation.

Cysteine concentrations (Cys and CySS) in the BALF of critically ill patients were higher than in healthy controls. The loss of integrity of the alveolar-capillary barrier, which is a component of ARDS and VAP, and is often exacerbated by mechanical ventilation, permits fluids to leak from the circulation to the airspaces of the lung. The great difference in Cys concentrations between the circulation and the epithelial lining fluid would mean that leak into the airspace would increase epithelial lining fluid Cys. Cysteine concentrations could be considered to be primarily an indication of impaired barrier function, except that plasma Cys was also increased in critically ill patients. Another possible source for increased Cys in both the BALF and plasma is increased γ-glutamyl-transpeptidase (GGT) activity. GGT is an enzyme that initiates the liberation of cysteine from glutathione and has been used as an indicator of oxidative stress. GGT also functions to increase intracellular GSH synthesis by the transport of cystine across cell membranes (83). One study has found increased GGT activity in the BALF of ARDS patients (85). Yet another possible cause for increased plasma Cys in critically ill patients is muscle wasting. Malnutrition and inactivity, common among critically ill patients, lead

to a loss of lean muscle mass. Muscle is broken down to free amino acids for protein synthesis elsewhere.

6.1.2 Future Directions

Lower expression of enzymes involved in hepatic GSH synthesis has been found in patients with alcoholic hepatitis, as well as greater efflux of hepatic GSH with chronic alcohol (38). This approach could be used to investigate the effect of chronic alcohol on GSH synthetic enzyme activity or expression in pulmonary epithelia and macrophages. GGT activity could be assayed to determine the source of additional Cys. Impaired transport of Cys into cells may be partly responsible for increased Cys concentrations. Alterations in expression of these transporters should be investigated. Mixed disulfides in the extracellular fluids could also be analyzed to determine how GSH availability is altered by glutathionylation.

One of the drawbacks to the use of BALF to quantify the contents of the epithelial lining fluid is the variable dilution by the instillate. One proposed method to address this issue is the use of a microlavage for normalizing BALF (101). Before the BAL procedure is performed, a small volume of the epithelial lining fluid is quickly sampled to measure urea and albumin concentrations. This procedure is performed quickly and with minimal alveolar distention, two problems associated with using urea and/or albumin to evaluate dilution.

HPLC measurement of thiols is not conducive to use in a clinical setting. Preparation of samples is involved and time-consuming. Johnson et al recently published a method using LC-FTMS for the analysis of plasma thiols (102), offering an alternative to HPLC more suitable for use in a clinical setting.

In a study of bleomycin-induced lung fibrosis, Iyer et al found that plasma GSH was more oxidized during the proinflammatory phase and plasma Cys was more oxidized during the fibrotic phase (103). Similarly, a longitudinal study of thiol status in critically ill patients could provide insight into the mechanisms involved in disease progression. A concurrent analysis of clinical outcomes could determine the prognostic value of thiol status.

6.2 Comparable Oxidation of Bronchoalveolar Lavage Fluid GSH in Healthy Alcohol Abusers and VAP patients

Oxidant concentrations in the BALF and plasma of VAP patients were significantly higher than healthy subjects, with or without a history of alcohol abuse. Concentrations of Cys and CySS were also raised in the VAP patients. Whereas there was no difference in any of these measures associated with alcohol abuse. On the other hand, we found that GSH in the BALF of healthy subjects with a history of alcohol abuse was depleted to the same extent as for patients diagnosed with ventilator-associated pneumonia (VAP). BALF GSH was also comparably oxidized (%GSSG) in these groups with respect to the healthy subjects with no history of alcohol abuse.

This oxidation of the primary pulmonary antioxidant in seemingly healthy alcoholics could be responsible for their increased risk for ARDS and VAP. They may be on the tipping point, vulnerable to the next oxidative assault. A simple screening tool for alcohol abuse could help identify who might be best served by antioxidant therapy.

The ratio of tCys/tCys was higher ($p=0.053$) in VAP patients who did not survive compared to survivors. Although the difference was not significant, these results in spite of small sample sizes, indicate the possibility of using this ratio as a prognostic indicator. More studies, with larger sample sizes, will need to be conducted to validate its use.

6.3 Chronic Alcohol Abuse History Associated with Oxidized GSH but Reduced Cys in Exhaled Breath Condensate

A history of alcohol abuse was associated with an oxidation of GSH, but with more reduced Cys. Smoking was not associated with any differences in GSH status, but the Cys of smokers was significantly more oxidized. It is unclear why Cys and GSH follow such different trends. These observations suggest that GSH may be a better discriminator of alcohol-induced oxidative stress, but that Cys may be more sensitive to smoking-induced oxidative stress. If this observation is validated in larger studies, it will indicate that Cys may be the best thiol for monitoring smoking-induced oxidative stress and conditions associated with smoking (such as COPD), while GSH may be preferred for alcohol-induced oxidative stress.

We found that the ratio tCys/tGSH for nonsmoking controls was approximately 1, much greater than the corresponding ratio in the epithelial lining fluid (approximately 0.1), where GSH is by far the predominant thiol in healthy adults (81, 82). This increase in the ratio may be due to the different volatilities and/or solubilities of the compounds in the microdroplets of BAL fluid captured and present in the EBC. Studies to address the association between thiol measures in BAL fluid and EBC are being pursued.

One of the advantages of the EBC procedure over BAL is that it can be performed easily with much greater frequency. This attribute would facilitate longitudinal studies investigating disease progression or efficacy of antioxidant therapies. A modified EBC apparatus could also be incorporated into mechanical ventilators, without additional stress on critically ill patients.

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