NAPHTHALENE INDUCED TOXICITY IN THE LUNGS OF JUVENILE, ADULT, AND GERIATRIC MICE POST ERGOTHIONEINE TREATMENT

By

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ABSTRACT

The impact of naphthalene (NA), an abundant volatile polycyclic aromatic hydrocarbon commonly found in wildfire smoke, cigarette smoke, and vehicle exhaust, is both site- and species-specific. NA toxicity in the lungs is well-defined to cause a dose-dependent Club cell toxicant in the conducting airway epithelium of mice, regardless of exposure route. Endogenous antioxidant, glutathione (GSH), detoxifies NA by creating a NA-GSH conjugate. Although NA and GSH are well studied, few scientists has examined exogenous antioxidants in this process and their ability to limit NA induced toxicity in the lungs. Ergothioneine (ET), a dietary antioxidant abundant in mushrooms, has been reported to protect cells from oxidant stress as a scavenger for free radicals and function as an immunoregulator in the presence of oxidative stress in several systems. ET transporter (SLC22A4) is the main regulator for the uptake of cellular ET and is known to concentrate in areas that experience high levels of oxidative stress. The overall aim of this dissertation is to understand the impact of ET pretreatment in NA exposed lungs of young (1 month), adult (2-3 months), and elderly mice (12-18 months). To test if ET pretreatment will protect the lung from NA toxicity, the mice will be treated with an oral dosage of 70 mg/kg of ET, or saline, daily for 5 days, and on day 8 mice are injected with NA or corn oil alone. At 2 and 24 hours post NA injection, the lungs, liver, and blood will be collected and processed for further analysis measuring oxidative stress and assessing detoxification. Chapter Two in this dissertation evaluates the impact of ET pretreatment and NA toxicity between juveniles and adult mice; Chapter Three examines the temporal pattern of molecular changes at 2 and 24 hours post NA exposure; and lastly, Chapter Four explores the impact of ET and NA in geriatric mice. This is the first time geriatric mice have been studied for NA toxicity. Collectively, this dissertation aims to advance our understanding of the mechanisms of NA

toxicity, normal cellular mechanism of protection, and the role of a potential dietary antioxidant, ET, in the lung, liver, and blood, across the life span.

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LIST OF ABBREVIATIONS

NA	Naphthalene
ET	Ergothioneine
ETT	Ergothioneine Transporter
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GSR	Glutathione S Reductase
GPx	Glutathione Peroxidase
GSTpi	Glutathione S Transferase
CCSP	Club Cell Secretory Protein
ETT (SLC22A4, OCTN1)	Ergothioneine Transporter (gene)
GCL	Glutamate cysteine ligase
GCLM	Glutamate cysteine ligase modulator subunit
GCLC	Glutamate cysteine ligase catalytic subunit
CYP	Cytochrome P450 monooxygenase

CHAPTER 1 - INTRODUCTION

Air pollution continues to be a global health challenge with wildfires, incineration of industrial waste, and a growing global population that has resulted in an increased use of fossil fuels all contributing to air pollution. There is a strong link between chemical exposures in air and lung diseases, especially for worsening of symptoms for those with existing disease (Holguin 2013; Rogers and Cismowski 2018). The correlation is also becoming stronger for lung disease causation as scientists continue to work towards understanding the risks of volatile chemical exposure in the lungs as well as exposures to other air pollutants (Li et al. 2021; Thurston et al. 2020). While there is abundant evidence that a diet high in antioxidants can have broad health benefits against chronic diseases (Thimmulappa et al. 2019), less is known about the specific impact dietary antioxidants have on the lung's ability to defend against air pollution. Underlying this goal to increase dietary antioxidants is the hypothesis that increasing the level of antioxidants may provide protection against lung damage and disease (Forman and Zhang 2021), although the success of prior interventions has been mixed (Boudi et al. 2019; Cook-Mills et al. 2013). With the lung heavily exposed to airborne toxicants, and also susceptible to ingested toxicants, the potential use of dietary antioxidants to protect the lungs could reap real benefits should an effective, or even partially effective, intervention be found. Such an intervention could delay the development and severity of acute lung damage and mitigate chronic lung diseases (Forman and Zhang 2021; Gangwar et al. 2020).

ANTIOXIDANTS

Antioxidants are found in a variety of foods but may also be synthesized from precursors within the body and within cells. The many functions of antioxidants include 1) maintaining cellular function by slowing or preventing oxidation, and 2) scavenging for free radicals and

oxidants (Kurutas 2016). Antioxidants can create an antioxidant network, interacting with other antioxidants, enhancing their original function (Adwas et al. 2019a; Kurutas 2016). Antioxidants are known to play an essential role in reducing oxidative stress. When a mammal consumes food, the digestive system breaks down the nutrient components, which are then mostly absorbed by the liver and further metabolized. The remaining nutrients, including antioxidants, or antioxidant building blocks, will travel through the circulatory system, reaching the lungs and heart. Lung epithelial cells can utilize available exogenous antioxidants and antioxidant components to combat oxidative stress and return the lung to homeostasis (Romieu et al. 2002) but it is important to also realize that the most abundant antioxidant in the lung, reduced glutathione, is one that is synthesized intracellularly within the cells of the lung.

Glutathione

Glutathione (GSH) is an abundant intracellular antioxidant created from three peptides: cysteine, glycine, and glutamic acid, with gamma glutamylcysteine ligase (GCL) as the rate limiting enzyme in the synthesis process that occurs inside cells (Pizzorno 2014; Rahman and MacNee 2000a; 2000b). GSH is oxidized by glutathione peroxidase (GPx) and reduced by glutathione s reductase (GSR), the reduced form of glutathione is abbreviated as GSSG. The primary function of GSH, oxidized form, is to detoxify xenobiotics by conjugating with a reactive form, such as an epoxide, via GSH-transferase, and eliminate the toxin through neutralization and excretion (Rahman and MacNee 2000a; 2000b). GSH is abundant in Club cells located in the conducting airway epithelium (Plopper et al. 2001b). These non-ciliated secretory cells also contain phase I and II metabolic enzymes including several isoforms of cytochrome P450 monooxygenases (CYP). Other functions of Club cells include being a progenitor cell of the distal airway, regulating local inflammatory responses, and secreting

proteins that protect the lungs such as Club Cell Secretory Protein or CCSP (Laucho-Contreras et al. 2016; Martinu et al. 2023; Plopper and Gram 1993; Royce et al. 2014; Van Winkle et al. 1995). Club cells' high expression of CYPs makes them a target for xenobiotics that are metabolized to reactive intermediates in the conducting airway (Oesch et al. 2019; Plopper et al. 1992; Rokicki et al. 2016). Although GSH can be conjugated to, and help with, elimination of metabolized toxins, metabolites can also be metabolized further by CYPs and other enzymes, continuing down a pathway that can be associated with toxicity (Buckpitt et al. 2002; Li et al. 2011; Shultz et al. 2001). These two pathways, detoxification and toxicity, are in competition. The detoxification pathway dominates until cellular stores of GSH are depleted below a critical level and at a rate that exceeds resynthesis (Forman and Zhang 2021).

Ergothioneine

Ergothioneine (ET) is a water-soluble sulfur containing thiol (Cheah and Halliwell 2021; Paul and Snyder 2009). Unlike GSH, ET is not synthesized in the body, rather it is solely obtained through diet, including the consumption of fungi, e.g., mushrooms (Cheah et al. 2016b; Halliwell et al. 2018; Tang et al. 2018). The mechanism of ET is still not completely defined; however, scientists have found that ET functions as an antioxidant, scavenger for free radicals, immunoregulator, and cytoprotectant (Cheah and Halliwell 2020). A unique transporter known as organic cation transporter novel type-1 (OCTN1), encoded by the gene SLC22A4, is vital for ET to enter cells. In addition, this ET transporter (ETT) functions as a regulator for the uptake of sodium, and similarly to ET, is exclusively found in tissues undergoing oxidative stress, e.g., the brain, eyes, lungs, and liver (Halliwell et al. 2018). A key limitation in other studies of antioxidant treatment has been bioavailability at the lung target site. A recent human study compared the ET accumulation in blood and plasma to ET clearance in urine (Cheah et al.

2016b). As the administered ET dose increased so did the circulating ET in the blood of the study participants. Low excretion of ET in the urine indicates that ET is being retained by the human body. Compared to most dietary antioxidants, ET has greater bioavailability (Cheah et al. 2016b). However, similar to GSH, in aging individuals (60 years of age and above), the expression of ETT decreases, resulting in a decline in ET uptake (Cheah et al. 2016a; Cheah and Halliwell 2021; Cheah et al. 2016b; Halliwell et al. 2018). Using a highly sensitive liquid chromatography tandem-mass spectrometry (LC-MS/MS) method, scientists found that elderly individuals with mild cognitive impairment had lower levels of ET compared to healthy individuals in their blood (Cheah et al. 2016a). Furthermore, they found that ET levels in the blood decrease in healthy individuals over 60 years of age. Little is known about the effect and possible benefits of ET on the lung in general but also by age.

OXIDATIVE STRESS

Reactive oxidant species (ROS) such as free radicals, superoxides, and hydrogen peroxide, maintained at a basal level, play an essential role in intracellular homeostasis (Forman and Zhang 2021; Holguin 2013). NADPH and many other ROS-generating enzymes expressed by cells will result in oxidant induced inflammation, DNA damage, cell death, endoplasmic reticulum stress, mitochondrial dysfunction, epigenetic alterations, pro-fibrotic signaling, and the oxidation of lipids and proteins (Thimmulappa et al. 2019). When there is an over expression of ROS due to environmental pollutant exposures, there is an increase in the oxidant cellular mechanism resulting in cell death. This cellular imbalance of oxidants and antioxidant defense creates an excess amount of free radicals, oxidized chemicals, and cellular components, also known as oxidative stress (Forman and Zhang 2021). Environmental pollutants that drive oxidative stress and the creation of free radicals include ozone, particulates,

pesticides/herbicides, and vapor phase polycyclic aromatic hydrocarbons (PAHs). When the lungs are exposed to environmental pollutants, creating high levels of oxidative stress, the lung epithelia will undergo necrosis and may also stimulate inflammation (Rahman and MacNee 2000a).

Naphthalene

Naphthalene (NA) is abundant in heavily populated urban areas. This volatile aromatic chemical is found in burning biomass, vehicle exhaust, tobacco smoke, and groundwater (Buckpitt et al. 2002; Carratt et al. 2016). In addition, it has been found in over 40% of human fat and 75% of human breast milk samples (Pellizzari et al. 1982; Wheeler et al. 2014; Yost et al. 2021). Once inhaled or ingested, NA disseminates throughout the body and is rapidly metabolized by Phase I metabolism involving CYPs in the lung and liver. Long term exposure to NA results in pulmonary disease in rodent models, including airway hyperresponsiveness and cancer (National Toxicology 1992; Yildirim et al. 2008). Studies have shown that long term exposures to vapor phase PAHs, such as naphthalene (NA), can lead to the development of olfactory and respiratory epithelial nasal tumors in rodent models (M. Abdo 2001; Program 1998), and may play a role in the development of cancer in humans (Mastrangelo et al. 1996). The effects of acute and chronic exposure to NA are less defined in humans; however, it has been suggested that chronic exposure can lead to cancer and the development of cataracts (Mastrangelo et al. 1996). With global warming, increased vehicle exhaust and wildfires, humans are increasingly being exposed to NA. Exposure to NA in adult mice causes injury to the nonciliated bronchiolar epithelium, specifically to Club cells, in as little as 4 hours at a concentration as low as 2 ppm (Buckpitt et al. 2002). The Occupational Safety and Health Administration's (OSHA's) workplace exposure standard for NA is 10 ppm and, although mice

are more susceptible to NA toxicity than humans, it is crucial that we understand the mechanisms of NA toxicity in mice in order to translate our findings to humans. Studying the mechanisms by which NA drives pulmonary disease will inform risk assessment and preventive measures. Acute NA toxicity is known to be site-, cell- and species- specific, as shown in **Table 1.1** (Buckpitt et al. 2002). Key influences on this toxicity that we will test in our proposed studies are shown in Figure 1. Club cells are abundant in distal airways of mice and humans where they are the chief secretory cell type in the terminal and bronchiolar epithelium. Club cells are the main target for NA toxicity, regardless of route of exposure, which is likely related to the Club cells' xenobiotic metabolism capabilities (Carratt et al. 2019). Club cells contain abundant CYP, CYP2F2 being the primary P450 isoform that initiates metabolism of NA in the mouse airway (Buckpitt et al. 2002; Carratt et al. 2016; Van Winkle et al. 1995). Club cells are progenitor cells of the distal airway, regulate local inflammatory responses, and secrete components of the lining layer of the airways that protect the lungs. Studies have shown that CYP metabolism is an obligate step in creating 1,2 NA epoxide which leads to NA toxicity although this may occur in either the lung or the liver (Buckpitt and Warren 1983; Carratt et al. 2016; Van Winkle et al. 1995).

In the absence of CYP, or with chemical inhibition of CYP, the initial 1,2 epoxide (**Fig. 1.1**) and downstream metabolites do not form, thus there is no toxicity (Plopper et al. 1992; Warren et al. 1982; Yildirim et al. 2008). Despite having lower levels of CYP due to the slow postnatal maturation of Club cells, neonatal and juvenile mice are more susceptible to NA exposures than adults (Carratt et al. 2019; Fanucchi et al. 1997). The mechanism of this susceptibility is not fully understood but may involve differential expression/regeneration of GSH and phase II metabolism needed to conjugate and detoxify oxidant metabolites of NA

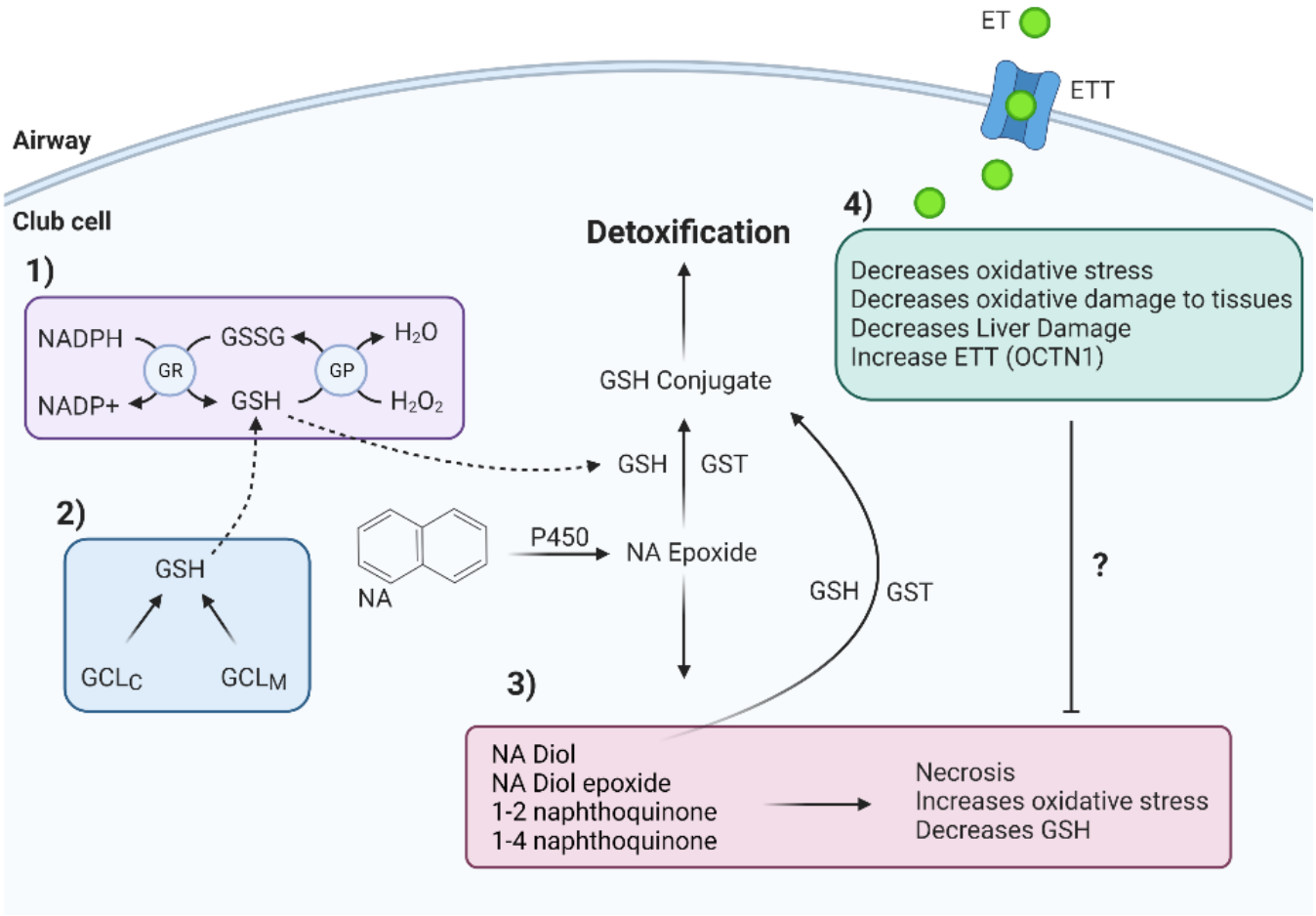

Fig.1.1 – Schematic of 1) GSH-GSSG Cycle, **2)** GSH Synthesis rate limiting enzyme, **3)** NA Toxicity, and **4)** Proposed ET mechanism. Created on BioRender.com. GSH, Glutathione; GSSG, Glutathione oxidized; GS, Glutathione synthetase; GCL, Gamma glutamylcysteine synthetase; GST, Glutathione transferase; NA, Naphthalene; ET, Ergothioneine; ETT, Ergothioneine transporter.

(Fanucchi et al. 1997; Li et al. 2011). NA metabolites are primarily detoxified by GSH in the lungs, as shown in **Fig. 1.1** (Chan et al. 2013; Fanucchi et al. 2000). In the presence of NA epoxide, GSH will form a NA metabolite conjugate via GSH-transferase and be eliminated. Alternately, the subsequent metabolites may continue down a toxicity-associated pathway to form products of naphthalene diol epoxide, 1-2 naphthoquinone and 1-4 naphthoquinone (Buckpitt et al. 2002; Pizzorno 2014; Warren et al. 1982). GSH depletion has been associated with reactive oxygen species (ROS) and is known to be an essential step in NA toxicity (Phimister et al. 2004; Plopper et al. 2001a; Shultz et al. 2001; West et al. 2001). In the presence of oxidative stress, ET will upregulate the activity of GPx, promote the function of NADP+ cycle, and even directly detoxify the oxidize stressor (Liu et al. 2023). When exposed to NA toxicity via inhalation or injection, it is important to consider the factor of time, affecting both the depletion and regeneration of GSH in the lungs (Phimister et al. 2004). During a 4-hour inhalation exposure in mice at 15 ppm of NA, GSH maximum depletion occurs at 2 hours in the whole lung, proximal and distal airways, and liver. This phenomenon worsens when a GSH depleting agent, diethylmalonate, was administer via ip. 1 hour prior to the inhalation exposure. Histopathology of the proximal airway in mice at 4 hours post NA inhalation exposure at 15 ppm, shows early signs of lung injury on the epithelium; swelling of the epithelium and vacuolation of Club cells (Phimister et al. 2004). At 24 hrs, the injury is advanced to frank toxicity in Club Cells which includes exfoliation. The relationship between NA activation and detoxification in the lungs may be complex, however having a base understanding will allow us to explore the impacts that dietary antioxidants and other contributing factors to susceptibility play in lung toxicity.

Species	Exposure	Dose	Trachea	Proximal airway	Distal airway	Parenchyma	Nasal Olfactory
Mouse	Intraperitoneal injection	50 mg/kg	–	+	+	–	–
		100 mg/kg	–	+	+	–	–
		150 mg/kg	–	++	++	–	–
		200 mg/kg	+	+++	+++	–	++
		400- 600 mg/kg	++	++++	++++	–	+
	Inhalation	2-5 ppm	++	+	+	–	+
		10 ppm	+++	++	++	–	n.d.
		20 ppm	++++	++	++	–	n.d.
	Oral	150 mg/kg	n.d.	+++	+++	–	n.d.
	Injection LD_{50}	380 mg/kg	n.d.	n.d.	n.d.	–	n.d.
	Oral LD_{50}	533 mg/kg	n.d.	n.d.	n.d.	–	n.d.
Rat	Intraperitoneal injection	200 mg/kg	–	–	–	–	++
		400 mg/kg	–	–	–	–	+++
		800 mg/kg	–	–	–	–	+++
		1600 mg/kg	–	–	–	–	+++
	Inhalation	3.4 ppm	–	–	–	–	++++
	Injection LD_{50}	1,600 mg/kg	n.d.	n.d.	n.d.	–	+++
	Oral LD_{50}	2200-2400 mg/kg	n.d.	n.d.	n.d.	–	n.d.

Table 1.1- Specificity of NA toxicity by site and species. Table is inspired and reconstructed from (Abdo et al. 1992; Carratt et al. 2016; Kelty et al. 2020; Lee et al. 2005; Plopper and Gram 1993; Plopper et al. 1992; West et al. 2001). Scoring for vacuolation and necrosis: –, no damage; +, low damage; ++, medium damage; +++, high damage; ++++, severe damage. Abbreviations: n.d., no data

DIETARY ANTIOXIDANTS

There are a number of preventive measures that have been attempted to reduce the impact of exposure to environmental toxicants. One of the most accessible ways is through diet. Many studies, in both traditional and modern medicine, have delved into the potential benefits that dietary antioxidants may play in decreasing or preventing oxidative stress in the lungs and systemically in the body. However, many of these studies are case reports or do not have rigorous controls. It is important to conduct rigorous mechanistic studies and also to note that not all dietary antioxidants have been successful in functioning as oxidative stress neutralizers. Much of this lack of efficacy can be attributed to lack of bioavailability in target sites. However, there are also some promising results that hint at strategies for preventing or reversing oxidative stress induced lung damage.

Traditional Medicine and Historical influence on Modern Medicine

Traditional medicine has been an inspiration and essential starting point for many modern medicines today. It is most commonly recognized as all-natural, plant based, non-synthetic options that have been historically used as healing agents in many cultures. Natural dietary antioxidants found in fruits and vegetables, e.g. flavonoids, vitamin C, vitamin E, coenzyme Q10, phenols, and polyphenols, are known to strengthen the endogenous antioxidants defenses from oxidative stressors and assist in restoring homeostasis in the cells (Adwas et al. 2019b). Most scientists who study traditional medicine and natural antioxidants will focus on a specific plant or bioactive compounds originating from Asia, North America, or Australian practices (Hano and Tungmunnithum 2020). A European study with a total of 680 participants in their mid-40's from 3 countries (Germany, Norway, UK) suggested that consumption of apples, banana, and tomatoes can slow down the decline of lung function over a 10 year span, especially

in those who are ex-smokers (Garcia-Larsen et al. 2017). In the presence of oxidative stressors caused by reactive oxygen or nitrogen species (ROS/RNS), plant derived antioxidants vary in their ability to neutralize ROS/RNS, some being more effective than others depending on the toxicant, the concentration and, the bioavailability of the antioxidant (Hano and Tungmunnithum 2020). Although dietary antioxidants hold many benefits, it is important to recognize the limitations to antioxidants and acknowledge clinical trials that have failed, notably vitamin E. Many of the dietary antioxidants listed above have been involved in clinical trials, however the most studied are vitamin C (L-ascorbic acid) and vitamin E (α-tocopherol). Although vitamin C is essential for collagen and protein production, it is not synthesized by the human body (Forman and Zhang 2021). Vitamin E on the other hand is biosynthesized and plays a critical role in maintaining the bioactivity and signaling of the lipid membrane. In the presence of an oxidative stressor vitamin C provides an electron to neutralize free radicals. Vitamin E will reduce peroxyl radicals and forms toxopheroxyl radicals (Fang et al. 2002). Some animal studies have shown that higher intake of vitamin C and vitamin promotes cancer development and metastasis in the lungs of mice models (Sayin et al. 2014). While some clinical studies have proposed concerns with the use of vitamin E acetate in e-cigarettes (Boudi et al. 2019), and vitamin E isoform a treatment for asthmatic children (Cook-Mills et al. 2013). There have been numerous studies focused on the impacts of dietary antioxidants on various lung diseases, as shown in **Table 1.2**, however there are still so much that needs to be done in order to improve modern medicine and created novel non-synthetic treatment alternatives.

CONTRIBUTING FACTORS TO SUSCEPTIBILITY

From within the womb to the geriatric stage of life, the body is routinely under oxidative stress induced from exposures to environmental toxins, resulting in potential lung damage (Valacchi et al. 2007). Evidence strongly supports that both juvenile and geriatric age groups are most susceptible to xenobiotics compared to adults (Kelly et al. 2003). However, this increased risk can be due in part by various factors such as: lung development, genetics, early life exposure (a critical window of susceptibility), secondhand exposure, co-exposures, sex, maturational stage or loss of protective factors or enzymes (in multiple organs) as well as pre-existing conditions (Bauer and Kleeberger 2010; Buckpitt et al. 2002; Carratt et al. 2019; Sutherland et al. 2012).

References	Therapeutic Target	Age (years)	Existing or pre-existing condition	Experimental design	Clinical/ Toxicological findings
(Omenn et al. 1996)	carotenoids and retinoids (Vitamin A)	45 - 69	Lung Cancer	73,135 patients, some with higher risk of developing cancer due to asbestos exposure and smoking, took a combination of beta carotene and vitamin A over a span of 4 years.	The active treatment group had 28% higher incidences of lung cancer than the placebo group, in addition to higher deaths from cardiovascular disease. This could have been a direct result from the supplements.
(Farazi et al. 2014)	Coenzyme Q10	Mid 60's	Community-acquired pneumonia (CAP)	141 patients were given 200 mg/ day of coenzyme Q10 or placebo orally for two weeks.	Patients treated with coenzyme Q10 had faster defervescence and hospital discharge and compared to placebo. There were few, but similar adverse events in both groups.
(Hunt et al. 1994)	Vitamin C	66 - 94	Lower respiratory tract infection	57 patients with acute respiratory infections (bronchitis and bronchopneumonia) were divided into subgroups of symptom severity and were treated orally with 200 mg of vitamin C or placebo for 2 and 4 weeks.	Vitamin C treatment improved symptoms in all subgroups and the most improvement was found in the patients with the most serve symptoms.
(Mahmoodpoor et al. 2018)	Selenium	42 - 63	Ventilator Associated Pneumonia (VAP), active smoker, COPD	99 patients (47 treatment group and 52 in placebo group) were infused with selenium or saline for 10 days.	23/99 participants developed VAP. There was no difference between the control and selenium treatments group. Although those treated with selenium increased GSH activity.
(Khazdair et al. 2018)	Carvacrol	27 - 30	Sulphur mustard (SM)-induced lung disorders	20 patients exposed to SM were treated for 2 months with carvacrol (1.2 mg/kg per day) or placebo.	Patients treated with carvacrol had improved pulmonary function tests, reduced inflammatory cells, and increased antioxidant biomarkers.

Table 1.2 Reference list of contributing factors to susceptibility in human lung. Table is inspired and reconstructed from (Rahman 2008).

References	Therapeutic Target	Age (years)	Existing or pre-existing condition	Experimental design	Clinical/ Toxicological findings
(Sagel et al. 2018)	Systemic antioxidant (β-carotene, coenzyme Q10, γ-tocopherol, and lutein)	10 - 30	Cystic fibrosis	73 patients were given a multivitamin supplement or placebo for 16 weeks. Endpoints were antioxidant concentration, biomarkers for inflammation and oxidative stress, clinical outcomes, safety, and tolerance.	Those treated with multivitamin had a significantly lower risk of first pulmonary exacerbation then the placebo. The treated group also had a modest decreased in inflammation after 4 weeks of treatment.
(Griese et al. 2013)	Glutathione	8 - 30	Cystic fibrosis	73 patients with cystic fibrosis inhaled 646 mg in 4 ml of glutathione, and 80 placebo using a nebulizer every 12 hours for 6 months.	There was no clinically relevant improvement seen in glutathione treated group compared to placebo.
(Dow et al. 1996)	Vitamin C and vitamin E	70 - 96	Self-reported respiratory symptoms	178 men and women were completed a questionnaire regarding their dietary intake of vitamin C and E and smoking habits. There lung function was also measured.	For every extra mg of vitamin E added to the daily diet, the FEV1 increased by 42 ml and FVC by 54 ml. Vitamin E intake may influence lung function in the elderly.
(Rerksuppaphol and Rerksuppaphol 2019)	Zinc	≤ 1	Acute respiratory tract infection	64 hospitalized children were treated with 30 mg of zinc/day or placebo.	Children who received Zinc treatment was discharged from the hospital faster than the placebo group. Zinc had no reported adverse effects.
(Pearson et al. 2004)	Vitamin E	18 - 60	Asthma	72 participants with asthma were given 500 mg of vitamin E or placebo for 6 weeks.	Those treated with vitamin E showed no improvement of asthma control.

Continuation of Table 1.2

Route of exposure

Historically in rodent models, NA toxicity in the lungs was evaluated via intraperitoneal (ip) injections, due to its easy to control dose delivered mechanism, but has also been studied in rodent models when given by inhalation or ingestion. The target cell type and the sequence of lung events involved in toxicity is the same whether the compound is inhaled, ingested, or injected. The benefits of studying potential toxicants via injection enables the scientist to manipulate the exposure dose and time following a bolus. Whereas inhalation exposures have a time element where some cells may respond early in the overall exposure and others may not respond until later. Alternatively, conducting an inhalation exposure, using dosage that are commonly found in the environment, can make results potentially more translatable to human exposure, although there are species specific considerations regarding deposition, bioactivation and cellular composition of the respiratory tract including the lung.

One study examined lung NA metabolites and toxicity in postnatal mice following inhalation exposure (Carratt et al. 2019). When comparing the results from this inhalation study to previously conducted ip injection studies, they found that juvenile mice 3 weeks of age in the inhaled study were most susceptible to NA vapor, while the previous ip study found that neonates 7 days old were most susceptible to NA toxicity in the lungs (Carratt et al. 2019; Fanucchi et al. 1997). NA exposure has yet to be conducted in neonates 3 weeks of age via ip, a data gap that we be address in this dissertation. Previous studies of adult mice exposed to NA vapor have shown greater damage in the proximal airway compared to the distal airway post inhalation exposure. A similar result, but with an opposing pattern of cellular injury distribution, was found in mice injected with NA; there was more damage in the distal airway compared to the proximal airway (Carratt et al. 2019; Fanucchi et al. 1997). Both routes of exposure target

Club cells in the epithelium but yield differing injury patterns with different locations targeted first with inhalation (proximal airways) than injection (terminal bronchioles). However, both routes contribute to the understanding of NA toxicity. Importantly, even NA given by inhalation escapes the lung and circulates as the parent molecule in the blood (Kelty et al. 2022) .It is essential to understand and study the impact of different routes and acute and chronic exposures if we want to truly understand the mechanisms of toxicity and find preventable measures for the toxicant of interest. In this dissertation we address the injection route of exposure and variables such as age, sex and antioxidant status. Future studies may address route of exposure.

Impact of Species

Non-human primates have a lung anatomy and cellular physiology that most closely resembles humans; however, primate pulmonary studies can be both time consuming and costly. Alternatively, scientists use rodent models, e.g., mice and rats, in order to understand the impacts of xenobiotics in the lungs. The most notable differences between human and non-human models include anatomy, function and structure of the respiratory system, cellular composition, and metabolism of xenobiotics. Yet many features, including key cell types and interrelation of organ systems involved in the response are also conserved between species allowing one to be used as a precursor for studies in the other.

The lung anatomy between humans, dogs, rodents, and primates all differ in size and structure. For example, when comparing humans and rodent models there are very distinct differences in the anatomy and total lung capacity, the total amount of air that the lung can hold. Both human and rodent lungs have 5 lung lobes (Irvin and Bates 2003). The human left lobe has two segments, an upper and lower lobe, and the right lobe is comprised of three segments, upper

middle and lower, with a total lung capacity of 6,000 ml or 6 liters (Delgado and Bajaj 2019; Patra 1986). The left lung lobe in rats and mice comprises of one large segment and the right lung lobe has a total of 4 segments of various sizes: the cranial lobe, middle lobe, caudal lobe, and accessory lobe (Patra 1986). Rats have a total lung capacity of 10 ml and mice at 1 ml (Irvin and Bates 2003). Mice and rats are both known obligate nose breathers, while humans are capable of breathing through both the mouth and nose. This crucial difference is important consider when studying toxicological studies of inhaled chemicals. In the section discussing naphthalene, **Table 1.1** summarizes the range of NA toxicity in different species and location. The important question here is why does the toxicity vary between species? In regard to NA, the answer is the location and activity of cytochrome P450, which is unusually well represented in Club cells located in the conducting airways of rodent, as well as other metabolites that maybe found predominantly in some species, and sparingly in others. For example, 49% of the cells found the mouse trachea are Club cells, while monkeys and humans have less than 1% (Hyde et al. 2006). Club cells make up 61% of the mouse proximal intrapulmonary airways and 60-80 % in the terminal bronchioles, while humans are about 20% (Dean and Snelgrove 2018). Each mammal has varying expression of P450 proteins and isoforms throughout the respiratory tract, directly influencing their susceptibility to localized oxidative stress and cell damage and this expression varies with age, airway location and possibly sex (Stelck et al. 2005; Van Winkle et al. 2002). Further the balance of activation and detoxification enzymes is also an important determination of injury at the target site and expression of Phase II metabolism also varies.

In summary, it is evident that there is a vast amount of difference between species. It is important to compare humans to non-human models in order to understand susceptibility, biology, contributing factors to toxicity, and to, ultimately, improve upon human health. Drug

development has begun to move away from animal research in order to accommodate animal activists and is now considering in vitro alternatives to in vivo testing. However, there are limitations to these approaches. For more than 80 years, the FDA has conducted drug safety and risk assessment on animals prior to conducting human trials, and with this upcoming change it will be interesting to see how translational research and drug development continue to study toxicants and susceptibility. Key limitations of in vitro approaches include poor preservation of in vivo metabolism and detoxification, an inability to test multi-organ and immune mediated toxicities and, so far, no real approach to study age specific effects. Thus, this approach will have limited, and specific, utility. The importance of prior animal work in interpretation of such studies remains.

Impacts of Age

The human lung fully matures around the age of 20 – 25 years old, and, from there, will gradually decline in pulmonary function and ability to overcome infections and stress over time. The pace of this decline varies due to 1) pre-existing pulmonary diseases, e.g., childhood asthma, 2) developing lung disease, e.g., COPD, 3) infectious pulmonary disease, e.g., COVID-19, and environmental toxicants that induce irreversible lung damage. Lung development has been heavily studied in mice, with prior studies of ip. naphthalene specifically comparing cytotoxicity in neonates at 7 and 14 days of age with adults (Fanucchi et al. 1997). Morphometric assessment of the bronchial and terminal epithelium showed the lowest cell expression at 7 days postnatal, and 14 days postnatal compared to adult. When exposed to an oxidative stressor, in this case 100 mg/kg of NA, neonates at postnatal day 7 had significant vacuolation in the bronchioles compared to adults. In the terminal bronchioles, both 7 days and 14 days postnatal mice had significant depletion of ciliated and non-ciliated cells post exposure compared to adults

(Fanucchi et al. 1997). This is just one of many studies that shows the role that age plays in lung susceptibility in mice models, with elevated susceptibility to NA by injection in younger mice than in adults at an equal dose.

Because the Club cell matures postnatally and CYP expression parallels that maturation, neonatal and juvenile animals are known to have lower levels of CYP, making it quite a conundrum of why they are more susceptible to some chemicals, such as NA, than adult mice (Carratt et al. 2019). The mechanism of this susceptibility is not fully understood but is thought to involve differential expression of phase II metabolism. Critically it is thought that it is the balance of activation and detoxification is skewed resulting in increased susceptibility in younger animals although this has not been adequately explained/investigated (Chan et al. 2013).

In children, as the lungs develop to maturity, it is not uncommon for some children to develop childhood asthma due to genetics and environmental exposures. A study in Mexico with moderate to severe asthmatics (8 – 9 yrs. old), shows the benefits of including supplements with antioxidants, 50 mg/day of vitamin E and 250 mg/day of vitamin C, in their diet, in order to modulate the impacts air pollution exposures, specifically PM10, ozone, nitrogen dioxide, and sulfur dioxide (Romieu et al. 2002). When comparing lung function between the placebo and supplement group post exposure to ozone, children taking daily supplements had minor improvement in FEV1 (forced vital capacity first measure) and FVC (forced vital capacity), and significant improvement in FEF (forced expiratory flow), and PEF (peak expiratory flow); overall indicating protection against air pollution (Romieu et al. 2002). Although this was a small study with a sample size of 158 participants, it is just one of many studies that shows the potential benefits of antioxidants in children (Rerksuppaphol and Rerksuppaphol 2019; Romieu et al. 2002; Sienra-monge et al. 2004).

When it comes to the decline in antioxidants, health, and aging, it is important to consider the impacts of environmental oxidative stressors and the role that it plays in lung health (Valacchi et al. 2007). A small clinical study in 1994, provided elderly patients with vitamin C supplements order to see if this would reduce symptoms of lower respiratory infections. They found that the participants in the severely ill subgroup had faster recovery from symptoms when treated with vitamin C supplements (Lee et al. 2020). Treatment with antioxidants is just one of many ways that improve lungs that are most susceptibility due to the impacts of age and exposure to xenobiotics.

INTRODUCTION TO DISSERTATION

Within this dissertation we will be exploring several key susceptibility factors: age, sex, and dose. In addition, we will be comparing the impact of different levels of antioxidants including endogenous, GSH, and dietary, ET, which may act to reduce toxicity induced by environmental oxidative stressors impacting the lungs. First, I will evaluate the morphologic impacts of ip NA exposure in adult and juvenile mice. In doing so we will be able to understand the developmental impacts that NA has on the lungs and the effects of ET pretreatment. This has not been established in both sexes at this age and the impact of ET on the susceptibility of the developing lung to NA has also not been established. Once we have an understanding of the histopathology, we will dive deeper into the cellular mechanisms underlie the morphologic changes and toxicity by comparing age, sex, and dose and the temporal response in the tissue to determine the sequence of events. Lastly, we will be evaluating the susceptibility of NA exposure and ET pretreatment in male and female geriatric mice. This study will be the first to conduct a NA dose response in geriatric mice.

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CHAPTER 2 - NAPHTHALENE TOXICITY IN THE JUVENILE AND ADULT MOUSE LUNG: IMPACT OF PRE-TREATMENT WITH ERGOTHIONEINE ON TOXICITY

It is known that lung development impacts secretory cell and Phase I and II xenobiotic metabolism maturation in the conducting airways, but few studies have examined how a dietary antioxidant, such as ergothioneine (ET), impacts naphthalene (NA) toxicity in the developing juvenile or adult mouse lung. The goal of this study was to define NA induced toxicity in the lungs of juvenile and young adult mice, at the same dose, with and without an oral antioxidant pretreatment with ET. NA toxicity in the lung has been well characterized in neonates and adults, but not in juvenile mice one month of age, post weaning. Juvenile and adult C57BL/6 mice, maintained on an ET-free synthetic diet from conception, were given ET at 70 mg/kg by oral gavage for five consecutive days. On day 8, the mice received 150 mg/kg of NA ip. and lungs were examined at 24 hours post exposure. The ET concentration, tissue morphometry of toxicity evident as vacuolated conducting airway epithelium, and expression of genes related to cellular detoxification were measured in the lung. Adult male mice pretreated with ET had significantly lower ($p \leq 0.0001$) NA-induced cellular vacuolation in the proximal and distal airways, compared to males not treated with ET. We found that ET pretreatment protects against NA induced lung toxicity in adult male mice but not juvenile mice.

INTRODUCTION

Naphthalene (NA), a volatile polycyclic aromatic hydrocarbon, is found abundantly in the environment, e.g. fossil fuels, wood smoke (Yost et al. 2021). The impact of NA toxicity in the developing lung has received little attention although prior work in mice has indicated that the immature lung, at ages ranging from one to three weeks, is more susceptible (Carratt et al. 2019; Fanucchi et al. 1997). The parent form of NA is not of concern until it is metabolized by

cytochrome P450 (CYP) to an epoxide, which is a toxic form and a precursor to other metabolites that result in oxidative stress and Club cell vacuolation and loss, as CYP are concentrated in Club cells. Club cells are non-ciliated secretory cells found in the conducting airway epithelium that mature in the postnatal period (Blackburn et al. 2023; Cardoso et al. 1993; Coppens et al. 2009; Rokicki et al. 2016). During postnatal maturation, Club cell secretory proteins (CCSP; SCGB1A1), cells, and phase I and II metabolic enzymes undergo non-synchronous maturation (Fanucchi et al. 2000b; Plopper and Fanucchi 2004; Plopper et al. 2001). Young animals are more susceptible to lung toxicity of metabolically activated xenobiotics than adult animals (Smiley-Jewell and Plopper 2003). There is a conundrum in the susceptibility of the developing epithelium which has less CYP present as the cells mature postnatally, yet the animals are more susceptible (Fanucchi et al. 2000b). Susceptibility in younger animals to oxidant stress is thought to be due to maturational events that are still in flux in the Club cell population; possibly due to an imbalance in phase I and phase II metabolism resulting in inadequate resources for detoxification (Chan et al. 2013). CCSP is a secretory protein secreted by Club cells that protects the bronchiolar epithelium, mitigates oxidant toxicity and can regulate macrophage responses; this molecule is also used as a marker of maturation of Club cell secretory function in addition to its role in mitigating toxicity and regulating inflammation (Reynolds et al. 2007; Snyder et al. 2010; Stripp et al. 2002).

Reduced glutathione (GSH) is a key component of the phase II antioxidant response in the lung. Regulation of GSH intracellular concentration is at the level of the GCS enzyme, the rate limiting step in GSH synthesis, which is thought to vary in throughput with age and prior exposures as do the key conjugation enzymes glutathione S-transferases (GST) (Chan et al. 2013; Plopper et al. 2001; Wu et al. 2004). GST comprises a multifunctional family of enzymes,

e.g., alpha, mu, pi, that primarily function in the detoxification of xenobiotics and can vary in concentration and expression pattern based on age and tissue location. The expression of GST, and the ability to detoxify xenobiotics, is dynamic and impacted by age and lung development.

Club cells also contain abundant amounts of reduced glutathione, which is critical to mitigating NA toxicity although there is heterogeneity in Club cell levels of GSH (Fanucchi et al. 2000b; Plopper et al. 2001; West et al. 2000). The protective role of GSH is underscored by experiments where GSH was depleted and where it was bolstered, which were shown to modify NA toxicity (Phimister et al. 2004; Warren et al. 1982). Overproduction of the reactive NA metabolites, as occurs within 2-4 hrs following an acute bolus exposure, will result in a depletion of endogenous antioxidants such as glutathione both due to direct binding/conjugation of reactive metabolites to these antioxidants in the cell and to loss of Club cells (Buckpitt et al. 2002). The impact of NA toxicity and the degrees of depletion of endogenous antioxidants are expected to differ by sex (Van Winkle et al. 2002) and age (Fanucchi et al. 1997), but they have not been comparatively defined for juveniles of one month of age as used in this study. Juveniles one month of age are important to study because the mice are post weaning and the alveolar growth by septation is subsiding at this time in the lung (Massaro and Massaro 2000; Shi et al. 2007; Tschanz et al. 2014).

This study will explore the ability of a dietary antioxidant, ergothioneine (ET), to protect the Club cell from NA induced toxicity in juvenile and adult mice of both sexes. ET is synthesized by bacteria and fungi and is concentrated in mushrooms (Cheah and Halliwell 2021). It has been used to prevent or reduce oxidative stress in the skin, brain, and gut of humans but is obtained solely through diet (Halliwell et al. 2018). This study will be the first to examine ET as

a dietary pretreatment for lung toxicity in juveniles one month of age compared to adult mice at 24 hours post naphthalene exposure.

METHODS

Animals

The experiment was conducted on two age groups of C57BL/6 mice: juvenile males (19-23 grams) and females (15-17 grams) that were 1 month old, and adult males (23-26 grams) and females (17-21 grams) that are 2-3 months old. Mice were placed on a special diet (AIN-93G, Research Diets) ad libitum from birth to minimize dietary exposure to ergothioneine. Litter size was normalized to 6 to 8 pups. The University of California Davis Animal Facility where all experiments were performed under protocols approved by the University of California Davis IACUC in accordance with National Institutes of Health guidelines housed the mice with a scheduled 12-hour light/dark cycle. Prior to the start of the study, animals were given a unique access number for tracking purposes. Sentinel mice were housed in the same facility and tested negative for respiratory virus for the duration of the study.

Chemical Sources

Naphthalene (NA) was purchased from Fisher Scientific (CAS-91-20-3) and was diluted in Mazola corn oil (vehicle control for NA treatment). Ergothioneine (ET) was purchased from BLDpharm (CAS-497-30-3) and was diluted in saline. Araldite 502 epoxy resin, DDSA, and DMP-30 were purchased from Ted Pella (Redding, CA). All other chemicals were reagent grade or better.

Experimental Design

Male and female juvenile mice were selected from 5 dams and weaned at 21 days of age. The mice were then separated into four experimental groups (n=5 /group): Saline (SA)/ Corn oil (CO), SA/NA, ET/CO, and ET/NA. The juvenile mice were gavaged with either saline or 70 mg/kg of

ET for 5 consecutive days, 2 days post weaning. On the 8th day, the mice were injected with either CO or 150 mg/kg of NA intraperitoneally (ip) at an equivalent amount per body weight. At 24 hours post injection the mice were killed by an overdose of pentobarbital ip, and samples were collected for further analysis. Similar to the juvenile mice, the male and female adult mice were separated in the same four experimental groups. Then for 5 consecutive days all mice were gavaged with either saline or 70 mg/kg of ET. On day 8 the mice were injected with CO or 150 mg/kg of NA at an equivalent amount per body weight. At 24 hours post injection, the mice were killed by an overdose of pentobarbital ip, and samples were collected for further analysis. To minimize the potential influence of diurnal differences in GSH levels on the response, mice were exposed to NA at the same time of day in relation to the light/dark cycle.

Ergothioneine Detection

Frozen blood, liver, and lung samples were shipped to University of Arizona where they were homogenized and prepped for detection of ET using LC-MS system which consist of an Agilent model 1290 HPLC (Agilent, Santa Clara, California), and a Qtrap 6500 plus mass spectrometer (Sciex, Ontario, Canada) equipped with a Turbo IonSpray source. Method of detection for ET was modified from published studies (Cheah et al. 2016a; Cheah et al. 2016b).

High resolution microscopy and stereology

The whole lung was fixed at 30 cm of constant pressure with Karnovsky's fixative, and cut into 4 separate pieces, exposing the airways. The samples were processed in Zetterquist's buffer and embedded into Araldite 502 epoxy resin. Two of the four lung samples were randomly selected for sectioning at 1 μm on a microtome with glass knives. The sectioned tissue was then placed on a gelatin slide, stained with methylene blue/Azure II, and imaged on a high-resolution bright field Olympus BH-2 microscope at 20x magnification. Stereological assessment of the surface per

volume in the lungs were conducted on the imaged slides (Hsia et al. 2010; Murphy et al. 2013). The mass (Vs) was measured using a counting system of points (P) and intercepts (I) on a cycloid grid in Stereology toolbox software (Kelty et al. 2020). Mass Vs was calculated using the following formula: Vs= (l/p)(∑Pts/∑Int)(1/2), where Vs is the airway thickness (μm3/μm2), (l/p) is the length and sum of curves divided by the sum of points, and (∑Pts/∑Int) is the sum of points of either all, vacuolated, or non-vacuolated conducting airway epithelial cells divided by the sum of the intercepts of the basal lamina of the epithelium. The results of (∑Pts/∑Int) is represented as a T_{epi}. The volume fraction percentage Vv was calculated using the equation: Vv= ∑Pts / P_t , where the sum of vacuolated points, ∑Pts, is divided by the total cells counted, P_t, which includes both vacuolated and non-vacuolated cells.

Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The extracted lung was fully inflated with RNA-later (Ambion/Applied Biosystems; Foster City, CA) and stored at -20 ºC. RNA was isolated from microdissected lung sub compartments: proximal airway, distal airway, and parenchymal tissue (Baker et al. 2004). The proximal airway includes the intrapulmonary bronchi and larger intrapulmonary bronchioles; the distal airway includes the more distal bronchioles and terminal airways; and the parenchyma is primarily composed of connective tissues and alveoli. RNA was isolated using a RNeasy Plus Mini kit with a gDNA elimination column (Qiagen; Hilden, Germany), and quantified with a NanoDrop spectrophotometer (ThermoFisher; Waltham, MA). The real-time PCR system (StepOnePlus; Applied Biosystems) was utilized to analyze the following genes of interest using Taqman assays (ThermoFisher; Waltham, MA): RPL13a, ribosomal protein (assay ID- mm01612987_g1; housekeeper gene), CCSP, Club cell secretory protein (assay ID- Mm00442046_m1), SLC22A4,

ET transporter (assay ID- Mm00457739_m1), and GSTpi, glutathione S transferase pi (assay ID- Mm00496606_m1)

Statistics

Data and statistical analyses were computed in GraphPad Prism. Each group had an sample size of 3-5 mice. Differences between age and treatment groups were calculated using two-way analysis of variance. Statistical significance between groups was based on Tukey's multiple comparisons test (Tukey's HSD) with significance set at $P< 0.05$. ET concentration was analyzed using one-way analysis of variance. All datasets were analyzed for outliers through the Grubbs' Test, resulting in significant outliers (p-value = 0.05) being omitted. Error bars are presented using standard error of mean, or SEM.

RESULTS

Gene expression in juvenile and adult sham treated lungs

To better understand the site and sex specific differential of Club cell component maturation between juvenile and adults, we examined the expression of genes related to Club cell maturation (CCSP), and Phase II detoxification in microdissected lungs. CCSP in the proximal ($p \leq 0.0001$) and distal ($p \leq 0.05$) airway of juvenile males was significantly greater in expression compared to the parenchyma (**Fig. 2.1A**). The expression of GSTpi varied by sex, age, and location, with juvenile male distal airways having significantly greater expression compared to juvenile females ($p \leq 0.05$) (**Fig. 2.1B**). The proximal airway of juvenile males ($p \leq 0.001$), juvenile females ($p \leq 0.01$), adult male ($p \leq 0.0001$), and adult female ($p \leq 0.05$) had significantly greater expression of foxj1 in comparison to the parenchyma (**Fig. 2.1C**). SLC22A4 in adult males had greater expression in the proximal airway compared to the distal airway ($p \leq 0.001$) and

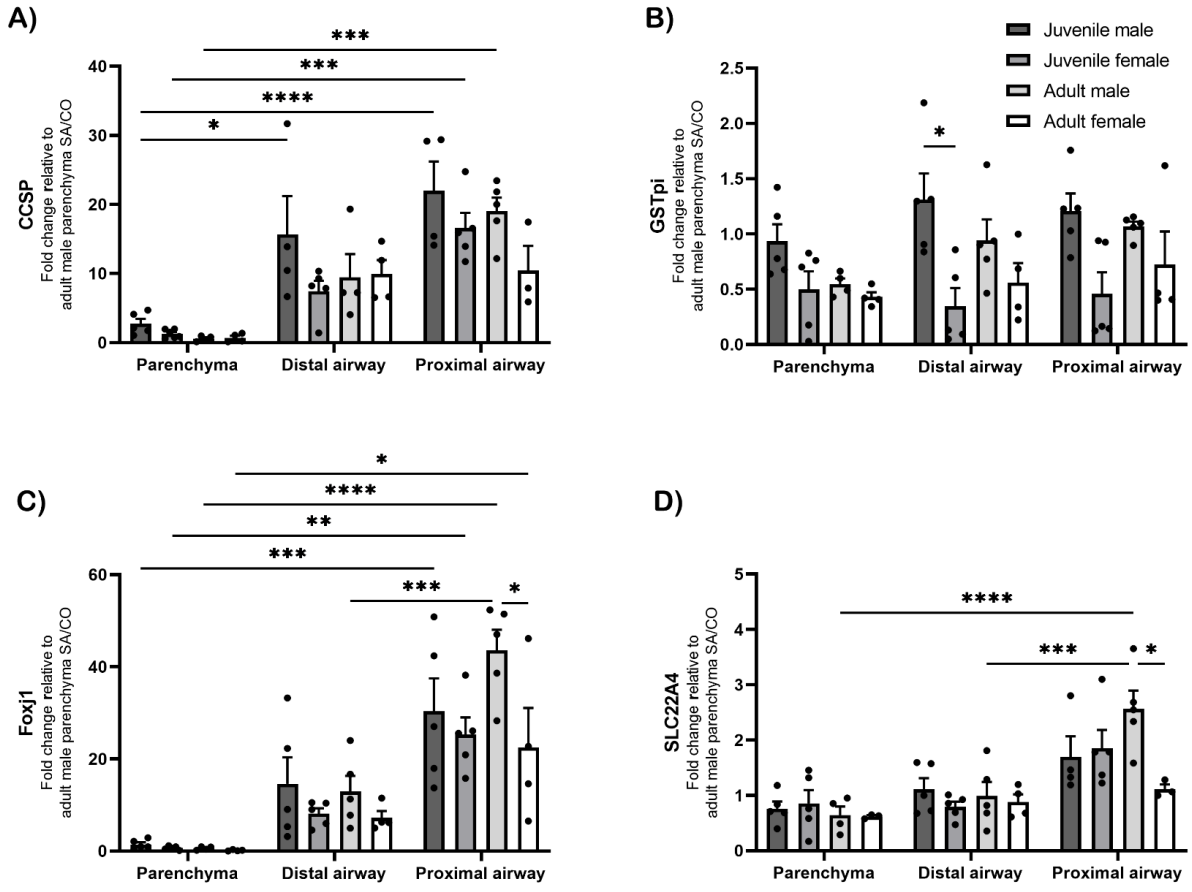


Figure 2.1- Site and sex specific differential expression of genes in the lungs of juveniles and adults. The parenchyma, a lung region containing no airways, was collected, and compared to the proximal and distal airways. CCSP **[A]**, GSTpi **[B]**, Foxj1 **[C]**, and SLC22A4 **[D]** were measured in lung samples from untreated and unexposed juvenile and adult mice (n=3-5). Gene expression was determined by qRT-PCR in relation to Rpl13a as a housekeeping gene. Values are fold changes with standard error of the mean, normalized to the adult male parenchyma region of the SA/CO group. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

parenchyma ($p \leq 0.0001$) and was significantly greater in the proximal airway compared to adult females ($p \leq 0.05$) (**Fig. 2.1D**).

Ergothioneine accumulation in the lung

ET accumulation was measured in the lung (**Fig. 2.2A, 2.2B**), liver (**Fig. 2.2C, 2.2D**), and blood (**Fig. 2.2E, 2.2F**) of juvenile and adult mice, with a limit of detection of ~0.02 μg/mL in blood and ~0.4 μg/g in tissues. There was no ET detected in mice treated only with saline, whereas those treated with ET had detectable levels in the lung, liver, and blood (**Fig. 2.2**). There was a significant age difference in ET levels, with adults having significantly greater ET concentrations in the lung ($p \leq 0.01$) and liver ($p \leq 0.001$) compared to juveniles (**Fig. 2.2A - 2.2D**). Male juvenile and adult mice had no significant difference in blood ET level, while adult female had significantly more ($p \leq 0.0001$) ET compared to juvenile females in the blood (**Fig. 2.2E, 2.2F**).

Stereology of vacuolated cells in proximal airway and terminal bronchioles

Using morphometry, we measured vacuolated cells in the proximal airway and bronchial terminal of adult mice since NA toxicity specifically targets club cells, which are fully developed at 2 months of age in mice; reason why juvenile mice were not included. Mice treated with SA or ET and exposed to 150 mg/kg NA, had significantly greater vacuolated cells (and lesser non-vacuolated cells) in the proximal airway in comparison to the CO exposed groups (**Fig. 2.3A, 2.3C**). But there were significantly less ($p \leq 0.0001$) vacuolated cells (though no significant difference in non-vacuolated cells) in males in the ET/NA group compared to the SA/NA group (**Fig. 2.3A**). There was no significant difference in the total thickness of the lung epithelium in the adult proximal airway when comparing sex and exposure groups (**Fig. 2.3E**).

Vacuolated cell in the terminal bronchioles of adult male and female mice, when exposed to NA in both the SA and ET treated groups, had significantly more ($p \leq 0.0001$) vacuolated cells

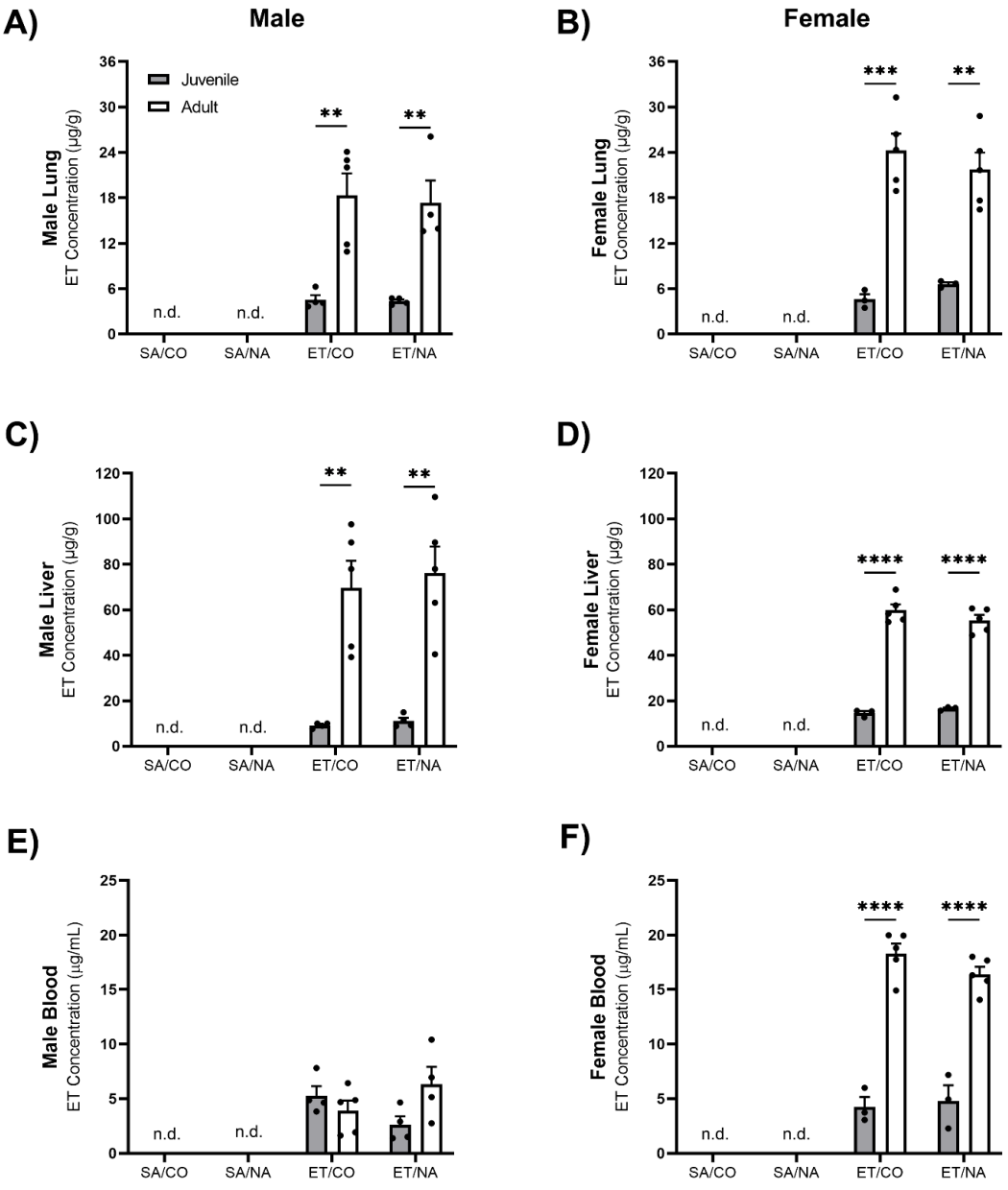


Figure 2.2- Ergothioneine accumulation in juvenile and adult mice, in whole lung, liver, and whole blood after NA exposure. Juvenile (n=3-4) and adult mice (n=4-5) were treated with 70 mg/kg ET for 5 consecutive days. The lung **[A,B]**, liver **[C,D]**, and blood **[E,F]** were analyzed for ET concentration 24 hours post exposure to 150 mg/kg of NA using HPLC-MS. The limit of detection was ~0.02 µg/mL in blood and ~0.4 µg/g in tissue samples. Error bars represent standard error of mean. **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: n.d., not detected; SA, saline; CO, corn oil; ET, ergothioneine; NA, naphthalene.

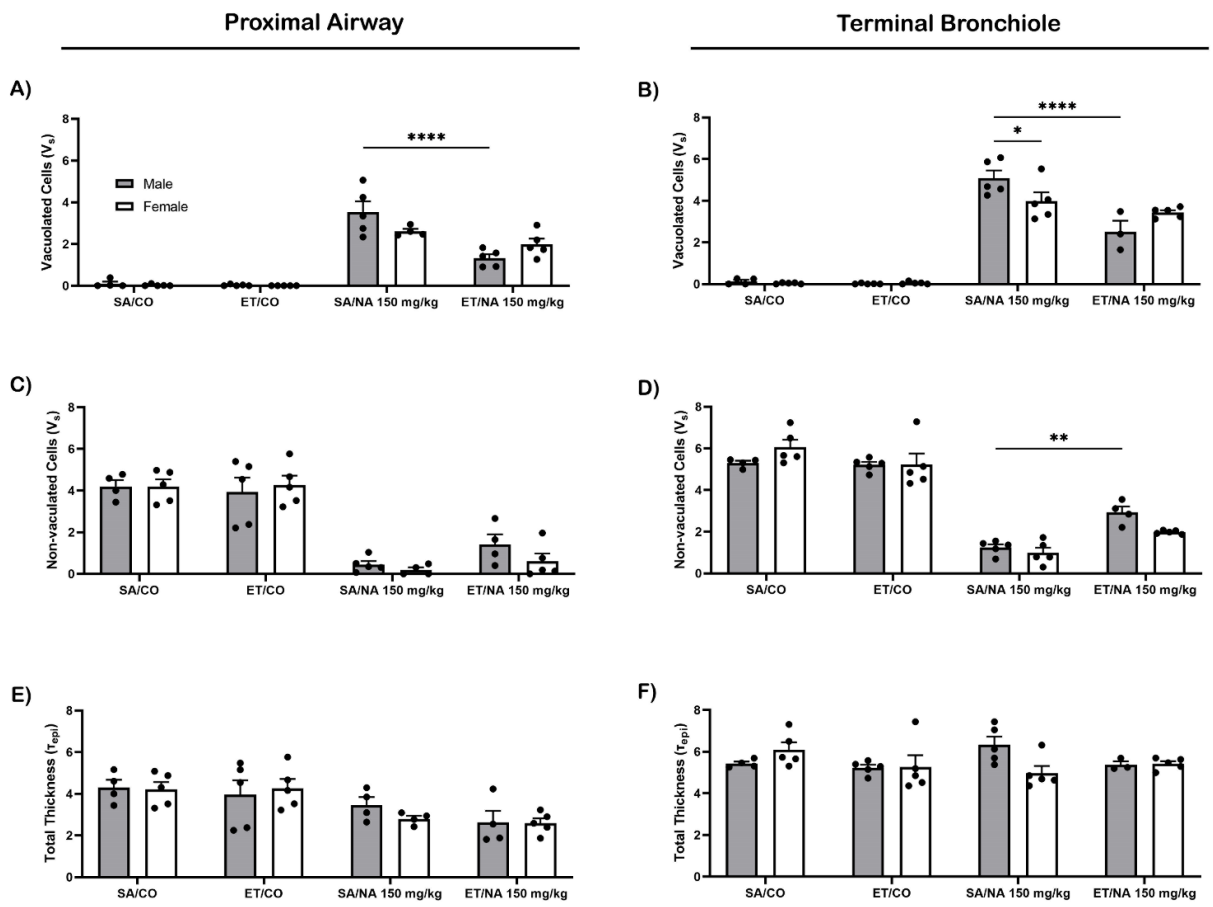


Figure 2.3- Stereology of NA toxicity in the proximal airway [A,C,E] and terminal bronchiole [B,D,F] of adult mice. Vacuolated cells **[A,B]**, non-vacuolated cells **[C,D]**, and total epithelial thickness **[E,F]** were measured in adult mice using the average volume of epithelial cells per basal lamina surface area. Error bars represent standard error of mean. **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: SA, saline; ET, ergothioneine; NA, naphthalene; CO, corn oil.

compared to unexposed groups (**Fig. 2.3B**). Adult females in the SA/NA group had significantly ($p \leq 0.05$) less vacuolated cells compared to adult males (**Fig. 2.3B**). Post NA exposure, cell vacuolation was significantly lower ($p \leq 0.0001$) in the male mice treated with ET compared to those treated with SA (**Fig. 2.3B**). Non-vacuolated cells were less abundant ($p \leq 0.0001$) in SA and ET treated group exposed to NA compared to unexposed group (**Fig. 2.3D**). Males had significantly more ($p \leq 0.01$) non-vacuolated cells in the ET treated group exposed to NA compared to the SA treated group (**Fig. 2.3D**). There was no significant difference between the total thickness of the lung epithelium in the adult terminal airway (**Fig. 2.3F**).

Gene expression of CCSP, SLC22A4, and GSTpi in juvenile and adult mice proximal and distal airway

The gene expression of CCSP (found in mature Club cells, **Fig. 2.4**), SLC22A4 (**Fig. 2.5A, 2.5B, S2.1A, S2.1B**), and GSTpi (**Fig. 2.5C, 2.5D, S2.1C, S2.1D**) was measured in the proximal and distal airway of juvenile and adult mice that were treated with SA or ET and exposed to CO or NA for 24 hours. The gene expression of CCSP in the SA/NA group of male ($p \leq 0.01$) and female ($p \leq 0.05$) juvenile mice was significantly lower in the proximal airway (similar trend though not significantly different in the distal airway) compared to the juvenile SA/CO group (control) (**Fig. 2.4A, 2.4C**). Adult females, when exposed to NA and treated with SA, had significantly less ($p \leq 0.05$) CCSP in the proximal airway compared to males (**Fig. 2.4B**). In addition, similar to juvenile females, adult female had significantly less ($p \leq 0.01$) CCSP in the proximal airway compared to the control group, SA/CO (**Fig. 2.4B**). The distal airway in adult showed a similar trend in expression of CCSP as the proximal airway but not significant (**Fig. 2.4D**).

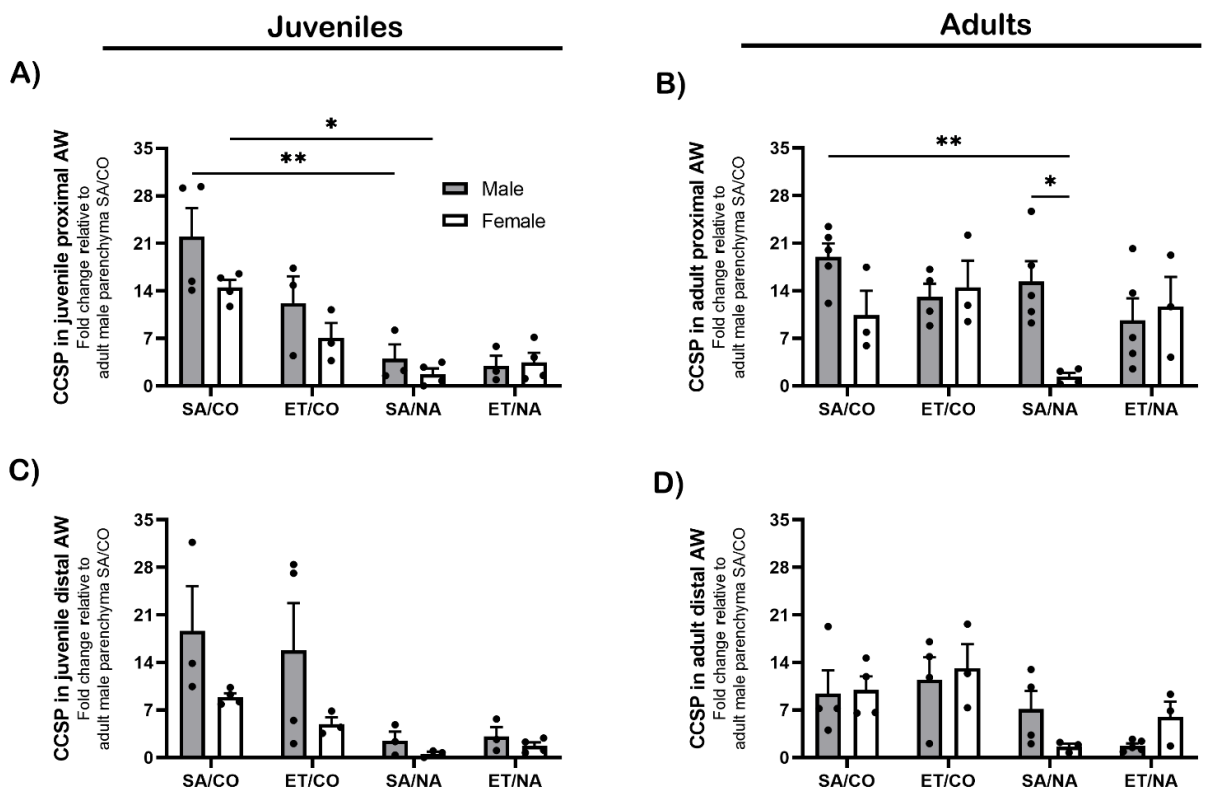


Figure 2.4- Gene expression of CCSP in the proximal and distal airway. CCSP located in the proximal **[A,B]** and distal airway **[C,D]** were measured in juvenile and adult mice treated with ET and exposed to 150 mg/kg NA for 24 hrs. Gene expression was determined using qRT-PCR in relation to Rpl13a as a housekeeping gene. Values are standard error of the mean fold change normalized to adult male parenchyma region of the SA/CO group (n= 3-5). *, $p \leq 0.05$; **, $p \leq 0.01$. Abbreviations: SA, saline; ET, ergothioneine; NA, naphthalene; CO, corn oil.

The ergothioneine transporter, SLC22A4, had no significant sex differences in the juvenile proximal and distal airway despite ET treatment or NA exposure (Fig. 2.5A, S2.1A). Adult male and females in the proximal airway had significantly more SLC22A4 ($p \leq 0.05$) in the ET/NA group compared to the ET/CO group (**Fig. S2.1B**). Adult female distal airway had the greatest expression ($p \leq 0.05$) of SLC22A4 in the SA/NA group compared to males, female ET/NA group, and female SA/CO (**Fig. 2.5B**).

When examining GSTpi in the proximal and distal airway of juveniles, there was no significant sex difference despite ET treatment or NA exposure (**Fig. 2.5C, S2.1C**). GSTpi expression in adult males was significantly greater ($p \leq 0.01$) in the ET/NA group compared to the ET/CO group (**Fig. S2.1D**). Adult female proximal airway and male distal airway had significantly more ($p \leq 0.05$) GSTpi in the SA/NA group compared to the SA/CO, control, group (**Fig. S2.1D, 2.5D**). The distal airway had a significant sex difference in the SA/NA group with females having significantly less ($p \leq 0.01$) expression of GSTpi compared to males (**Fig. 2.5D**). Lastly, GSTpi in male distal airway significantly decreased ($p \leq 0.05$) in the ET/NA group compared to the SA/NA group (**Fig. 2.5D**).

DISCUSSION

Juvenile mice did not have a reduction in NA toxicity in the lung with ET pretreatment, although adult males did have reduced toxicity evident as decreased cellular vacuolation and protection from toxicity. We suspect that juvenile mice may have cells in the lung epithelium that are still undergoing development, have a lack of expression of a protective element, or may just have greater susceptibility to NA toxicity at the same dose. Adult mice have significantly greater accumulation of ET compared to juvenile mice, despite having no age difference in ET transporter expression, SLC22A4, in the proximal and distal airway. ET levels specifically in female juveniles

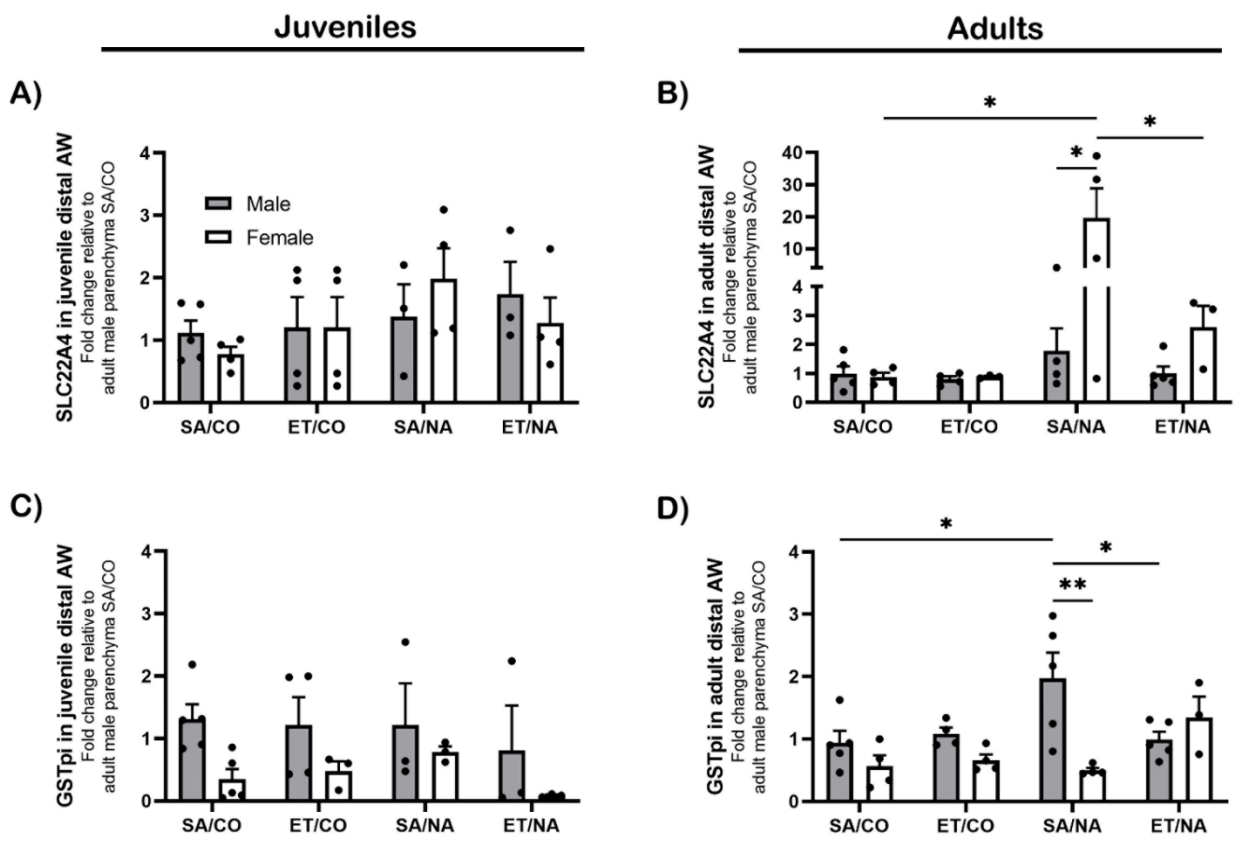
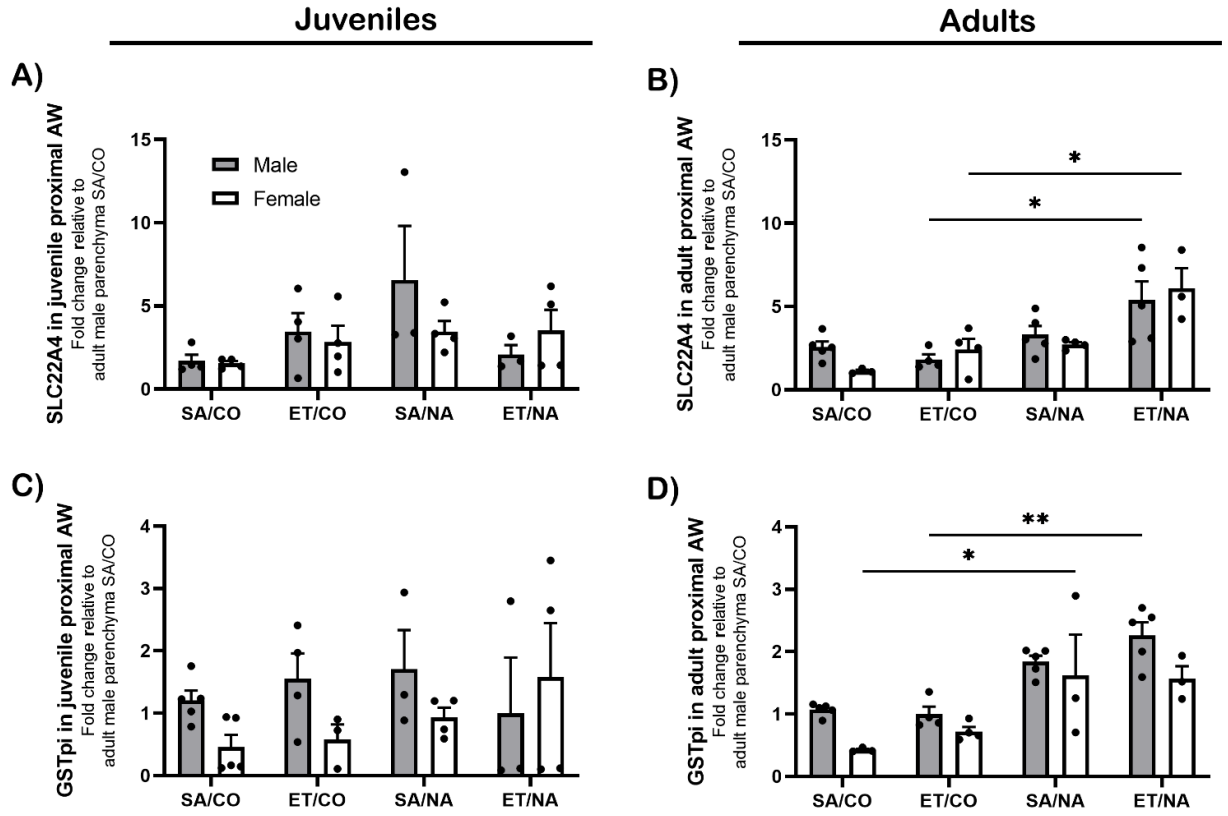


Figure 2.5 – Gene expression of SLC22A4 and GSTpi in the distal airway. Genes located in the distal airway, SLC22A4 **[A,B]** and GSTpi **[C,D]**, were measured in juvenile and adult mice treated with ET and exposed to 150 mg/kg NA for 24 hrs. Gene expression was determined using qRT-PCR in relation to Rpl13a as a housekeeping gene. Values are standard error of the mean fold change normalized to adult male parenchyma region of the SA/CO group (n= 3-5). *, $p \leq 0.05$; **, $p \leq 0.01$. Abbreviations: SA, saline; ET, ergothioneine; NA, naphthalene; CO, corn oil.



Supplemental Figure 2.1 – Gene expression of SLC22A4 and GSTpi in proximal airway. Genes located in the proximal airway, SLC22A4 **[A,B]** and GSTpi **[C,D]**, were measured in juvenile and adult mice treated with ET and exposed to 150 mg/kg NA for 24 hrs. Gene expression was determined using qRT-PCR in relation to Rpl13a as a housekeeping gene. Values are standard error of the mean fold change normalized to adult male parenchyma region of the SA/CO group (n= 3-5). *, $p \leq 0.05$; **, $p \leq 0.01$. Abbreviations: SA, saline; ET, ergothioneine; NA, naphthalene; CO, corn oil.

were low in the blood and tissues, suggesting poor retention of ET overall. Thus while juvenile and adult mice may have the same capacity to take up ET through the transporters, since transporter expression is similar, juveniles may lack other key mechanisms required to absorb, utilize, and retain ET, or may excrete it more rapidly (Cheah et al. 2016b; Tang et al. 2018).

Our stereology results suggest that ET pretreatment is effective in reducing NA induced toxicity in the proximal and distal airway of adult males, while females had no difference in vacuolated cells with or without ET pretreatment. At 24 hours after 150 mg/kg NA ip exposure, male mice are able to successful recover and/or resist NA toxicity in the lungs. Female mice are more susceptible to NA, as seen in other studies (Carratt et al. 2019; Van Winkle et al. 2002), and this could contribute to a lack of ET effectiveness as a pretreatment. A limitation of our current data is that we only conducted stereology on adult mice and not on juvenile mice. Although it is already known that juveniles are more susceptible, and stereology on immature mice has been evaluated in prior studies (Carratt et al. 2019).

Evaluation of toxicity and the response to NA treatment via analysis of gene expression of CCSP, SLC22A4, and GSTpi in juvenile and adult mice highlighted the maturation of the lungs, the susceptibility of females and juvenile mice to NA toxicity, and the limited impact of ET pretreatment in in developing or susceptible groups. CCSP gene expression in juveniles, when exposed to NA, was significantly decreased in the proximal and distal airway **(Fig. 2.4A, 2.4C)**, showing similar susceptibility patterns as in adult females **(Fig. 2.4B, 2.4D)**. Loss of CCSP gene expression can be attributed, at least in part, to Club cell loss (Martinu et al. 2023). However, when pretreated with ET, CCSP levels in the proximal and distal airway of adult females trended near control levels, despite 24 hour NA exposure, possibly indicating some sparing of Club cell loss. In contrast, there was no difference in CCSP levels between juvenile mice that were pretreated

with SA and those pretreated with ET post NA exposure, suggesting that Club cells were not spared from NA toxicity.

ET transporter, SLC22A4, is known to increase in tissues that are experiencing high levels of oxidative stress, so the observation that the adult female distal airway had statistically more ($p \leq 0.05$) SLC22A4 in the SA/NA group compared to males **(Fig. 2.5B)** was consistent and supportive of previous studies mention of female susceptibility to NA toxicity compared to males (Winkle et al. 2002). It is also important to note that the transporter expression was decreased in the females exposed to NA when treated with ET, providing indirect evidence that the females experienced less oxidative stress in the presence of ET. Lastly, GSTpi, crucial in the detoxification of NA, had a trend towards an increase in NA exposed adult female distal airway when treated with ET, compared to those treated with saline (not significant), while the males had the opposite result, a decrease ($p \leq 0.05$) **(Fig. 2.5D)**. The proximal airway had no significant sex or treatment difference post NA exposure in GSTpi levels. What was striking was that, while adult females responded to NA exposure by increasing SLC22A4 expression **(Fig. 2.5B)**, adult males responded to NA exposure by increasing GSTpi expression **(Fig. 2.5D)**; in both cases, the increased expression was reversed by ET pretreatment. The molecular mechanisms for this apparent sex difference in responses to NA exposure remains to be determined. Functionally, it is unlikely that GSTpi is responsible for the protective effects of ET against NA toxicity observed in males **(Fig. 2.3)**, given the reduction in GSTpi levels in the ET-treated group **(Fig. 2.5D)** but perhaps there is a co-regulatory relationship still to be discovered. GST developmental expression has been shown in previous studies to be highly variable with age (Buckpitt et al. 2002; Fanucchi et al. 2000a; Fanucchi et al. 2000b). GST alpha and mu expression was at low levels before birth, at high levels on postnatal day 7, at low levels again on postnatal days 14 and 21, at high levels on postnatal day

28, and then at low levels in adults. GSTpi had the greatest expression on postnatal day 4, was undetectable on day 21, and then was high again in adult mice. Since our study is a snapshot in time at 24 hours post exposure, it is likely that we are observing a post-injury recovery in gene expression that is akin to the juveniles maturation of GSTpi to near adult levels. Overall, it seems as though ET pretreatment has selective impact on the amelioration of NA toxicity in both juveniles and adults, targeting those most susceptible being adult females and those still undergoing lung development.

In conclusion our study showed that juveniles at one month of age have similar expression of genes related to development and detoxification compared to adults. In addition, we observed that ET is capable of ameliorating NA induced toxicity in adult male mice in both the proximal and distal airway, but did not have an equal impact in female mice, who were more susceptible to NA. This study supports the importance of continuing to explore potential utility of dietary antioxidants such as ET that can be beneficial to susceptible groups.

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CHAPTER 3 - SEX DIFFERENCES IN GLUTATHIONE AND GLUTATHIONE RELATED MOLECULES IN ADULT MICE PRETREATED WITH ERGOTHIONEINE AND EXPOSED TO NAPHTHALENE

It is known that the temporal pattern of response to injury and exposure plays a key role in both activation and detoxification in the conducting airways, but few studies have examined how a dietary antioxidant, ergothioneine (ET), impacts naphthalene (NA) toxicity in adult mice over acute timescales. In this study, C57BL/6 mice, maintained on an ET-free synthetic diet from conception, were given 70 mg/kg of ET via oral gavage for five consecutive days. On day 8, the mice were exposed to 150 mg/kg of NA, or corn oil (CO) vehicle, ip for 2 or 24 hours. The lung, liver, and blood were extracted and analyzed for ET, GSH/GSSG, and NA-GSH levels, as well as gene expression related to ET and glutathione (GSH). GSH concentration and GSH/GSSG ratio in the lungs were higher at 2 hours compared to 24 hours in both male and female mice in essentially all groups, implicating a large effect of the corn oil vehicle on GSH oxidation. In addition, females had significantly greater NA-GSH levels in plasma ($p \leq 0.001$), and gene expression of GLCM ($p \leq 0.05$) and GSR ($p \leq 0.001$) in the airways compared to males. These finding further support female susceptibility to NA toxicity in the lung and also support that there are major differences by sex in key elements of phase II responses to NA toxicity.

INTRODUCTION

Produced by combustible sources, NA is a volatile polycyclic aromatic hydrocarbon known to specifically target Club cells where it is metabolized by cytochrome P450 monooxygenase (P450) into an epoxide. Club cells are targeted regardless of route of exposure (Carratt et al. 2016; Plopper et al. 2001; Van Winkle et al. 1996). Detoxification of the NA epoxide involves conjugation with glutathione (GSH) with the assistance of glutathione-S-transferases (GST)

(Fanucchi et al. 2000). Time is a crucial variable in naphthalene (NA) toxicity and detoxification post exposure as cellular injury only occurs following metabolism of NA and subsequent local depletion of GSH in the target Club cell. The production of intracellular GSH is determined by glutamate cysteine ligase (GCL), which has two subunits: the modifier (GCLM) and catalytic (GCLC) subunits, and is essential in the detoxification of xenobiotics (Phimister et al. 2005; West et al. 2000; West et al. 2001). GSH, the reduced form, is oxidized by glutathione peroxidase (GPx) to generate GSSG which is the oxidized form, GSSG is reduced to GSH by glutathione S reductase (GSR). In the mouse lung exposed acutely to NA the process of GSH conjugation to the oxidative NA metabolites occurs rapidly 2-4 hours post exposure, depleting GSH and resulting in Club cell injury and loss at 24hrs (Plopper et al. 1992; Plopper et al. 2001). GSH levels and depletion/repletion are influenced by time of day, age, sex, toxicity dose, and exposure duration.

This study aims to understand how both time and an antioxidant pretreatment impact NA induced toxicity in adult C57BL/6 mice by looking at both the immediate acute phase of the response to NA at 2 hrs and the more traditional toxicity timepoint at 24 hrs and, critically, in both male and female mice. The endogenous antioxidant, GSH, is well studied in the lung and is abundant in both Club cells and conducting airways (West et al. 2002). Little is known about the impact that exogenous dietary antioxidants have on NA induced toxicity in the lung (Stohs et al. 2002). Ergothioneine (ET), a soluble amino acid synthesized by bacteria and fungi (e.g., mushrooms) has shown evidence of cellular protection in tissues that experience high levels of oxidative stress (Cheah and Halliwell 2021; Ey et al. 2007). ET, obtained solely through diet in mammals, tends to accumulate in tissues exposed to high levels of oxidative stress (Cheah and Halliwell 2020; Cheah and Halliwell 2021; Cheah et al. 2016b; Halliwell et al. 2018; Tang et al. 2018). Uniquely, ET has a specific transporter (OCTN1 or ETT) encoded by the gene SLC22A4.

The transporter is also upregulated in tissues that experience high levels of oxidative stress (Cheah et al. 2016a; Cheah and Halliwell 2021; Halliwell et al. 2018). There is still much to learn about the physiological function of ET and how this dietary antioxidants interacts with GSH in the lung post NA exposure. This study aims to explore the impacts that sex and exposure time has in lungs pretreated with ET and exposed to NA, while also understanding ET's role in ameliorating NA toxicity in the lungs.

METHODS

Chemical Sources

Naphthalene (NA), purchased from Fisher Scientific (CAS-91-20-3), was diluted in Mazola corn oil (vehicle control for NA treatment) prior to intraperitoneal injection. Ergothioneine (ET), purchased from BLDpharm (CAS-497-30-3), was diluted in saline (vehicle control for ET exposures) prior to gavage. All other chemicals were reagent grade or better.

Animals

Purchased from Envigo, C57BL/6J mice were used to create a breeding colony while maintained on ET-free synthetic diet (AIN-93G, Research Diets), which resulted in undetectable basal levels of ET in mice by HPLC-MS (< 0.4 μg/g), for the duration of the experiment. Mice were bred and housed at UC Davis on a scheduled 12-hour light/dark cycle from birth up to 2-3 months of age, litter sizes were normalized to 6 to 8 pups. Prior to the start of the study, adult mice were placed in 4 experimental groups and were given a unique access number for tracking and randomization/blinding purposes. Sentinel mice were housed in the same facility and tested negative for respiratory virus for the duration of the study. All animals were euthanized with a lethal injection of pentobarbital ip All animal experiments were performed under protocols

approved by the University of California Davis IACUC in accordance with National Institutes of Health guidelines.

Experimental Design

Mice were randomly separated into four experimental groups (*n*=5 /group): saline (SA)/ corn oil (CO), SA/ naphthalene (NA), ergothioneine (ET)/CO, and ET/NA. On day one, mice were gavaged with either saline or 70 mg/kg of ET for five consecutive days in the morning. This dose of ET was selected because it was the highest dosage that was used in prior studies and has been shown to be both safe and effective in mice (Tang et al. 2018). Two days after the last ET gavage, mice were given corn oil (CO) or 150 mg/kg of NA ip. We elected to use ip injection because this allows control of dose delivered and facilitates studies of temporal responses in the Club cell target population. At 2 or 24 hours post injection the lung, liver, and blood were extracted and processed for further analysis.

Sample preparation and LC-MS conditions for analysis of GSH/GSSG, ET and NA-GSH

Lung, liver, and blood samples were collected at 2 or 24 hours post NA exposure and flash frozen in liquid nitrogen. Tissue samples were homogenized in Tris-acetate buffer in an ice bath using a Polytron Bio-Gen Series PRO200 powered homogenizer (speed level 3 for 10 seconds). GSH/GSSG levels in liver and lung were determined by processing tissue homogenates using protein precipitation and liquid-liquid extraction. Method for detection of GSH/GSSG was adopted from prior studies (Kelty et al. 2022). ET and NA-GSH levels were analyzed both in whole blood (plasma for NA-GSH) and tissue samples. Method for detection of ET was modified from (Cheah et al. 2016a; Cheah et al. 2016b). 20 µL blood (plasma for NA-GSH) or tissue homogenates were mixed with 10 µL internal standard (Acetaminophen (AP)-GSH, 1 µg/mL, Toronto Research Chemicals) and 500 µL methanol, and then vortexed for 10s at RT and centrifuged in an Eppendorf

5424R centrifuge with 14000 rpm at 4 ℃ for 10 min. Aliquoted 50 µL of the supernatant and diluted with 450 µL water, the mixture were further processed using IsoluteC18 25mg/1-mL solid phase extraction (SPE) cartridges (Biotage, Charlotte, North Carolina). The column was activated with 1 mL methanol, equilibrium with 1 mL water, then the diluted sample was loaded, followed by collection of the unbound fraction and an additional wash with 500 µL of 10 % methanol. The collected fraction was then centrifuged at 14000 rpm for 10 min at 4 ℃ and 2 uL of the resultant supernatant was injected into the LC-MS/MS for ET and NA-GSH quantification.

Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The lung was cannulated at the trachea and fully inflated with RNA-later (Ambion/Applied Biosystems; Foster City, CA) and stored at -20 ºC. The lung was microdissected into three lung sub compartments: proximal airway, distal airway, parenchymal tissue (Baker et al. 2004). The proximal airway included the intrapulmonary bronchi and larger intrapulmonary bronchioles; the distal airway included the smaller bronchioles and terminal airways; and the parenchyma was primarily composed of connective tissues and alveoli. RNA was isolated from homogenized lung samples using a RNeasy Plus Mini kit with a gDNA elimination column (Qiagen; Hilden, Germany), and quantified with a NanoDrop spectrophotometer (ThermoFisher; Waltham, MA). The real-time PCR system (StepOnePlus; Applied Biosystems) was utilized to analyze the following genes of interest using Taqman assays (ThermoFisher; Waltham, MA) shown in **Table 3.1**. Genes were normalized to the housekeeper, RPl13a, and then to the male parenchyma of the SA/CO group. The rationale for normalizing to the males rather than females is because it is known that females are more susceptible to NA toxicity compared to males (Van Winkle et al. 2002). Out of the 3 lung regions we chose to normalize to the parenchyma region due to the absence of

Table 3.1- ThermoFisher Scientific TaqMan Assays used

Target	**Gene aliases**	**Gene name**	**Assay ID**
RPl13a	tum-antigen	ribosomal protein L13A	mm01612987_g1
Slc22a4	Octn1	solute carrier family 22 (organic cation transporter), member 4	Mm00457739_m1
Gstp1	GstpiB	glutathione S-transferase, pi 1	Mm00496606_m1
Gclm	AI649393, Gcmc, Glclr	glutamate-cysteine ligase, modifier subunit	Mm01324400_m1
Gclc	D9Wsu168e, Glclc	glutamate-cysteine ligase, catalytic subunit	Mm00802655_m1
Gsr	AI325518, Gr-1, Gr1	Glutathione S reductase	Mm00439149_m1
GPx	CGPx, GPx-1, GPx1	glutathione peroxidase 1	Mm04207457_g1

airways, compared to the proximal and distal conducting airways; further, this facilitates direct comparison of males and female responses to each other.

Statistics

Statistical analyses were conducted in GraphPad Prism. Differences between age, treatment, and time were evaluated using two-way analysis of variance. Significance found between groups was based on Tukey's multiple comparisons test (Tukey's HSD) with significance set at $P< 0.05$. All datasets were analyzed for outliers through the Grubbs' Test, resulting in significant outliers (p-value = 0.05) being omitted. Error bars are presented using standard error of mean, or SEM.

RESULTS

ET concentration in lung, liver, and whole blood

In order to understand basic temporal changes in the accumulation of ET in tissues and blood, we treated adult mice with ET and exposed them to 150 mg/kg of NA for 2 and 24 hours (**Fig. 3.1, S3.1**). Mice treated with SA had undetectable levels of ET in the lung, liver, and blood, while those treated with ET had detectable levels of ET (**Fig. 3.1, S3.1**). There was no significant difference in ET concentration at 2 or 24 hours in the lung and liver (**Fig. 3.1A - 3.1D**). Specifically at 2 hours we saw no significant sex difference in ET levels in the lung, liver, and blood (**Fig. S3.1A - S3.1C**). We only saw significant time difference in ET levels in the blood of males and females within the ET/CO and ET/NA group. ET levels in males were significantly lower ($p \leq 0.001$) at 24 hours compared to 2 hours, while females significantly increased ($p \leq 0.01$) in ET levels at 24 hours compared to 2 hours (**Fig. 3.1E - 3.1F**).

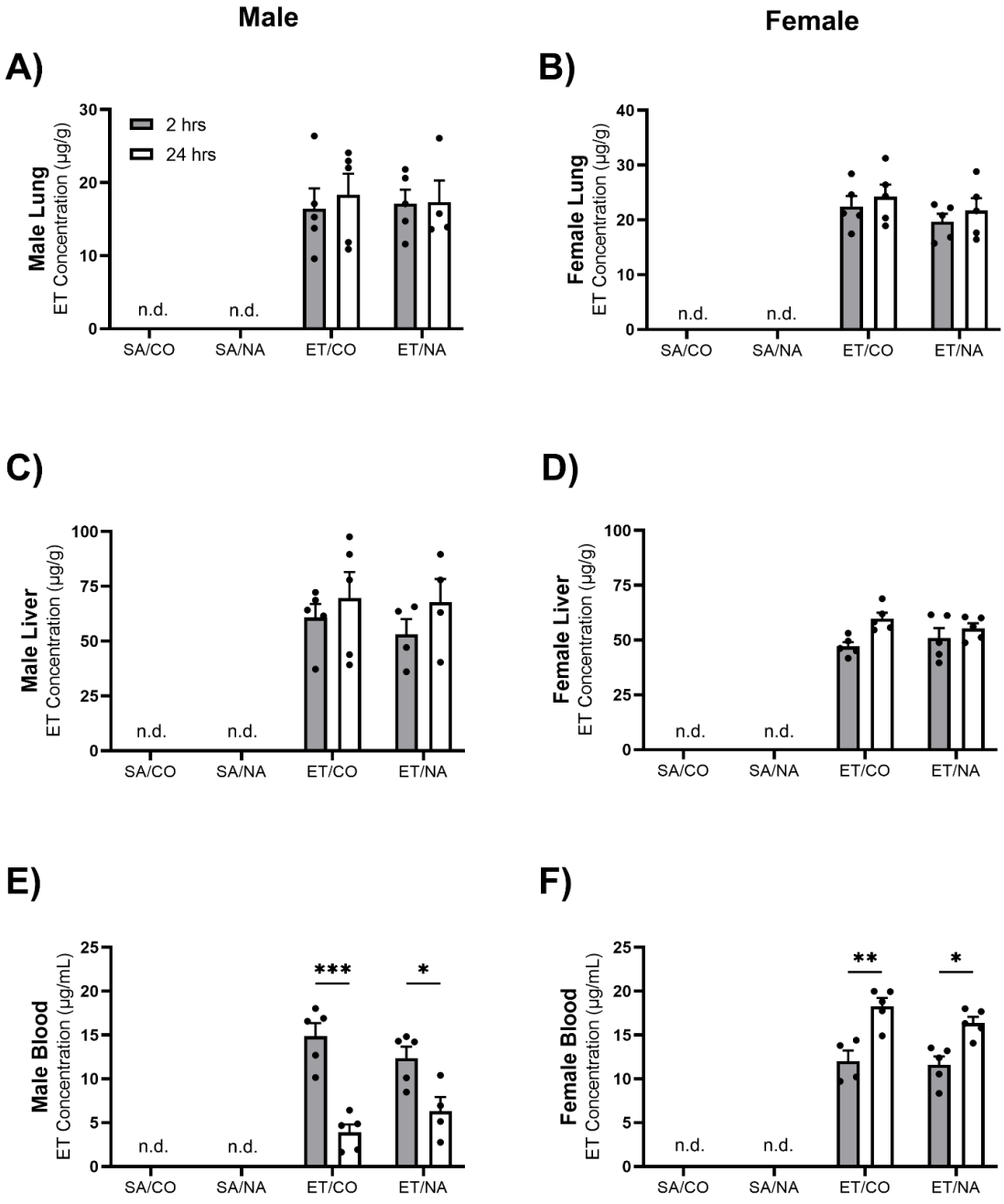
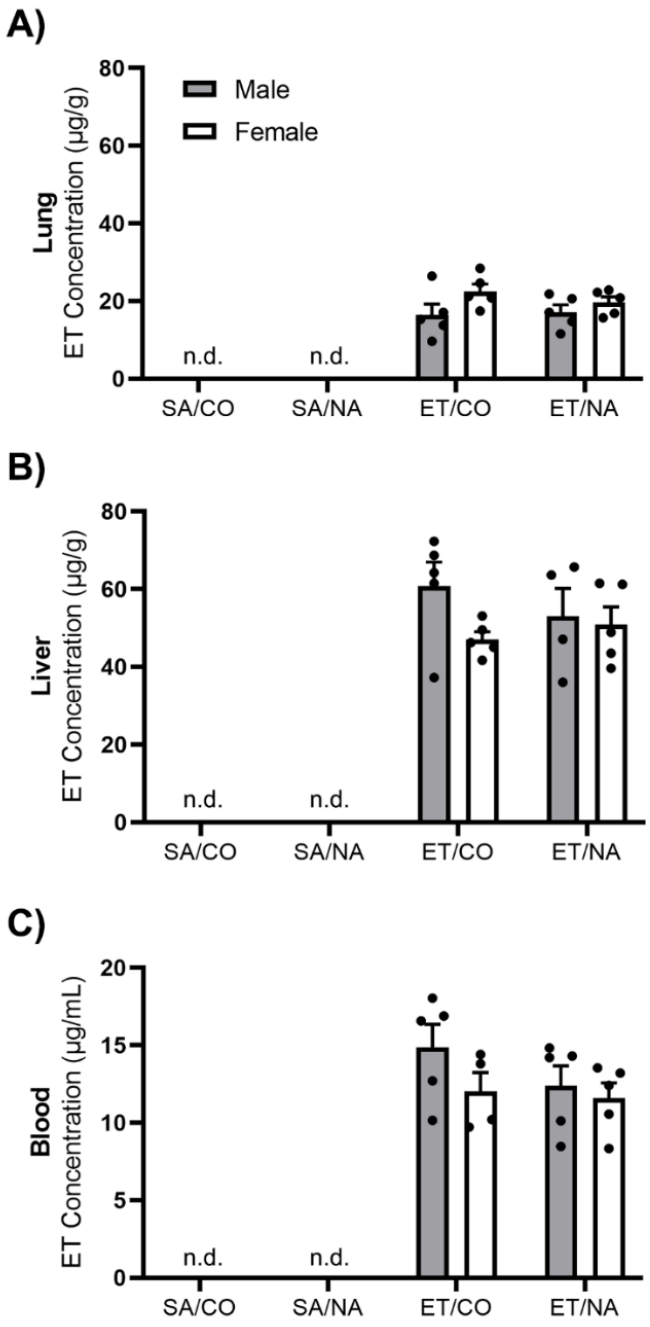


Figure 3.1- Time comparison of ET concentration in lung, liver, and whole blood. Male and female adult mice were treated with 70 mg/kg ET for 5 consecutive days. The lung **[A,B]**, liver **[C,D]**, and blood **[E,F]** were analyzed for ET concentration 2 and 24 hours post exposure to 150 mg/kg of NA via ip. The level of detection is >0.02 µg/mL in blood and >0.4 µg/g in tissue samples. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Abbreviations: n.d., not detected; SA, saline; CO, corn oil; ET, ergothioneine; NA, naphthalene.

Sup. 3.1- Sex comparison of ET concentration in lung, liver, and whole blood post 2 hours NA exposure. Male and female geriatric mice were treated with 70 mg/kg ET for 5 consecutive days. The lung **[A]**, liver **[B]**, and blood **[C]** were analyzed for ET concentration 2 hours post exposure to 150 mg/kg of NA via ip. using LC-MS. Abbreviations: n.d., not detected; ET, ergothioneine; NA, naphthalene. The level of detection is >0.02 µg/mL in blood and >0.4 µg/g in tissue samples. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: SA, saline; CO, corn oil; ET, ergothioneine; NA, naphthalene.



Endogenous antioxidants and detoxification in the lung, liver, and plasma

When measuring the GSH levels in the lungs at 2 and 24 hours post NA injection, the level of GSH significantly decreased ($p \leq 0.0001$) at 24 hours in both male and females, despite ET treatment or NA exposure (**Fig. 3.2A, 3.2B**). The GSSG levels in males significantly increased at 24 hours in each exposure group, while females had no significant changes between 2 and 24 hours NA exposures (**Fig. 3.2C, 3.2D**). The GSH/GSSG ratio was examined to determine the overall health of the lung. Both male and female mice had significantly higher ($p \leq 0.0001$) GSH/GSSG ratios at 2 hours post NA exposure compared to 24 hours in each exposure group (**Fig. 3.2E, 3.2F**). In addition, both male ($p \leq 0.01$) and female ($p \leq 0.0001$) mice exposed to NA at 2 hours had increased GSH/GSSG ratio when treated with ET compared to SA treated (**Fig. 3.2E, 3.2F**).

There was no difference with time post exposure in the levels of GSH in liver of both male and female mice, except for the male ET/CO group, which was significantly lower ($p \leq 0.01$) at 24 hours compared to 2 hours NA exposure time (**Fig. 3.3A, 3.3B**). Male GSSG concentration in the liver was significantly higher ($p \leq 0.0001$) at 24 hours compared to the 2 hour timepoint in each exposure group, while female GSSG levels had no significant change (**Fig. 3.3C, 3.3D**). The GSH/GSSG ratio in male liver was significantly lower ($p \leq 0.0001$) at 24 hours compared to 2 hours in each exposure group, while females had no significant exposure time difference (**Fig. 3.3E, 3.3F**).

We compared male and female GSH, GSSG, and GSH/GSSG ratio levels in the lung and liver of mice treated with ET and exposed to NA for 2 hours (**Fig. S3.2**). There was no significant sex difference in GSH concentration in the lung or liver (**Fig. S3.2A, S3.2B**). GSSG concentration in the lung had no significant sex change, while the liver SA/CO ($p \leq 0.001$) and SA/NA ($p \leq 0.05$) group had significantly more GSSG in female mice compared to males (**Fig. S3.2C, S3.2D**). While there was no significant sex difference for GSH/GSSG ratio in the lung, there were significant sex

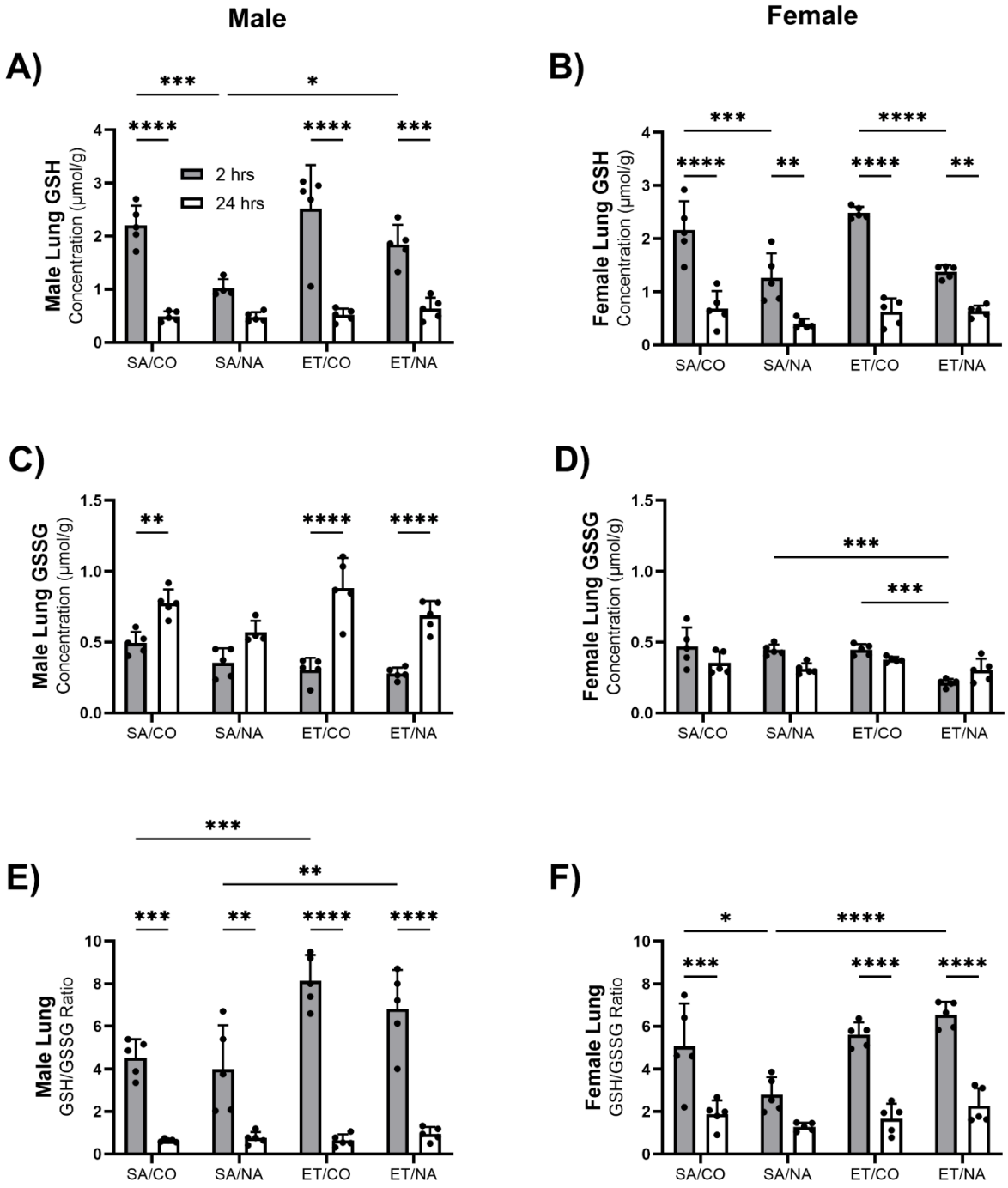


Figure 3.2 – Time comparison of GSH, GSSG, and GSH/GSSG ratio in adult lung. Adult mice were treated with ET and exposed to 150 mg/kg of NA for 2 and 24 hours. The concentration of GSH **[A,B]**, GSSG **[C,D]**, and GSH/GSSG ratio **[E,F]**, were analyzed using LC-MS in lung samples. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: SA, saline; CO, corn oil; ET, ergothioneine; NA, naphthalene.

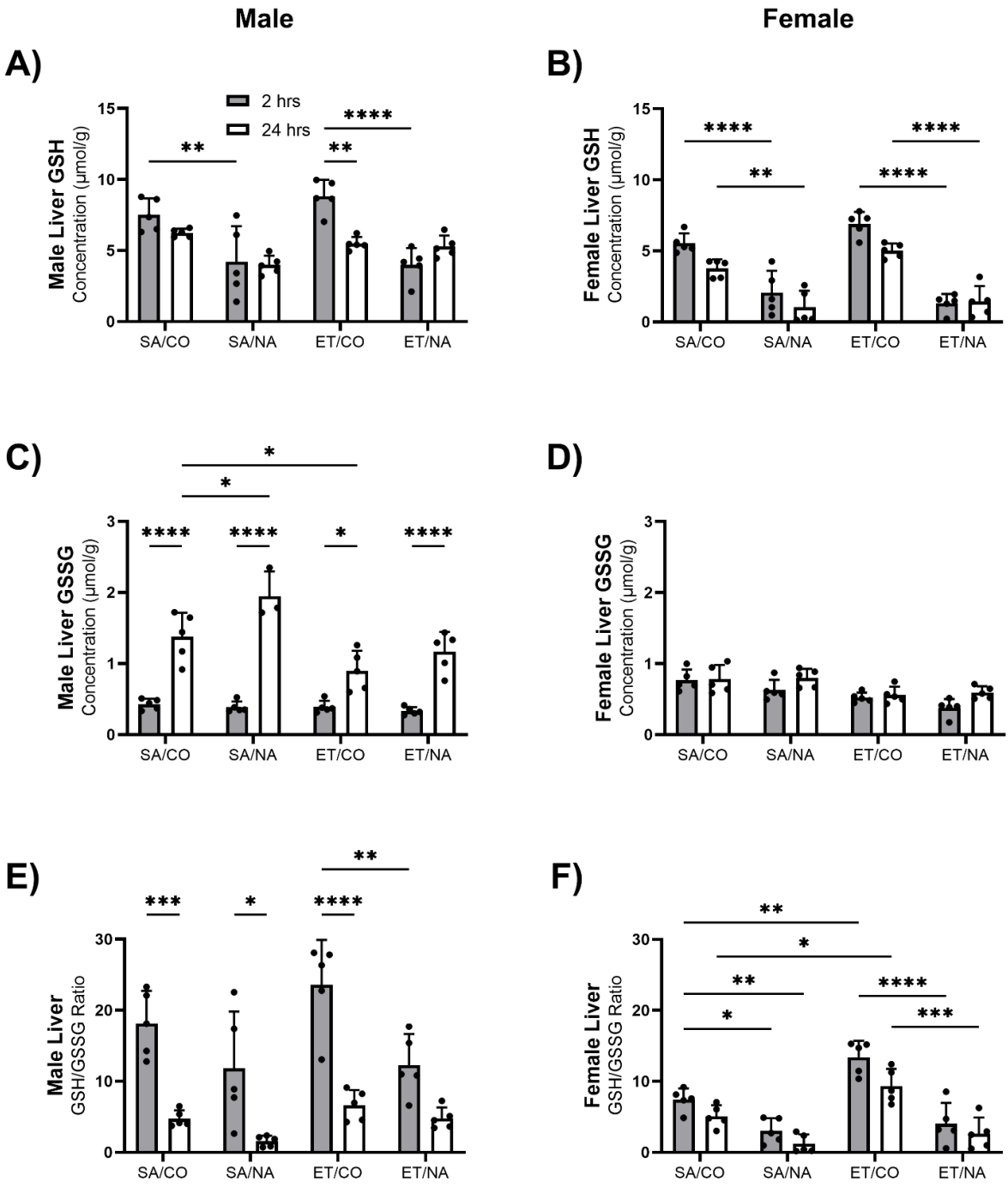
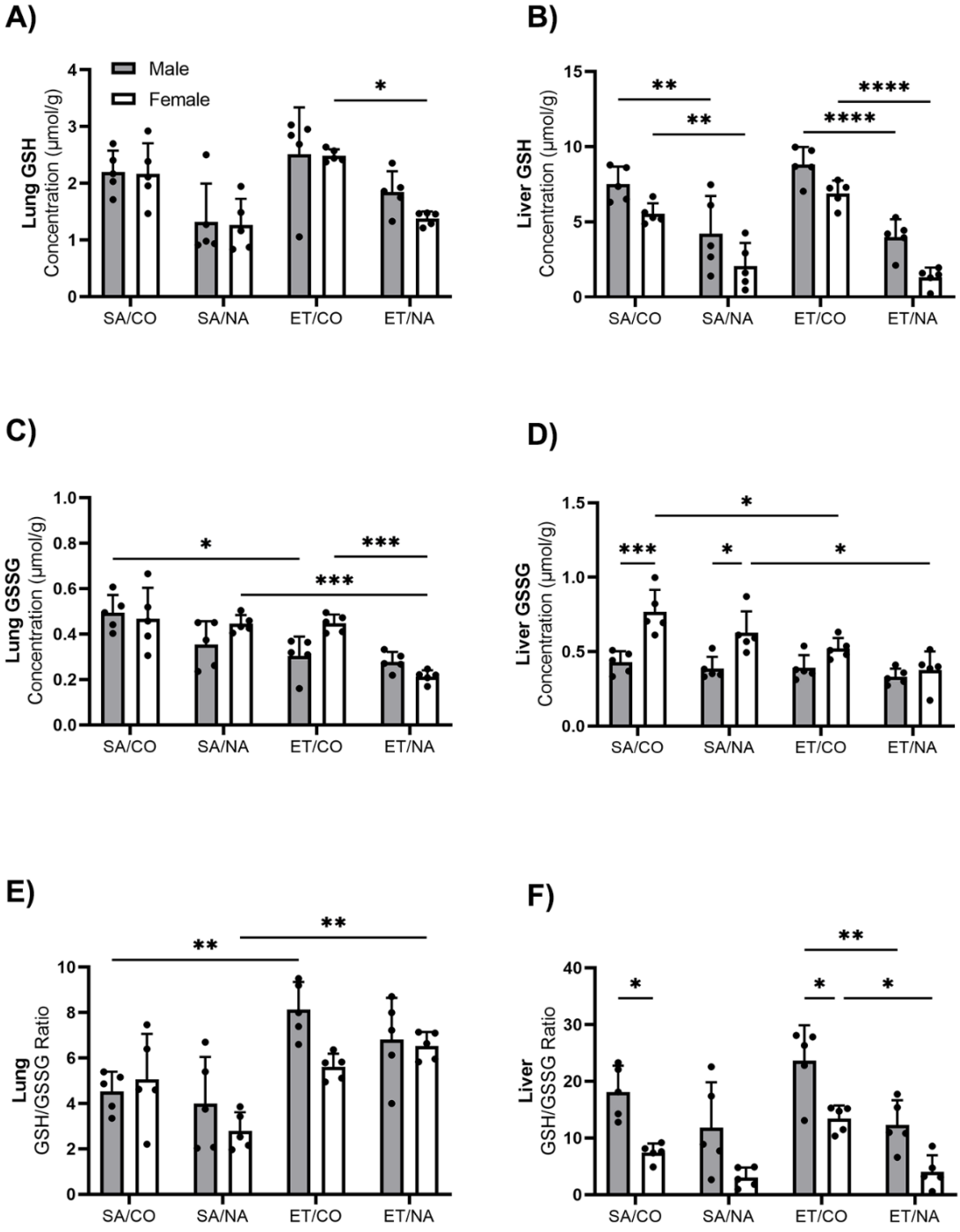


Figure 3.3 – Time comparison of GSH, GSSG, and GSH/GSSG ratio in adult liver. Adult mice were treated with ET and exposed to 150 mg/kg of NA for 2 and 24 hours. The concentration of GSH **[A,B]**, GSSG **[C,D]**, and GSH/GSSG ratio **[E,F]**, were analyzed using LC-MS in liver samples. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: SA, saline; CO, corn oil; ET, ergothioneine; NA, naphthalene.



Sup. 3.2- Sex comparison of GSH, GSSG, and GSH/GSSG ratio lung and liver post 2 hours NA exposure. Male and female geriatric mice were treated with ET and exposed to 150 mg/kg of NA ip. for 2 hours. The concentration of GSH **[A, B]**, GSSG **[C, D]**, and GSH/GSSG ratio **[E, F]**, were analyzed using LC-MS in lung and liver samples. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: SA, saline; CO, corn oil; ET, ergothioneine; NA, naphthalene.

difference in the liver of the SA/CO and ET/CO group, with females having significantly less ($p \leq 0.05$) compared to males (**Fig. S3.2E, S3.2F**).

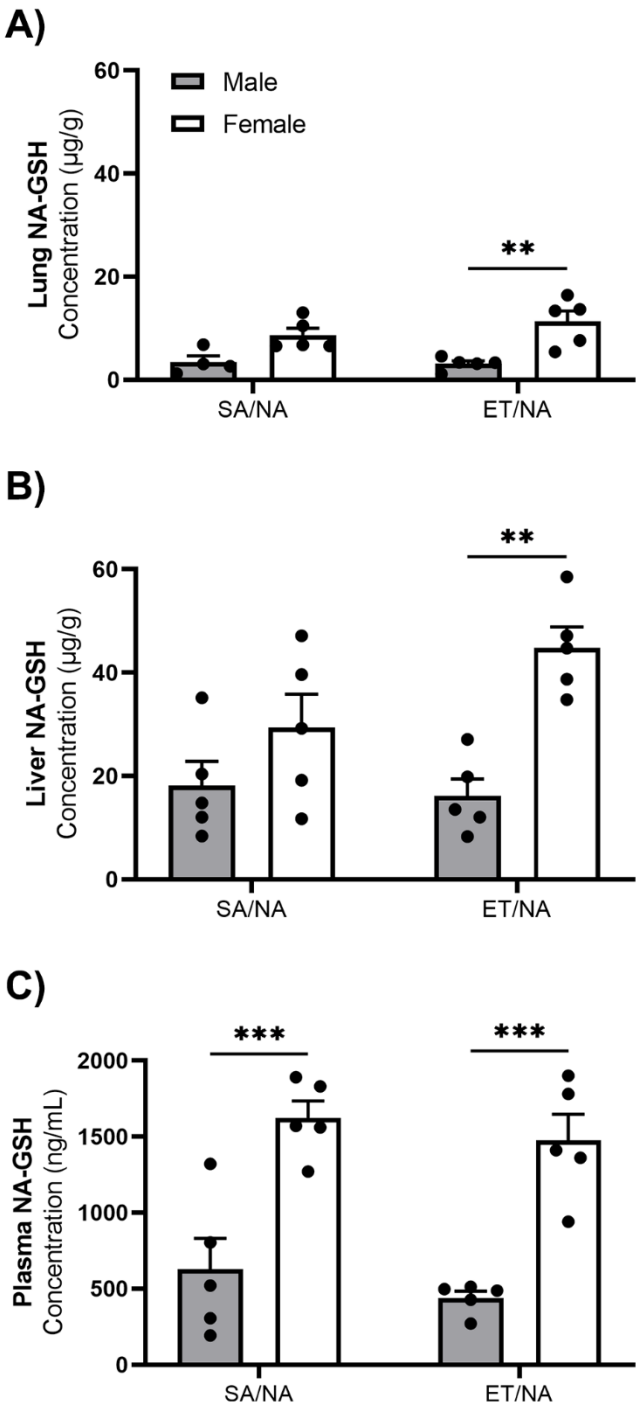
To begin the detoxification process in the lungs, GSH will bind with NA, creating a NA-GSH conjugate, which is a measurable marker of the output of NA metabolism and when GSH concentrations are high in the cell the majority of NA that is metabolized to an epoxide subsequently forms NA-GSH adducts. When exposed to NA for 2 hours, female mice had significantly more ($p \leq 0.01$) NA-GSH, regardless of ET treatment, in the lung, liver, and plasma compared to males (**Fig. 3.4A - 3.4C**).

Gene expression in microdissected lungs

Site specific expression of genes involved in GSH and ET are important to measure regionally because Club cells are the target cell type for NA and are only found in the conducting airways. To understand the GSH synthesis in the presence of an oxidative stressor, we measured the gene expression of GCL subunits, GCLM and GCLC, in the proximal and distal airway at 2 hours post NA exposure; normalized to adult male parenchyma region of the SA/CO group (**Fig. 3.5**). GCLM was significantly greater ($p \leq 0.01$) in female proximal and distal airway compared to males in all exposure groups (**Fig. 3.5A, 3.5B**). This trend was seen in the proximal AW with females having significantly more ($p \leq 0.01$) GCLC than males, specifically in the SA/CO group (**Fig. 3.5C**). GCLC in the distal airway had no significant difference between sex and exposure groups (**Fig. 3.5D**).

We then measured key enzymes involved in oxidation and reduction of GSH in the proximal and distal airway of the lungs (**Fig. 3.6**). The expression of GSR and GPx in the proximal airway was significantly greater ($p \leq 0.001$) in females compared to males in the SA/CO group (**Fig. 3.6A, 3.6C**). When treated with ET and exposed to NA for 2 hours we saw no significant

Figure 3.4 - Sex comparison of NA-GSH in lung, liver, and plasma post 2 hours NA exposure. Male and female geriatric mice were treated with ET and exposed to 150 mg/kg of NA ip. for 2 hours. The concentration of NA-GSH was analyzed in the lung **[A]**, liver **[B]**, and plasma **[C]** using LC-MS. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: SA, saline; CO, corn oil; ET, ergothioneine; NA, naphthalene.



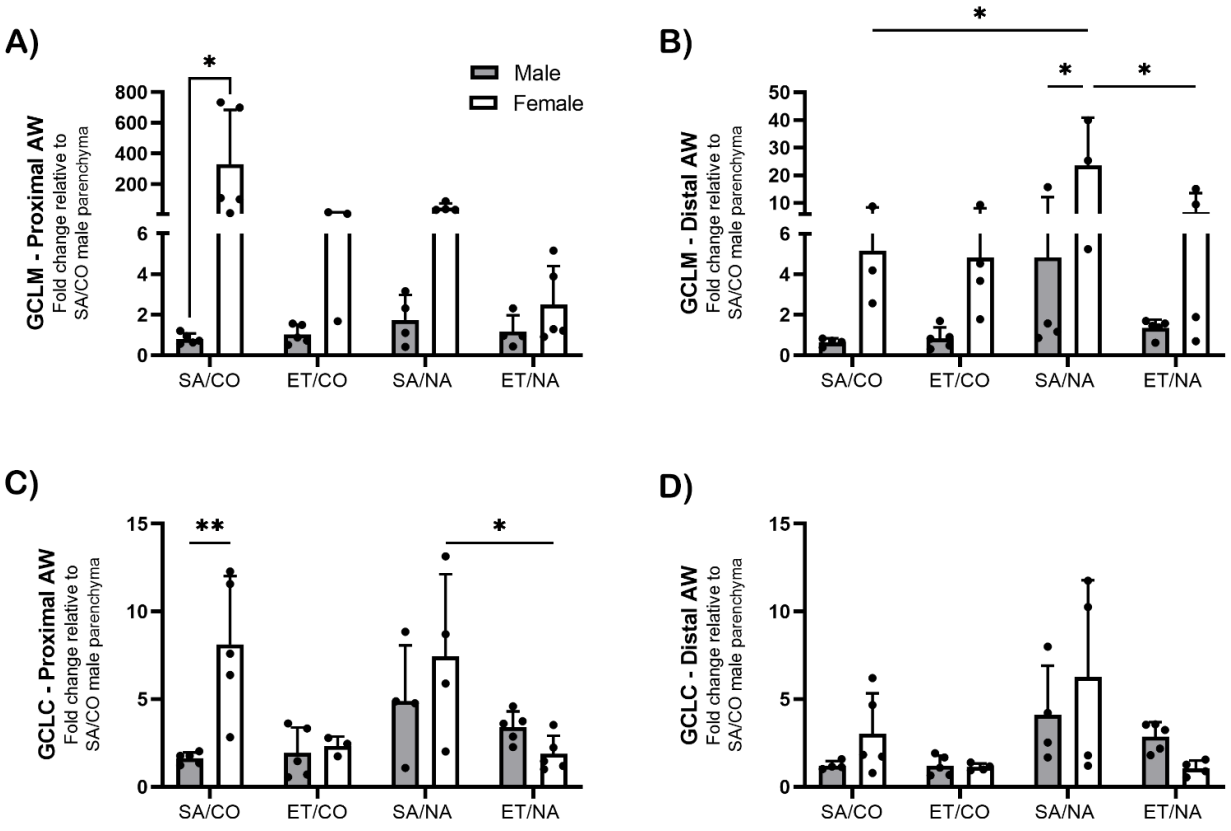


Figure 3.5 - GCLM and GCLC gene expression 2 hours post naphthalene treatment in microdissected proximal and distal conducting airways. Genes located in the proximal and distal airway, GCLM **[A-B]** and GCLC **[C-D]**, were measured in juvenile and adult mice treated with ET and exposed to 150 mg/kg NA for 2 hours. Gene expression was determined using qRT-PCR in relation to Rpl13a as a housekeeping gene. Values are standard error of the mean fold change normalized to adult male parenchyma region of the SA/CO group (n= 3-5). *, $p \leq 0.05$; **, $p \leq 0.01$. Abbreviations: SA, saline; ET, ergothioneine; NA, naphthalene; CO, corn oil.

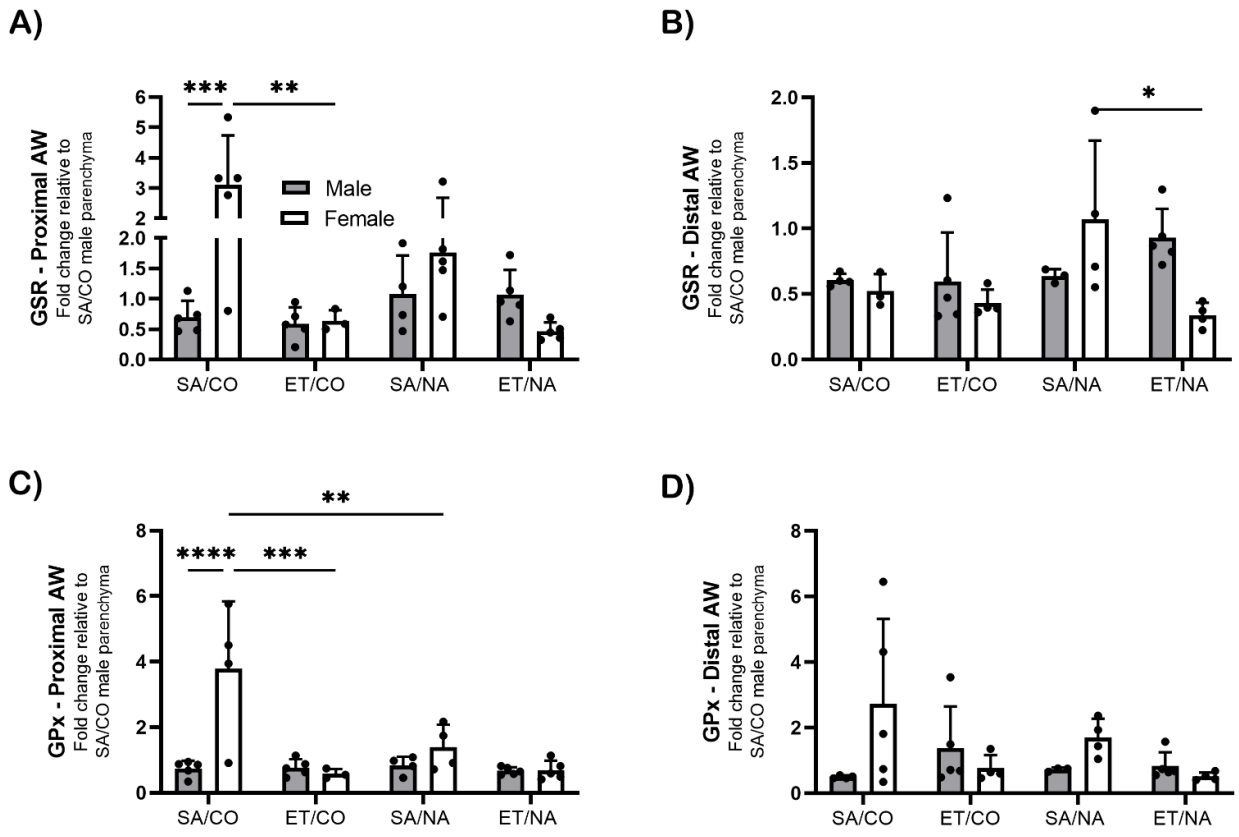


Figure 3.6- GSR and GPx gene expression 2 hour post naphthalene treatment in microdissected proximal and distal conducting airways . Genes located in the proximal and distal airway, GSR **[A-B]** and GPx **[C-D]**, were measured in juvenile and adult mice treated with ET and exposed to 150 mg/kg NA for 2 hours. Gene expression was determined using qRT-PCR in relation to Rpl13a as a housekeeping gene. Values are standard error of the mean fold change normalized to adult male parenchyma region of the SA/CO group (n= 3-5). **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: SA, saline; ET, ergothioneine; NA, naphthalene; CO, corn oil.

difference in GSR and GPx expression in the proximal or distal airway between males and females in each exposure group (**Fig. 3.6A - 3.6D**).

In order to understand the detoxification process, we looked at ET transporters, SLC22A4, and GSTpi in the proximal and distal airway of the lungs (**Fig. 3.7**). Females in the SA/CO group had significantly more ($p \leq 0.0001$) SLC22A4 in the proximal and distal airway compared to males (**Fig. 3.7A, 3.7B**). There was no significant change in SLC22A4 between sex or exposure group, except for a significant decline ($p \leq 0.0001$) in expression in the proximal airway of females in the SA/NA and ET/CO group compared to the SA/CO group (**Fig. 3.7A, 3.7B**). GSTpi in the proximal airway had significantly more expression in females compared to male in the SA/CO group (**Fig. 3.7C**). There was no significant sex difference in expression of GSTpi in the distal airway (**Fig. 3.7D**). Both the proximal and distal airway of female mice exposed to NA had significantly lower expression ($p \leq 0.001$) of SLC22A4 when treated with ET compared to mice treated with SA (**Fig. 3.7C, 3.7D**).

DISCUSSION

Since NA is known to critically deplete GSH in as little as 2 hrs, this study used bolus exposure of NA to dissect out the rapid responses, and differences in these responses, with sex and time post exposure (Phimister et al. 2004; Plopper et al. 2001). We found striking sex differences in baseline gene expression of key phase 2 enzymes in the conducting airway target zones with female mice having more GCLM, GST pi, GSR and GPX. Paradoxically female mice, which have been reported to be more susceptible to NA toxicity than male mice, had more NA-GSH formed in the lung, liver, and plasma than males indicating possibly more throughput for the female mice to this metabolite. This result is consistent with prior reports of female mice having elevated susceptibility to NA toxicity compared to males (Van Winkle et al. 2002). Elevated formation of

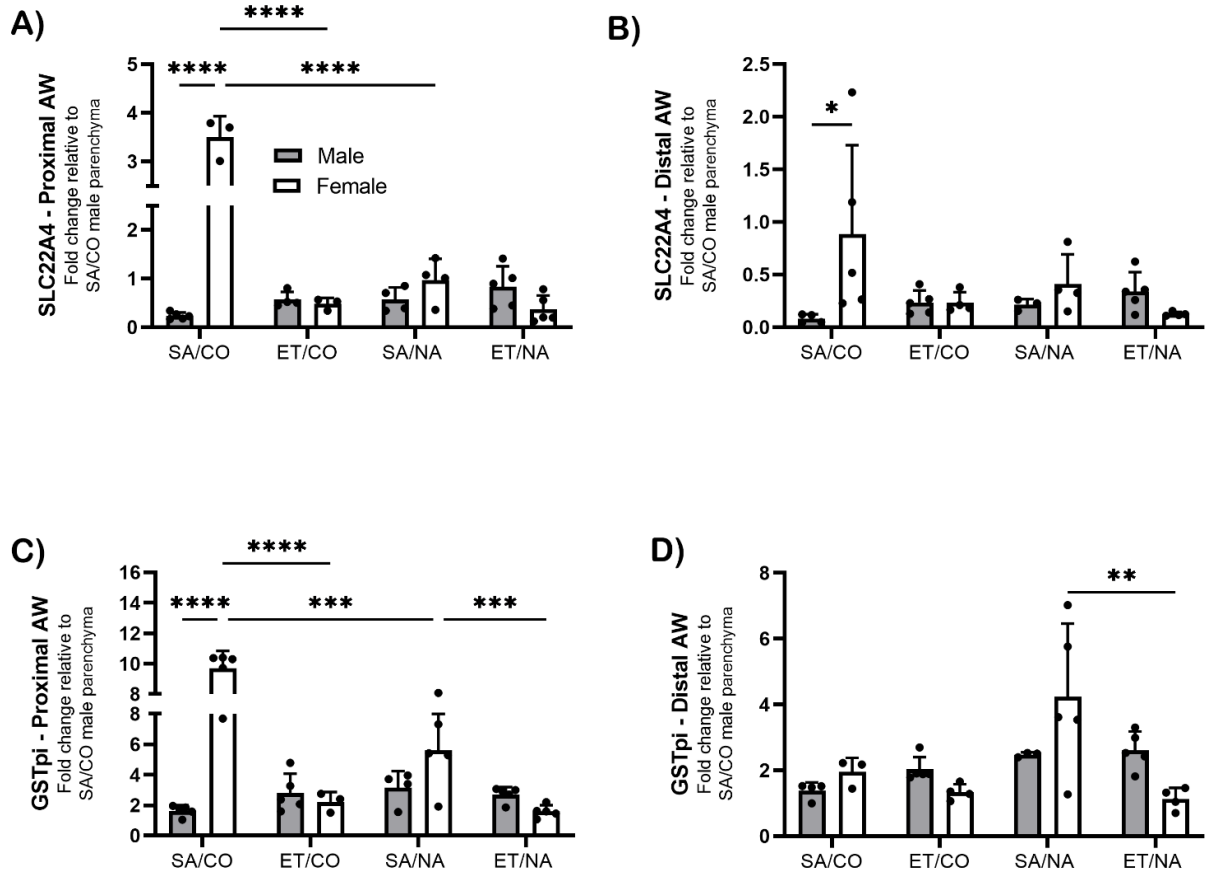


Figure 3.7- SLC22A4 and GSTpi gene expression 2 hour post naphthalene treatment in microdissected proximal and distal conducting airways . Genes located in the proximal and distal airway, SLC22A4 **[A-B]** and GSTpi **[C-D]**, were measured in juvenile and adult mice treated with ET and exposed to 150 mg/kg NA for 2 hours. Gene expression was determined using qRT-PCR in relation to Rpl13a as a housekeeping gene. Values are standard error of the mean fold change normalized to adult male parenchyma region of the SA/CO group (n= 3-5).**, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: SA, saline; ET, ergothioneine; NA, naphthalene; CO, corn oil.

NA-GSH may be facilitated by the females having greater upregulation of GCLM than males at 2 hrs post exposure to NA allowing females to readily increase GSH synthesis. There were also sex differences in response to ET pretreatment. A previous study with female C57BL6 mice exposed to NA for 1 hour had GSH levels about 1 μmol/g (Kelty et al. 2022). While our study at 2 hours had GSH levels around 2 μmol/g at 2 hrs and below 1 μmol/g at 24 hours. Females had less favorable impact of ET pretreatment on GSH levels than males. Male mice did show an impact of ET pretreatment on GSH where the ET pretreated mice preserved more GSH at 2 hrs post NA exposure. ET pretreatment in both males and females resulted in an increased GSH/GSSG ratio in the ET/NA treated group at 2 hrs compared to the SA/NA treated group, likely reflecting reduced formation of GSSG in the female combined with slightly elevated GSH in the ET/NA group at 2 hrs. This impact was not apparent at 24 hrs, underscoring the temporal nature of this acute response. There was a significant increase in GSH/GSSG ratio at 2 hours in the ET/NA compared to the SA//NA groups supporting the benefits of ET pretreatment for NA induced toxicity.

Most importantly, ET accumulation in the lung and liver were the same for males and females at both 2 and 24 hours. The exception was in whole blood which had significantly more ET in males at 2 hours and in females at 24 hours **(Fig. 3.1E, 3.1F)**. Based on the blood ET temporal pattern of accumulation in males we can see that at 2 hours ET was still circulating and distributing throughout various organs, while at 24 hours the decrease in the blood and increase in the lung and liver is a perfect illustration of how time plays a role in the distribution of ET (Cheah et al. 2016b; Tang et al. 2018). This decrease at 24 hrs may be due to tissue uptake of circulating ET. However this was not the same by sex. At 2 hours, females and males have the same level of ET in the blood, then at 24 hours females increased ET levels. This could be a result of female

susceptibility, with ET need to continue to compensate via release from some internal stores or a decrease in transporters at other sites or both.

The proximal and distal airway gene expression of GCLC, GSR, GPx, SLC22A4, and GSTpi had no significant difference by sex or exposure group post ET treatment and NA exposure in this study. However, GCLM had significant sex differences in all exposure groups with females having a greater expression of GCLM compared to males in both the proximal and distal airway with exposure. This is in contrast to a prior study conducted in NIH Swiss mice, 10 weeks of age, where gene expression of GCLM in the terminal bronchioles was significantly greater in males compared to females in the control group (CO for 7 days), and when challenged with 200 mg/kg and 300 mg/kg NA ip (Sutherland et al. 2012). However, a tolerance model may indicate additional impacts of adaptation following repeated exposures and acute single exposures weren't studied. In another study, 3-5 month old C57BL/6 mice 24 hours post aspiration of multiwalled carbon nanotubes had greater whole lung mRNA expression of GCLM in males compared to females, while GCLC expression was greater in females compared to males (Cartwright et al. 2016). Gene expression changes at 2 and 24 hours post exposure to NA are in essence a snapshot of where the expression was at that point in time, highlighting the extremely dynamic detoxification process that occurs in the lungs contingent on age, sex, target region of toxicity and toxicant. It is difficult to get the entire story of that role that ET plays in phase two metabolism in the lungs with gene expression alone. In the future we would need to conduct a study with additional time points using methods to examine protein expression and function in order to see how the molecules related to the endogenous and exogenous antioxidants are impacted.

It is interesting that CO treatment caused a decline in GSH in the lung at 24 hrs **(Fig 3.2A, 3.2B)**. A prior study conducted in rats that compared normal diet to a hyperlipidic diet primarily

composed of corn oil (CO) found that rats on the hyperlipidic diet had significantly lower TBARS levels, measuring lipid peroxidation, and low GSH levels (although not significant) in the liver compared to control diet (Valls et al. 2003). This data may seem conflicting as it shows high CO diet to be both able to reduce lipid peroxidase, while also depleting GSH. If this is the case in our study, with CO decreasing GSH, this may be contributing to the reduced efficacy of ET in the lungs. It is also important to note that ET in prior studies reduced lipid peroxidation in human skin (Dong et al. 2007), but in rat liver was unable to react with superoxide or hydrogen peroxide, and it did not inhibit microsomal lipid peroxidation in the presence of iron ions (Akanmu et al. 1991). It may seem that the efficacy of ET and the role that CO plays in the implementation of GSH redox cycle highly depends on species, exposure dose, time, and route of exposure. We'd like to also point out that our study measured GSH and GSSG in whole lung whereas many prior studies used microdissected airways only (Plopper et al. 1991; Plopper et al. 1992; Plopper et al. 2001), this may contribute to a lack of detection of this effect previously. Further, recent metabolomics studies found changes in lipids in response to NA in CO throughout the lung and this included non-target regions of the lung such as the alveolar compartment (Stevens et al. 2021). In addition, the most significant metabolic changes in the lungs were seen at earlier time points (2 hr and 6 hrs), compared to the 24 hr timepoint (Stevens et al. 2021). This confounding effect of the CO vehicle was unexpected and highlights a future research direction that needs to be pursued: use of inhalation exposures and investigation of the role of fatty diet. We would predict that ET would have more efficacy in an inhalation study due to removal of the confounding impact of CO on GSH depletion which may create a scenario in the current study where the CO and the NA effect are additive and more difficult to ameliorate with ET pretreatment.

In conclusion our study adds further support to the susceptibility of NA induced toxicity in the lungs of female mice, in addition to emphasizing the potential impact that ET pretreatment may have on endogenous antioxidant, GSH, at an earlier exposure timepoint. Sex, exposure time, and dietary antioxidant pretreatment play essential roles in the detoxification process within the lung and liver, making this study an important step in understanding how these variables impact the detoxification process of NA toxicity and ET ability to assist.

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CHAPTER 4 - IMPACT OF AGING AND ERGOTHIONEINE PRE-TREATMENT ON NAPHTHALENE TOXICITY IN LUNG

Aging increases susceptibility to lung disease, but the topic is understudied, especially in relation to environmental exposures. This study aims to define the pulmonary toxicity of naphthalene (NA) and the impacts of a dietary antioxidant, ergothioneine (ET), in the lungs of geriatric mice. NA causes a well-characterized pattern of conducting airway epithelial injury in the lung in young adult mice but NA's toxicity has not been characterized in geriatric mice. ET is a dietary antioxidant that is synthesized by bacteria and fungi. The ET transporter (ETT), SLC22A4, is upregulated in tissues that experience high levels of oxidative stress. In this study, geriatric male and female C57BL/6J mice, aged 1 - 1.5 years, maintained on an ET-free synthetic diet from conception, were fed through gavage with 70 mg/kg of ET for five consecutive days. On day 8, the mice were exposed to a single intraperitoneal NA dose of 50, 100, 150, or 200 mg/kg. At 24 hours post-injection samples were collected and analyzed for ET concentration and reduced (GSH) and oxidized glutathione (GSSG) concentrations. Histopathology, morphometry, and gene expression were examined, which revealed NA-induced lung toxicity and cellular responses. Histopathology of mice exposed to 100 mg/kg of NA suggests reduction in toxicity in the terminal airways of both male ($p \leq 0.001$) and female ($p \leq 0.05$) geriatric mice by the ET pretreatment. Our findings are the first to document the toxicity of NA in geriatric mice and show some efficacy of ET in preventing NA toxicity in the lungs.

INTRODUCTION

Americans age 65 and older comprise an estimated population of 54.1 million, with 80.8 million projected by 2040 (Ansah and Chiu 2022). Many factors contribute to susceptibility in these individuals, including medical and exposure history, declining immune response, and

reduced protection by antioxidants; all resulting in an increased risk of disease (Smithard and Yoshimatsu 2022). Lung disease can be both exacerbated and initiated by environmental exposures (Rajendra et al. 2018). This is a concern as wildfires are becoming more frequent with climate change, especially in the western and southwestern US. Chemicals in wildfire smoke include polycyclic aromatic hydrocarbons in both the vapor and particulate phases, and these can impact lung health (Lu et al. 2005; Preuss et al. 2003). Naphthalene (NA) is an abundant vapor phase chemical, found in wildfire smoke, that has a well described injury and repair pattern in young adult (Van Winkle et al. 1995; Van Winkle et al. 2002). However, NA injury and repair in aging lungs has not been previously investigated.

NA, abundant in urban areas, is metabolized by cytochrome P450 monooxygenase enzymes resulting in increased oxidative stress and cytotoxicity from reactive metabolites, e.g., naphthalene epoxide, 1,2-naphthoquinone (1,2-NQ) and 1,4- naphthoquinone (1,4-NQ). In the mouse lung, the conducting airways contain nonciliated bronchiolar epithelial Club cells which have abundant cytochrome P450 enzymes (CYP) and metabolize toxicants such as NA (Phimister et al. 2005b). NA targets Club cells in the lung regardless of the route of exposure (Plopper et al. 1992; Van Winkle et al. 1995). Typical routes of exposure to NA include inhalation and ingestion; both routes, as well as ip. injection, have been used in the mouse, demonstrating a similar lung cellular target in all cases, the nonciliated bronchiolar epithelial Club cell (Buckpitt et al. 2002; M. Abdo 2001; Plopper et al. 1992; Shopp et al. 1984). NA causes oxidative stress-related damage to the conducting airway epithelium of the lung in both sexes with a slight sex difference in susceptibility; adult females are more susceptible (Carratt et al. 2016; Stelck et al. 2005; Van Winkle et al. 2002). However, the impact of NA on a geriatric lung in either sex has not been investigated.

Endogenous antioxidants are important in protecting the lungs from oxidative stress. Glutathione (GSH) is a key player in NA detoxification as demonstrated in a series of studies using GSH depletion and/or blocking its repletion (Phimister et al. 2005a; Phimister et al. 2005b; West et al. 2000; West et al. 2001a). GSH, the reduced form, is oxidized by glutathione peroxidase (GPx) to generate GSSG, which is reduced to GSH by glutathione S reductase (GSR). When in the reduced form, GSH can detoxify reactive NA metabolites by creating NA-GSH conjugates, a process assisted by glutathione S transferase (GST) (Fanucchi et al. 2000; Forman and Zhang 2021). The production of GSH is determined by glutamate cysteine ligase (GCL), which has two subunits: the modifier (GCLM) and catalytic (GCLC) subunits. The GCL enzyme is essential to GSH homeostasis inside the cell as it catalyzes the rate-limiting step in GSH synthesis. A decline in GSH levels due to aging has been seen in rodent models as well as humans (Lu 2009; Polonikov 2020; Wang et al. 2003; Wu et al. 2004; Zhu et al. 2006). Thus, the process of aging can result in increased susceptibility to xenobiotic toxicants (Kurutas 2016; Lange et al. 2012). This could result in increased injury when challenged with environmental toxicants. Understanding the effects of oxidative stress on the geriatric population requires knowledge of antioxidant efficacy in the context of aging.

Human studies have shown that ergothioneine (ET), a dietary antioxidant, protects from oxidant stress in the aging brain (Cheah et al. 2016a). ET is derived from bacteria and fungi, with the highest concentrations found in selective species of mushrooms, e.g. king bolete, and oyster mushrooms (Ey et al. 2007). ET, which is obtained solely through diet in humans and other animals, accumulates in tissues that are exposed to high levels of oxidative stress (Cheah and Halliwell 2020; Cheah and Halliwell 2021; Cheah et al. 2016b; Halliwell et al. 2018; Tang et al. 2018). There is still much to learn about the physiological function of ET, especially in the lung.

Uniquely, ET has a specific transporter (OCTN1 or ETT) encoded by the gene SLC22A4, which is upregulated in tissues that experience high levels of oxidative stress (Cheah et al. 2016a; Cheah and Halliwell 2021; Halliwell et al. 2018).

Given that the impact of NA in aging mice is unknown, and the role of oxidant/antioxidant balance in epithelial biology of the aging lung is relatively unclear, our goal in the current study was to evaluate 1) basal antioxidant enzyme expression in aging mice, 2) responses of aging mice to NA exposure, and 3) susceptibility of the aging lung to NA-induced cytotoxicity when pretreated with ET.

METHODS

Chemical Sources

Naphthalene (NA) was purchased from Fisher Scientific (CAS-91-20-3) and was diluted in Mazola corn oil (vehicle control for NA treatment). Ergothioneine (ET) was purchased from BLDpharm (CAS-497-30-3) and was diluted in saline (vehicle control for ET exposures). Araldite 502 epoxy resin, dodecenyl succinic anhydride, and 2,4,6-Tris-(dimethylaminomethyl)phenol (DMP-30) were purchased from Ted Pella (Redding, CA). All other chemicals were reagent grade or better.

Animals

C57BL/6J mice were purchased from Envigo and used to create a breeding colony. Mice were bred and housed at UC Davis on a scheduled 12-hour light/dark cycle from birth up to 1.5 years of age. Pregnant dams and offspring were placed on an ET-free synthetic diet (AIN-93G, Research Diets), which resulted in undetectable basal levels of ET in mice by HPLC-MS (< 0.4 μg/g), for the duration of the experiment. Because the goal of the overall study was to compare mice by age, the diet used was kept constant for all ages; this defined diet was selected because it

promotes normal mouse growth and is suitable for long term studies. Prior to NA treatment, aging mice were placed in groups of 4-6 and were given a unique access number for tracking and randomization/blinding purposes. All animals were euthanized with a lethal injection of pentobarbital ip. Sentinel mice were housed in the same facility and tested negative for respiratory virus for the duration of the study. All animal experiments were performed under protocols approved by the University of California Davis IACUC in accordance with National Institutes of Health guidelines.

Experimental design

Mice from different breeding pairs were randomly placed into experimental groups. Groups (n=5) were either treated with saline (SA) or 70 mg/kg of ET by gavage for 5 days (**Fig. 4.1** – Experimental design). This dose of ET was selected because it was the highest dosage that was used in prior studies and has been shown to be both safe and effective in mice (Tang et al. 2018). Mice were then, two days after the last ET gavage, given Corn oil (CO) or NA at 50 mg/kg, 100 mg/kg,150 mg/kg, and 200 mg/kg ip. Doses were selected from prior studies in juvenile and adult mice to facilitate comparisons with the prior work and future work with mice of different ages where respiratory rate and body size would complicate dose delivered equivalencies (Carratt et al. 2019a; Fanucchi et al. 1997; Plopper et al. 2001; Van Winkle et al. 1995; West et al. 2001b). We elected to use ip injection because this allows control of dose delivered and facilitates studies of temporal responses in the Club cell target population. At 24 hours. post injection, the lungs were extracted, and the left lung was stored inflated at 30 cm of pressure in Karnovsky's fixative for embedment in araldite resin for high resolution light microscopy and stereology (Carratt et al. 2019b; Plopper et al. 1991; Sutherland et al. 2010; Van Winkle et al. 1996). The right lung from the same animal was used for RNA preparation for qRT PCR after inflation with RNALater. The

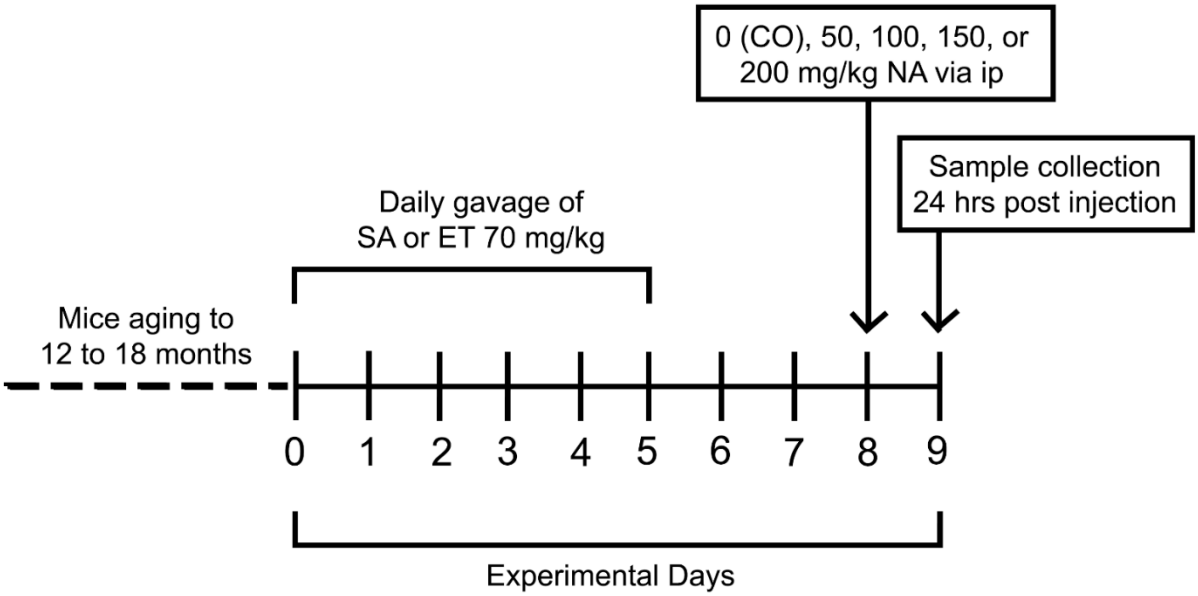


Figure 4.1- Experimental design. This figure represents the experimental design with mice aging to 12 to 18 months while on a defined diet. Abbreviations: SA, saline; CO, corn oil; ET, ergothioneine; NA, naphthalene.

time point of 24 hours post NA exposure enables observation of maximal cytotoxicity at the lung epithelium, as has been demonstrated in prior studies (West et al. 2002).

Lung microdissection and real-time PCR

The right lung lobe was cannulated at the trachea and fully inflated with RNA-later (Ambion/Applied Biosystems; Foster City, CA). The liver was diced into small cubes and stored in RNA-later. All samples were stored at -20 ºC. RNA was isolated from homogenized lungs and microdissected into three lung sub compartments: proximal airway, distal airway, parenchymal tissue (Baker et al. 2004). The proximal airway included the intrapulmonary bronchi and larger intrapulmonary bronchioles; the distal airway included the smaller bronchioles and terminal airways; and the parenchyma was primarily composed of connective tissues and alveoli. RNA was isolated using a RNeasy Plus Mini kit with a gDNA elimination column (Qiagen; Hilden, Germany), and quantified with a NanoDrop spectrophotometer (ThermoFisher; Waltham, MA). The real-time PCR system (StepOnePlus; Applied Biosystems) was utilized to analyze the following genes of interest using Taqman assays (ThermoFisher; Waltham, MA) shown in **Table 4.1**. Genes were normalized to the housekeeper, RPl13a, and then to the male parenchyma of the SA/CO or CO-only group. The rationale for normalizing to the males rather than females is because it is known that females are more susceptible to NA toxicity compared to males (Van Winkle et al. 2002). Out of the 3 lung regions we chose to normalize to the parenchyma region due to the absence of airways, compared to the proximal and distal conducting airways; further, this facilitates direct comparison of males and female responses to each other.

Table 4.1- ThermoFisher Scientific TaqMan Assays used

Target	**Gene aliases**	**Gene name**	**Assay ID**
Rpl13a	tum-antigen	ribosomal protein L13A	mm01612987_g1
Scgb1a1	CCSP, CC10, CC16	secretoglobin, family 1A, member 1 (uteroglobin)	Mm00442046_m1
Slc22a4	Octn1	solute carrier family 22 (organic cation transporter), member 4	Mm00457739_m1
Gstp1	GstpiB	glutathione S-transferase, pi 1	Mm00496606_m1
Gclm	AI649393, Gcmc, Glclr	glutamate-cysteine ligase, modifier subunit	Mm01324400_m1
Gclc	D9Wsu168e, Glclc	glutamate-cysteine ligase, catalytic subunit	Mm00802655_m1
Gsr	AI325518, Gr-1, Gr1	Glutathione S reductase	Mm00439149_m1
GPx	CGPx, GPx-1, GPx1	glutathione peroxidase 1	Mm04207457_g1

Resin Embedment and High-resolution light microscopy

The left lobes of the mice were fixed at 30 cm of constant pressure, stored in Karnovsky's fixative, and cut into 4 separate pieces, exposing the airways. The samples were processed in Zetterquist's buffer and embedded into Araldite 502 epoxy resin. Two of the four lung samples were randomly selected for sectioning at 1 μm on a microtome with glass knives. The sectioned tissue was then placed on a gelatin slide, stained with methylene blue/Azure II, and imaged on a high-resolution bright field Olympus BH-2 microscope at 20x magnification.

Stereology

Lung tissue sections were imaged by a high-resolution brightfield microscope for stereological assessment of the epithelium in the conducting airways (Hsia et al. 2010; Murphy et al. 2013). The mass (Vs or Volume per surface) of the epithelium was measured using a counting system of points (P) and intercepts (I) on a cycloid grid in Stereology toolbox software (Kelty et al. 2020). Volume fraction was calculated using the following formula: $Vs= (l/p)(\sum Pts/\sum Int)(1/2)$, where Vs is the mass ($\mu m^3/\mu m^2$), (l/p) is the length and sum of curves divided by the sum of points, and ($\sum Pts/\sum Int$) is the sum of points of either all, vacuolated, or non-vacuolated conducting airway epithelial cells divided by the sum of the intercepts of the basal lamina of the epithelium. The results of ($\sum Pts/\sum Int$) is represented as thickness or T_{epi}. The volume fraction (percentage Vv) was calculated using the equation: $Vv= \sum Pts / P_t$, where the sum of vacuolated points, $\sum Pts$, is divided by the total cells counted, P_t, which includes both vacuolated and non-vacuolated cells.

Mass Spectrometry

Frozen blood, liver, and lung samples were collected 24 hours post NA exposure. Tissue samples were homogenized in 19x volumes (v/w, i.e. 1 mg liver or lung added to 19 μL buffer) of Tris-acetate buffer (100 mM Tris-base, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 150 mM

potassium chloride (KCl), pH=7.4) in an ice bath using a Polytron Bio-Gen Series PRO200 powered homogenizer (speed level 3 for 10 seconds).

GSH/GSSG levels in liver and lung were determined by processing tissue homogenates using protein precipitation and liquid-liquid extraction as follows: 20 µL internal standard, GSH-$^{13}C2,^{15}N$ (6 µL/mL in 10 % acetonitrile; Sigma Aldrich), was added to 50 µL tissue homogenate and followed by the addition of 50 µL 1 N HCl. The samples were vortexed for 10s at room temperature (RT) and centrifuged in an Eppendorf 5424R centrifuge at 14000 rpm, 4 ℃, for 10 min. Then 50 µL supernatant was transferred to a new 1.5-mL plastic tube and combined with 350 µL water and 500 µL dichloromethane, vortexed for 30s at RT and centrifuged at 4 ℃ and 14000 rpm for 10 min. The supernatant was collected and centrifuged a second time; 5 µL of the resultant supernatant was injected into the LC-MS/MS for GSH/GSSG quantification. Method for detection of GSH/GSSG was adopted from prior studies (Kelty et al. 2022).

Method for detection of ET was modified from (Cheah et al. 2016a; Cheah et al. 2016b). To analyze ET levels, 20 µL of blood or tissue homogenates were mixed with 10 µL internal standard (hercynine-d9, 5 µg/mL, Tetrahedron) and 150 µL methanol, and then vortexed for 10 s at room temperature and centrifuged in an Eppendorf 5424R centrifuge at 14000 rpm and 4 ℃ for 10 min. The supernatant was then diluted with 800 µL water and processed using IsoluteC18 25-mg/1-mL solid phase extraction (SPE) cartridges (Biotage, Charlotte, North Carolina). The column was activated with 1 mL methanol, equilibrium with 1 mL water, then the diluted sample was loaded, followed by collection of the unbound fraction. The collected fraction was then centrifuged at 14000 rpm for 10 min at 4 ℃ and 2 µL of the resultant supernatant was injected into the LC-MS/MS for ET quantification. The LC-MS system consisted of an Agilent model 1290 HPLC (Agilent, Santa Clara, California), and a Qtrap 6500 plus mass spectrometer (Sciex, Ontario,

Canada) equipped with a Turbo IonSpray source. Analyst Software 1.6.3 was used for data acquisition and processing.

Statistics

Statistical analysis was conducted in GraphPad Prism. Differences between age and treatment groups were evaluated using two-way analysis of variance. Significance found between groups was based on Tukey's multiple comparisons test (Tukey's HSD) with significance set at $P < 0.05$. ET concentration was analyzed using one-way analysis of variance. All datasets were analyzed for outliers through the Grubbs' Test, resulting in significant outliers (p-value = 0.05) being omitted. Error bars are presented using standard error of mean, or SEM.

RESULTS

Gene Expression in geriatric sham treated lung

Gene expression in three microdissected regions from male and female lungs was analyzed for key elements of phase two metabolism involving conjugation and detoxification, as well as other cellular markers, with fold change relative to geriatric male parenchyma. Club cell secretory protein (CCSP) was expressed at significantly greater levels ($p \leq 0.01$) in the female proximal airway compared to the distal airway (**Fig. 4.2A**). In addition, there was a significantly greater expression of CCSP in the male ($p \leq 0.001$) and female ($p \leq 0.0001$) proximal airway compared to the parenchyma (**Fig. 4.2A**). Similarly, to CCSP expression, ergothioneine transporters (SLC22A4) in geriatric females was significantly greater in the proximal airway compared to the distal airway ($p \leq 0.01$) (**Fig. 4.2B**). There was no significant difference in regard to sex or location for expression of glutathione transferase (GSTpi) (**Fig. 4.2C**). Glutathione reductase (GSR) in the parenchyma ($p \leq 0.0001$), distal airway ($p \leq 0.001$), and proximal airway ($p \leq 0.01$) was significantly lower in females compared to males (**Fig. 4.2D**). In contrast, glutathione peroxidase

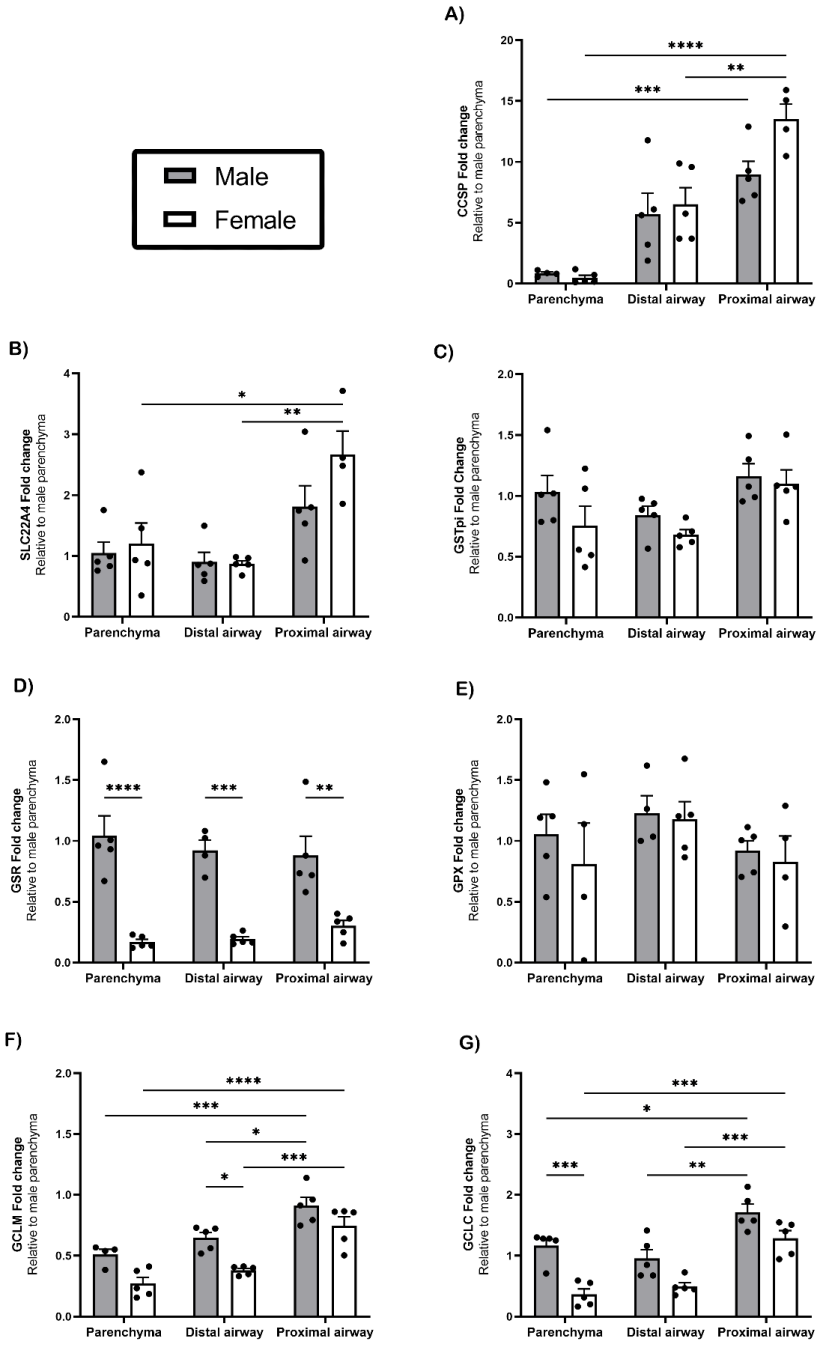


Figure 4.2- Site and sex specific differential expression of genes in the lung. Gene expression was determined by qRT-PCR in relation to Rpl13a as a housekeeping gene. CCSP **[A]**, SLC22A4 **[B]**, GSTpi **[C]**, GSR **[D]**, GPx **[E]**, GCLM **[F]** and GCLC **[G]** were measured in lung samples from untreated male and female geriatric mice (1 to 1.5 years) n= 4-5. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: SLC22A4, ergothioneine transporter; GSTpi, glutathione S transferase; GSR, glutathione S reductase; GPx, glutathione peroxidase; GCLM, glutamate-cysteine ligase modifier subunit; GCLC, glutamate-cysteine ligase catalytic subunit.

(GPx) showed no significant difference in regard to sex or location (**Fig. 4.2E**). Glutamate cysteine ligase subunit modulator (GCLM) (**Fig. 4.2F**), and catalytic (GCLC) (**Fig. 4.2G**) was significantly expressed in the proximal airway compared to the distal airway, having the greatest expression in males compared to females in all lung regions ($p \leq 0.001$).

Impact of aging on genes related to the abundance of oxidized and reduced GSH in lung tissue

To determine whether the reduction in GSR levels in geriatric female proximal and distal airways, compared to males, was due to aging, we compared the adult and geriatric control groups for gene expression of GSR and GPx, with fold change relative to adult male parenchyma (**Fig. 4.3**). We found that expression of GSR in the proximal and distal airways of adult mice was not significantly different by sex, unlike in the geriatric mice, and geriatric males (but not females) had greater expression of GSR than adult males in the parenchyma ($p \leq 0.0001$), proximal ($p \leq 0.01$), and distal airways ($p \leq 0.05$) (**Fig. 4.3A**). Expression of GSR was significantly lower in the parenchyma ($p \leq 0.0001$), proximal ($p \leq 0.001$), and distal airways ($p \leq 0.05$) of geriatric females than males (**Fig. 4.3A**). Strikingly, GPx was highly expressed in geriatric mice compared to adults, in all lung regions in both males and females (**Fig. 4.3B**).

ET levels in whole lung, liver, and whole blood

Geriatric mice treated with SA (n= 4-5) and maintained on an ET free diet had undetectable levels of ET in lung (**Fig. 4.4A**), liver (**Fig. 4.4B**), and blood (**Fig. 4.4C**). The minimum detectable level of ET in blood was 0.02 μg/mL, and 0.4 μg/g in tissue samples. All samples collected from mice treated with ET had detectable levels of ET. Liver had greater concentration of ET compared to lung. There was no significant difference between sex or exposure groups in the blood and tissue samples.

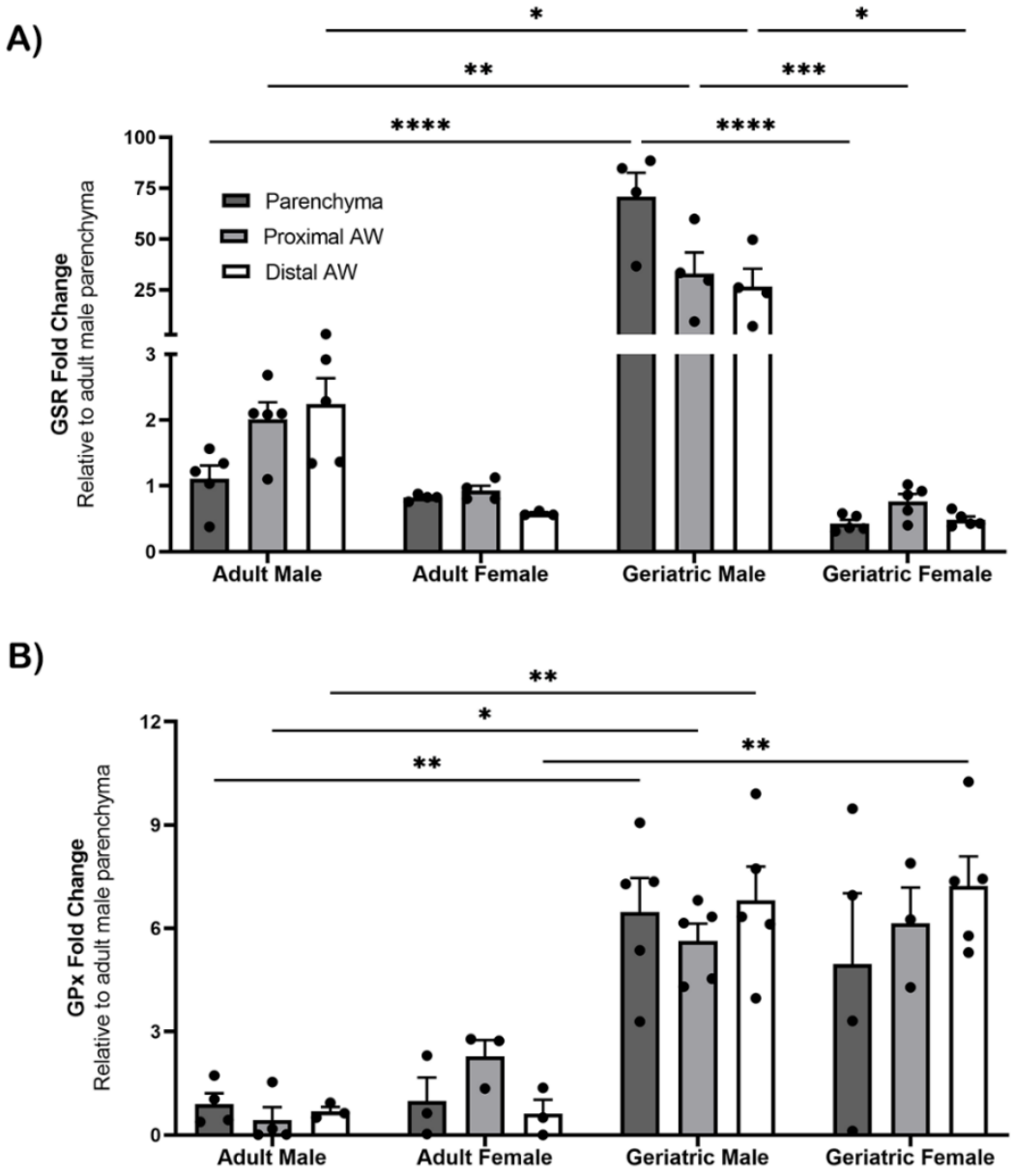


Figure 4.3- Comparison of adult and geriatric gene expression of GSR and GPx in the microdissected lung. The lungs of male and female adult (2 to 3 months) and geriatric (1 to 1.5 years) mice in the control group were microdissected and analyzed for GSR **[A]** and GPx **[B]** expression. Gene expression was determined by qRT-PCR in relation to Rpl13a as a housekeeping gene. Values are standard error of the mean fold change normalized to the adult male parenchyma region (n= 3-5). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: AW, Airway; GSR, glutathione S reductase; GPx, glutathione peroxidase.

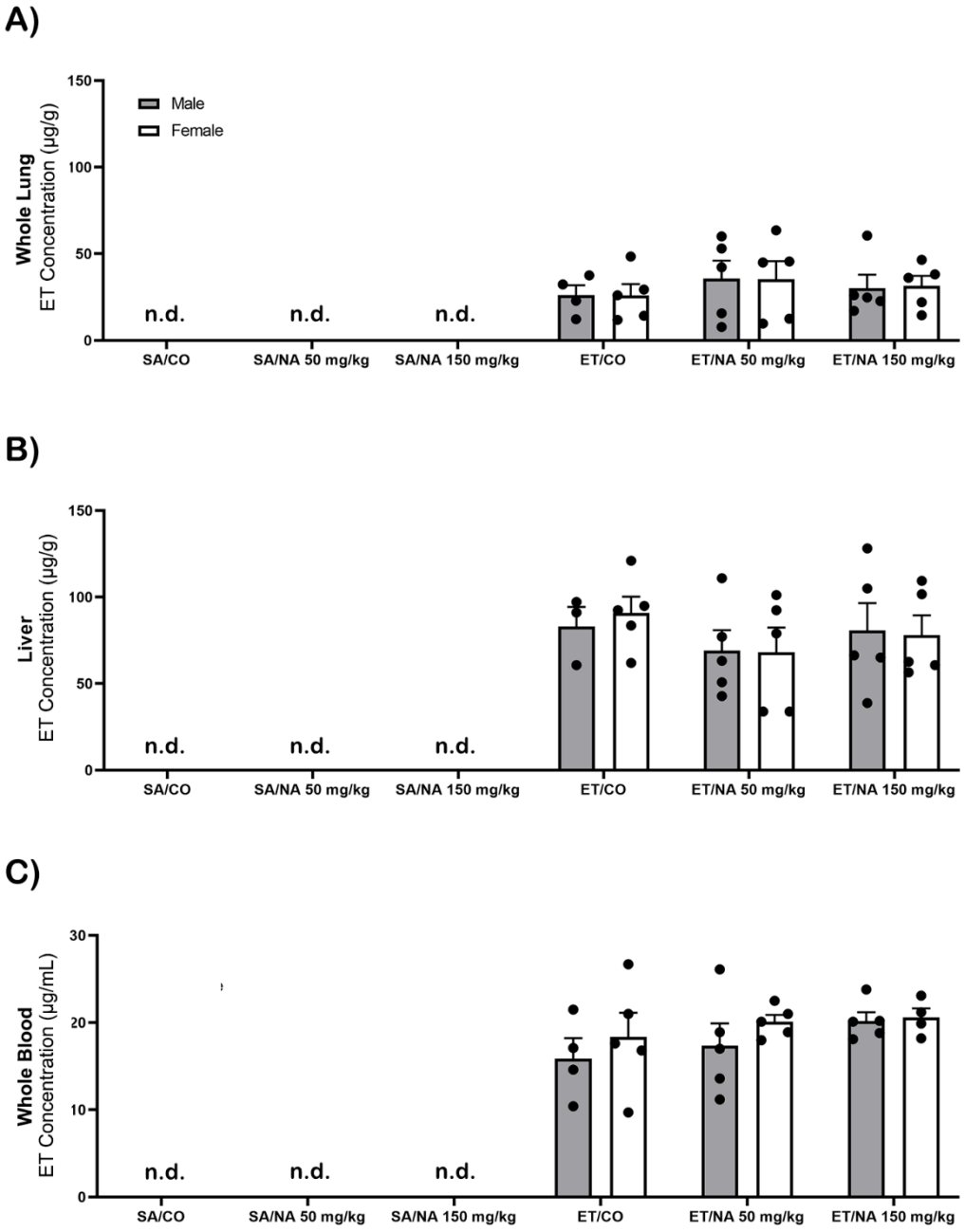
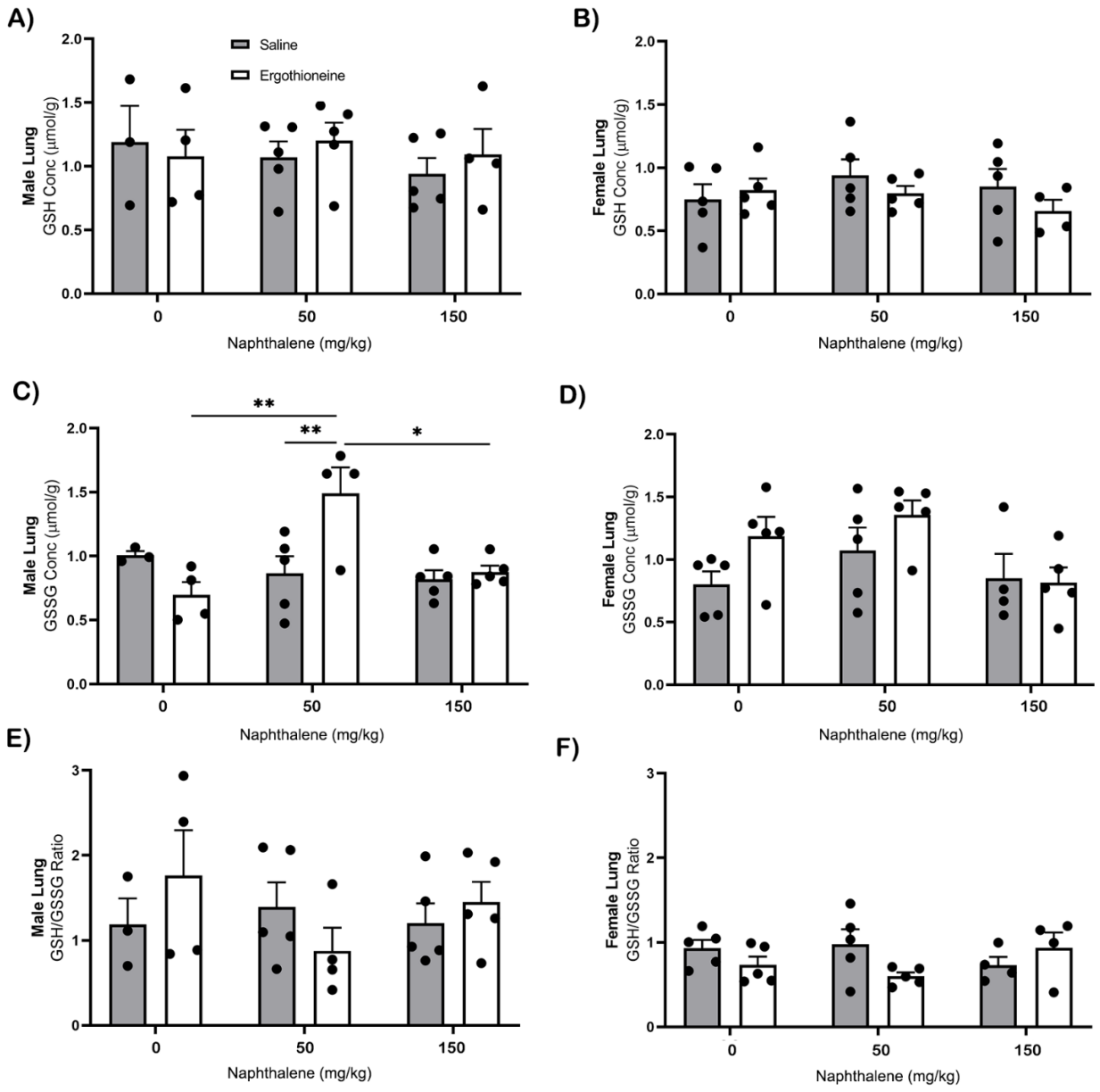


Figure 4.4- Accumulation of ET & lack of ET concentration changes in whole lung, liver, and whole blood after NA exposure. Geriatric mice (n=4-5) were treated with 70 mg/kg ET for 5 consecutive days. The lung **[A]**, liver **[B]**, and blood **[C]** were analyzed for ET concentration 24 hours post exposure to 150 mg/kg of NA using HPLC-MS. The level of detection is >0.02 µg/mL in blood and >0.4 µg/g in tissue samples. Two-way ANOVA showed no significance between male and females in the treated group. Abbreviations: n.d., not detected; SA, saline; CO, corn oil; ET, ergothioneine; NA, naphthalene.

GSH, GSSG and GSH/GSSG in the lung and liver

To assess the capacity of the prevalent endogenous antioxidant GSH to mitigate oxidative stress in geriatric mice, we measured the concentration of oxidized and reduced glutathione in both the lung and liver using HPLC-MS. In the lung, there was no significant difference in the GSH concentration in both male and female mice between saline and ET treated groups or among groups treated with 0, 50, and 150 mg/kg of NA , at 24 hours after exposure (**Fig. S4.1A, S4.1B**). When male mice were treated with ET and exposed to 50 mg/kg of NA, the GSSG level in the lungs increased by 2-fold ($p \leq 0.01$) compared to the control. Additionally, ET treated mice had a 2-fold greater ($p \leq 0.01$) concentration of GSSG at 50 mg/kg than untreated mice. As the concentration increased from 50 mg/kg to 150 mg/kg of NA the GSSG concentration significantly decreased by 2-fold in the ET treated male group (**Fig. S4.1C**). As for females, there was no significant difference in GSSG concentration between the treated and untreated groups (**Fig. S4.1D**). The GSH/GSSG ratio, an indicator of cellular health, had no significant differences between the groups of male and female mice (**Fig. S4.1E, S4.1F**).

GSH concentrations in the liver decreased by 2-fold in both males ($p \leq 0.05$) and females ($p \leq 0.001$) as NA concentration increased (**Fig. 4.5A, 4.5B**). ET treated female mice had a 2-fold decrease ($p \leq 0.01$) in GSH as NA concentration increased (**Fig. 4.5B**). GSSG in the liver showed no significant difference in males (**Fig. 4.5C**). Female GSSG levels significantly increased in both the ET treated and untreated groups ($p \leq 0.05$) as the NA concentration increased (**Fig. 4.5D**). Male and female GSH/GSSG ratio in the liver of the untreated group significantly decreased 2-fold ($p \leq 0.05$) from 50 mg/kg NA to 150 mg/kg NA.



Supplemental Figure 4.1- GSH, GSSG and GSH/GSSG ratio in the lung. Male and female geriatric mice (n=3-5) have GSH **[A-B]**, GSSG **[C-D]** levels measured using HPLC-MS post ET pretreatment and 24 hours after NA exposure to 50 and 150 mg/ kg ip. GSH/GSSG ratio, an indicator of lung health, was also evaluated in both males **[E]** and females **[F]**. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: ET, ergothioneine; NA, naphthalene.

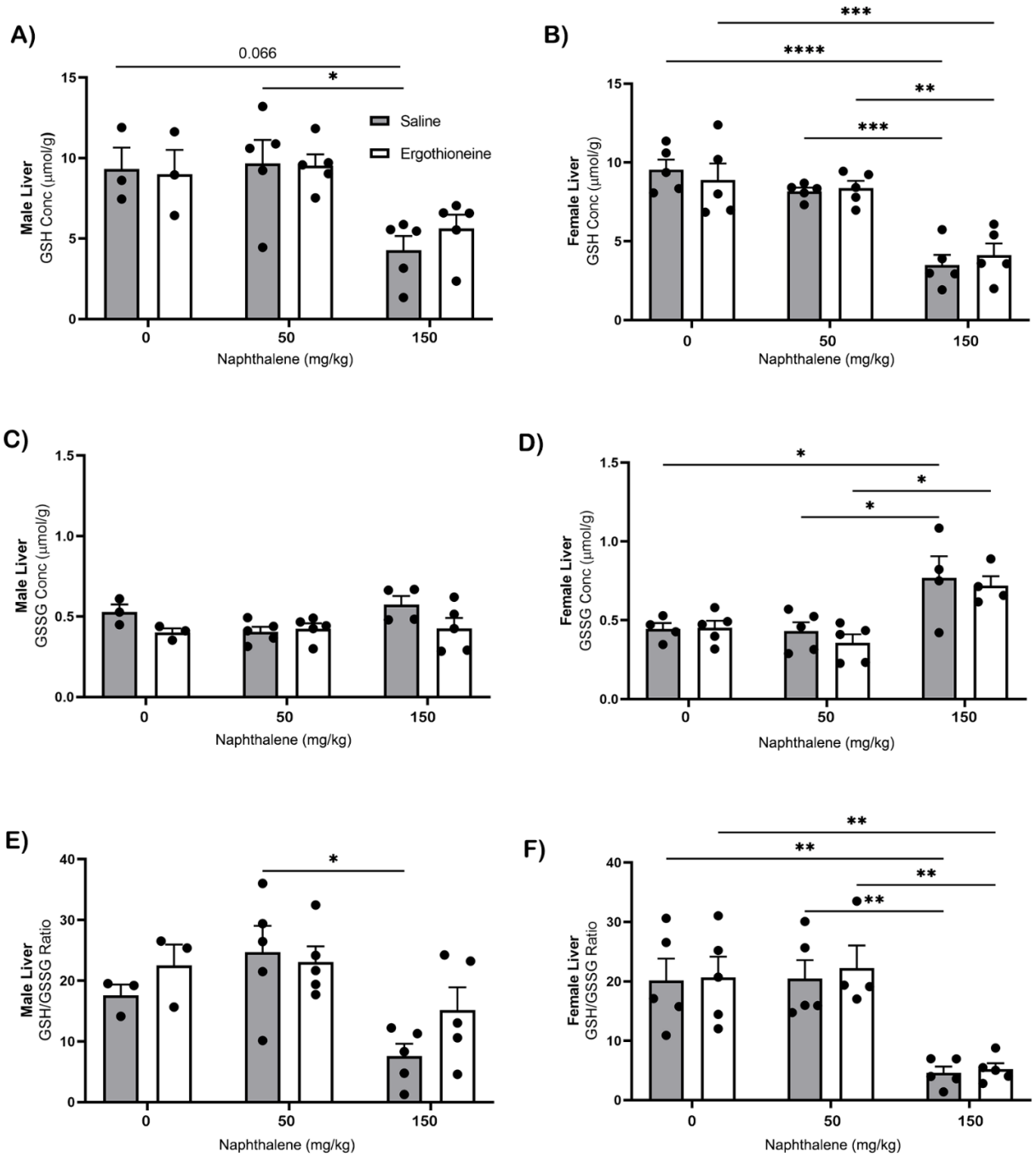


Figure 4.5- GSH, GSSG and GSH/GSSG ratio in the liver. Male and female geriatric mice (n=3-5) have GSH **[A-B]**, GSSG **[C-D]** levels measured using HPLC-MS post ET pretreatment and 24 hours after NA exposure to 50 and 150 mg/ kg ip. GSH/GSSG ratio, an indicator of liver health, was also evaluated in both males **[E]** and females **[F]**. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: ET, ergothioneine; NA, naphthalene.

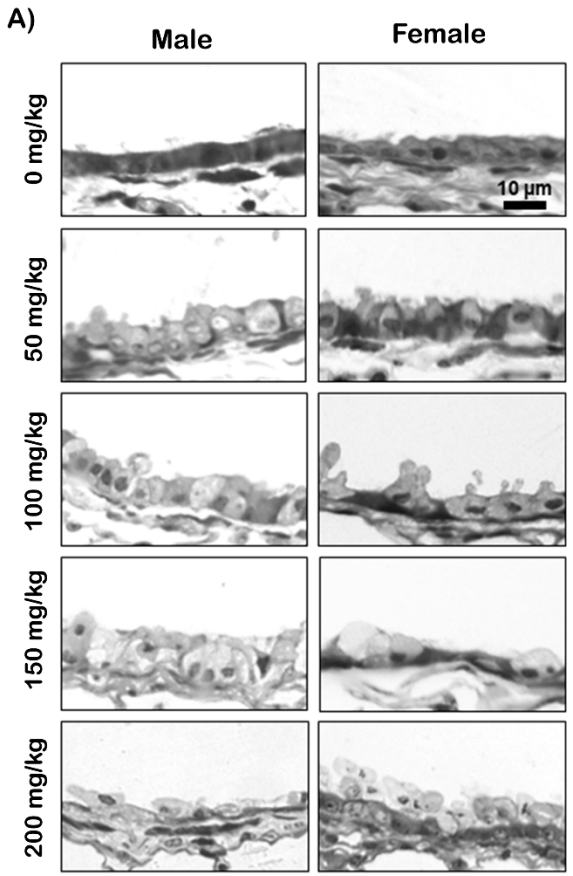
This was also observed in ET treated mice, however the ET treated male liver exposed to 150 mg/kg NA had greater ratios than the saline treated mice exposed to 150 mg/kg, although not significant (**Fig. 4.5E, 4.5F**).

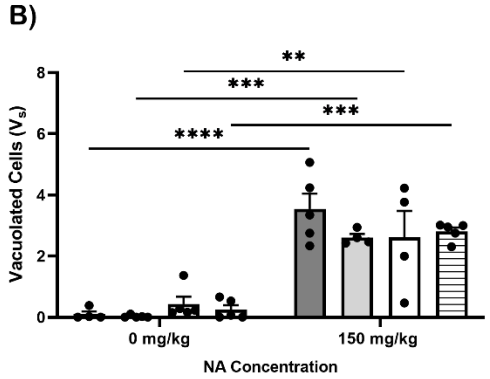
Histology and Stereology of NA toxicity in the proximal and terminal airway

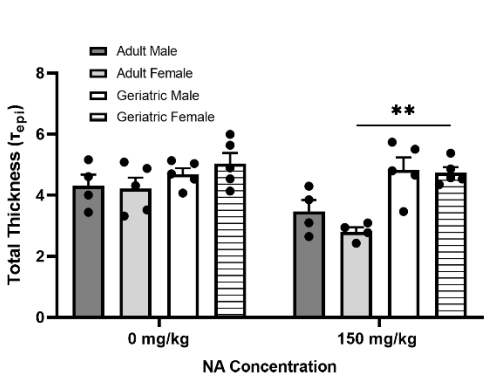
Male and female geriatric mice were given ip doses of 0 mg/kg (control), 50 mg/kg, 100 mg/kg, 150 mg/kg, or 200 mg/kg of NA in corn oil vehicle. Both the proximal (**Fig. S4.2A**) and terminal airway (**Fig. 4.6A**) were imaged for histopathology. The airway at 0 mg/kg NA represents lungs unexposed to NA. Mice exposed to 50 mg/kg of NA had the least damage on the lung epithelium, with ciliated cells, and some Club cells, in the airway appearing uninjured. Some Club cells that are beginning to swell are visible in the epithelium of the airways. At 100 mg/kg of NA, more damage was visible as cytoplasmic vacuoles compared to the 50 mg/kg dose and Club cells are increasing in size (swelling). At 150 mg/kg of NA, much more damage was apparent and prominent vacuolation was present in the airway epithelium. The proximal and terminal airway epithelium of the males contained an increased distribution and abundance of swollen and vacuolated epithelial cells compared to the females. The highest dose (200 mg/kg NA) caused severe damage to the lung epithelium in the airways of both males and females. All of the cells on the male terminal airway were vacuolated and the cell renewal process on the lung epithelium has begun with lost cells being replaced by squamated surviving cells (**Fig. 4.6A**). The female terminal airway epithelium also displays evidence of advanced vacuolation, cell damage and cell loss with squamation of remaining epithelia (**Fig. 4.6A**).

The mass (V_s) of vacuolated cells was measured in the proximal and distal airway of two different age groups: young adult, 2 – 3 months, and geriatric, 1 – 1.5 years. The mass of vacuolated cells at 150 mg/kg NA significantly increases ($p \leq 0.01$) in the proximal (**Fig. S4.2B**)

and distal (**Fig. 4.6B**) airways compared to 0 mg/kg for both sexes and ages. Specifically, in the distal airway, adult females have significantly lower ($p \leq 0.05$) vacuolated cells compared to adult male (**Fig. 4.6B**). When comparing by age, the distal airway of geriatric males has significantly lower ($p \leq 0.0001$) vacuolated cells compared to adult males (**Fig. 4.6B**). In regard to total thickness





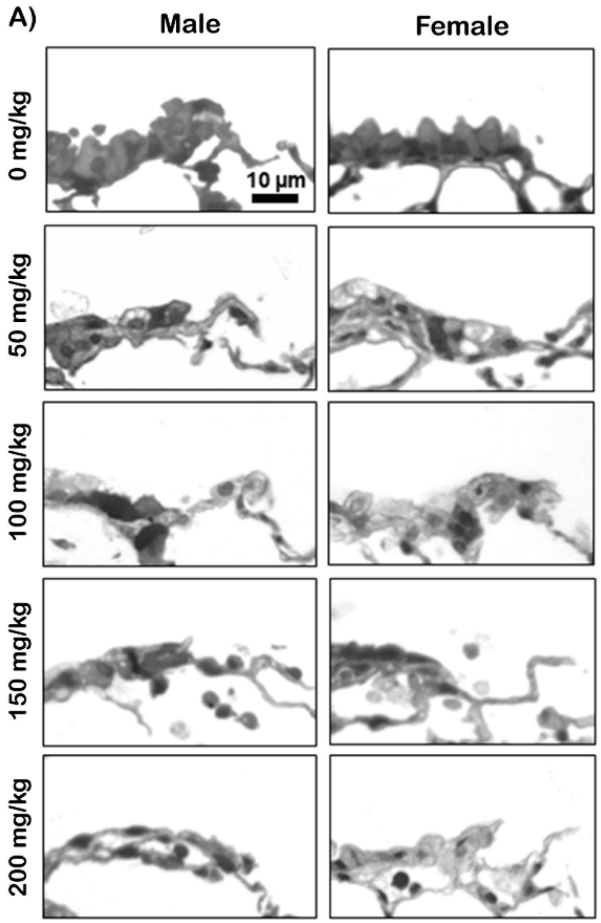


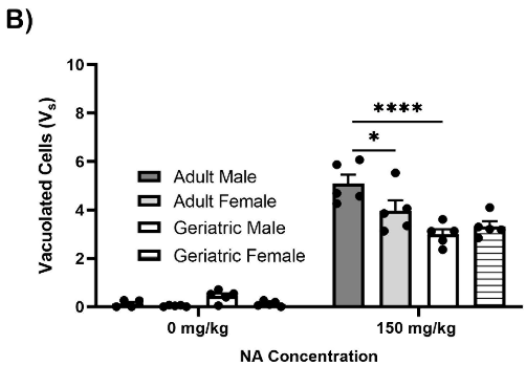
D)

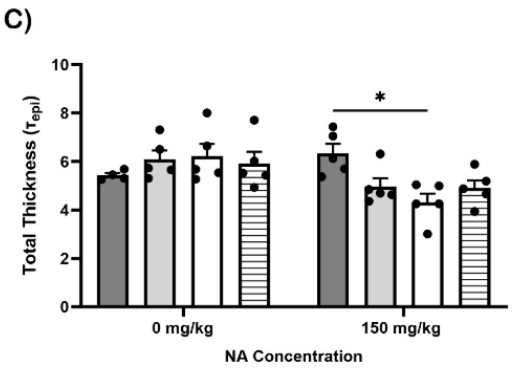
Age	Sex	Treatment	V_v of damaged cells (%)
Adult	Male	0 mg/kg NA	2.6 ± 1.8
Adult	Female	0 mg/kg NA	0.5 ± 0.4
Geriatric	Male	0 mg/kg NA	9.4 ± 5.0
Geriatric	Female	0 mg/kg NA	4.3 ± 2.4
Adult	Male	150 mg/kg NA	89.1 ± 2.6*
Adult	Female	150 mg/kg NA	93.3 ± 4.0*
Geriatric	Male	150 mg/kg NA	63.5 ± 16.5*
Geriatric	Female	150 mg/kg NA	59.6 ± 3.7*

*, $p \leq 0.05$, compared to 0 mg/kg NA of the corresponding age and sex (two-way ANOVA, followed by Tukey's multiple comparisons test).

Supplemental Figure 4.2- Histology and Stereology of NA toxicity in proximal airways. A NA dose response was conducted in geriatric mice and imaged at 20x in high resolution resin sections **[A]**. Vacuolated cells **[B]**, and total epithelial thickness **[C]** in adult and geriatric mice were measured using the average volume of epithelial cells per basal lamina surface area. The volume fraction (Vv) of damaged cells was also calculated, presented as mean ± SD (n= 4- 5) **[D]**. Both adult and geriatric C57bl6 mice were exposed to a single dose of 150 mg/kg of NA and sacrificed 24 hours post exposure. Error bars represent standard error. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: NA, naphthalene.







D)

Age	Sex	Treatment	V_v of damaged cells (%)
Adult	Male	0 mg/kg NA	1.5 ± 1.4
Adult	Female	0 mg/kg NA	0.6 ± 0.2
Geriatric	Male	0 mg/kg NA	7.6 ± 2.2
Geriatric	Female	0 mg/kg NA	2.0 ± 0.8
Adult	Male	150 mg/kg NA	80.3 ± 2.5*
Adult	Female	150 mg/kg NA	79.9 ± 5.3**
Geriatric	Male	150 mg/kg NA	71.7 ± 7.9**
Geriatric	Female	150 mg/kg NA	68.3 ± 4.2**

*, $p \leq 0.05$ and **, $p \leq 0.01$, compared to 0 mg/kg NA of the corresponding age and sex (two-way ANOVA, followed by Tukey's multiple comparisons test).

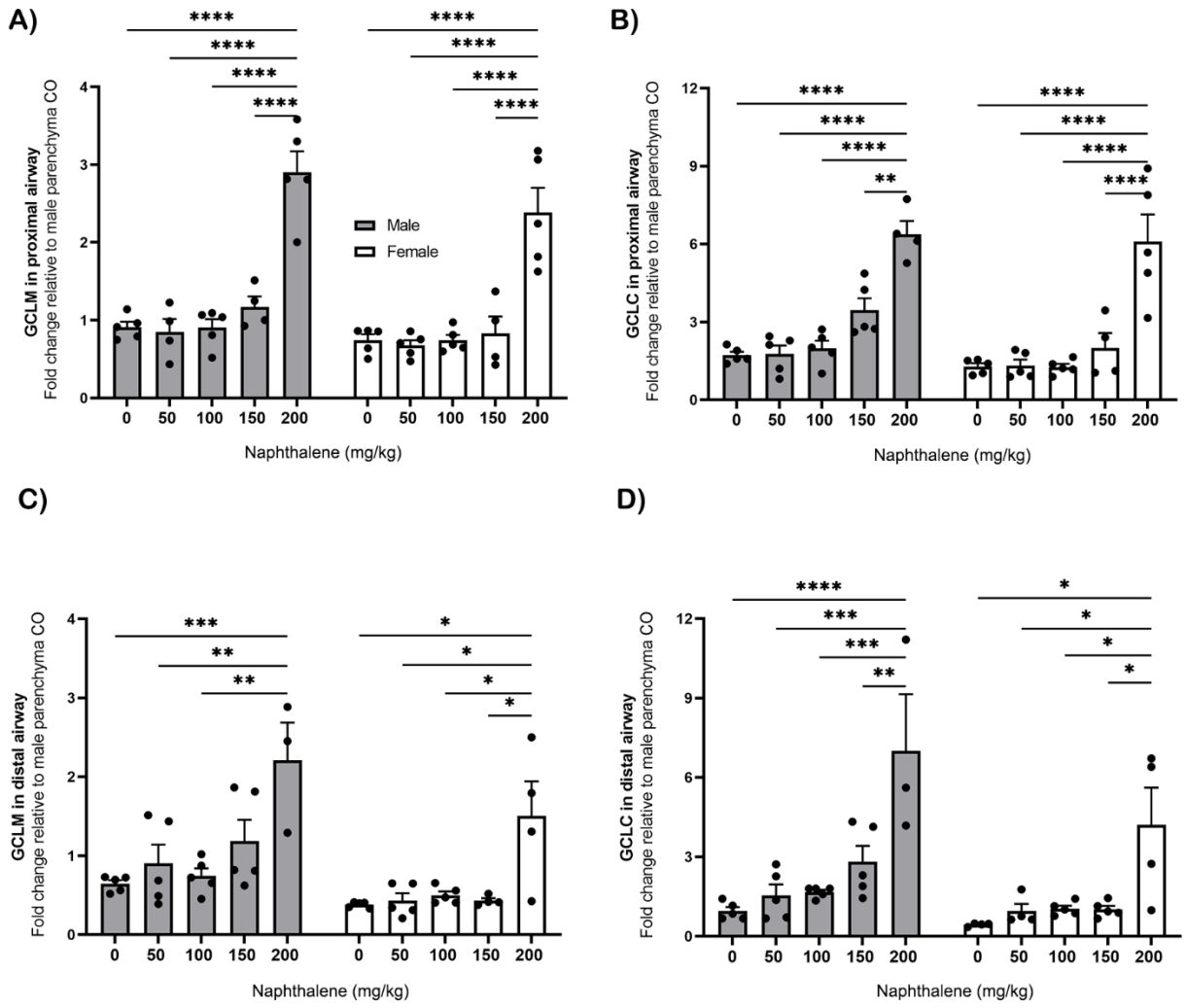
Figure 4.6- Histology and Stereology of NA toxicity in terminal bronchiolar airway. NA dose response of 0 mg/kg (CO), 50 mg/kg, 100 mg/kg, 150 mg/kg, or 200 mg/kg was conducted in geriatric mice and imaged at 20x **[A]**. Vacuolated cells **[B]**, and total epithelial thickness **[C]** in adult and geriatric mice were measured using the average volume of epithelial cells per basal lamina surface area. The volume fraction (Vv) of damaged cells was also calculated, presented as mean ± SD (n= 4- 5) **[D]**. Both adult and geriatric C57bl6 mice were exposed to a single dose of 150 mg/kg of NA and sacrificed 24 hours post exposure. Error bars represent standard error. *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$. Abbreviations: NA, naphthalene.

of the epithelium, adult females had a significantly lower ($p \leq 0.01$) total epithelial cell mass post NA exposure compared to geriatric females in the proximal airway (**Fig. S4.2B**). In the distal airway the total thickness of the epithelium significantly decreases ($p \leq 0.05$) in geriatric males post NA exposure and when compared to adult males (**Fig. 4.6C**). The volume fraction of damaged cells, significant increase in the adult ($p \leq 0.05$) and geriatric ($p \leq 0.01$) group exposed to 150 mg/kg of NA when compared to the control groups of the corresponding age and sex (**Fig. 4.6D**).

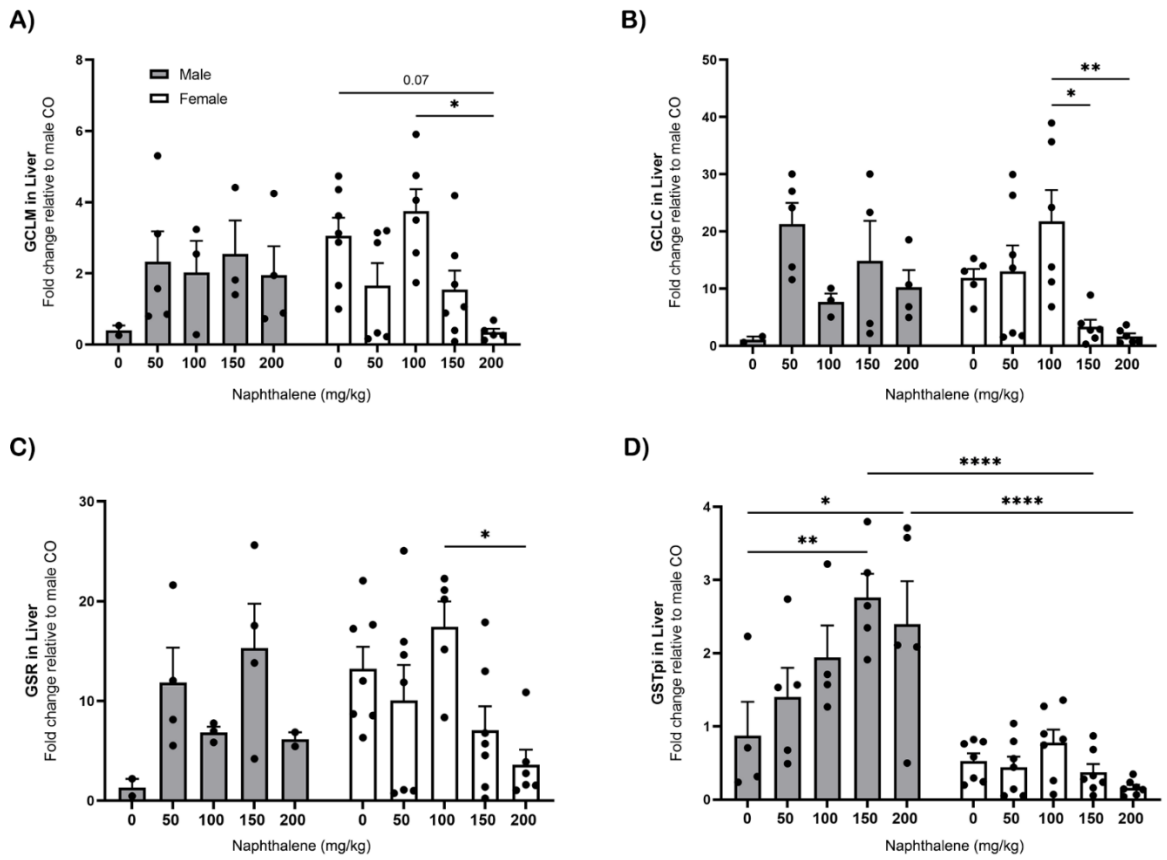
Gene expression related to GSH production and detoxification pathways in lung tissue

To establish if a NA exposure at various doses impacts GCLM and GCLC, we measured gene expression in both microdissected lung (**Fig. S4.3**) and whole liver (**Fig. S4.4A, S4.4B)** in geriatric male and female mice. Expression of GCLM and GCLC significantly increased at the maximum NA exposure dose of 200 mg/kg compared to 0 mg/kg, 50 mg/kg, 100 mg/kg, and 150 mg/kg in both the proximal ($p \leq 0.0001$) and distal airway ($p \leq 0.05$) (**Fig. S4.3**). There was no significant difference of GCLM and GCLC expression in the male liver after NA exposure. In the female liver, GCLM was significantly lower at 200 mg/kg compared to 0 mg/kg ($p = 0.07$) and 100 mg/kg ($p \leq 0.05$) of NA (**Fig. S4.4A**). GCLC gene expression in the female liver was significantly lower at 150 mg/kg ($p \leq 0.05$) and 200 mg/kg ($p \leq 0.01$) compared to 100 mg/kg NA (**Fig. S4.4B**).

GSTpi in the male liver was significantly greater at 150 mg/kg ($p \leq 0.01$) and 200 mg/kg ($p \leq 0.05$) of NA compared to 0 mg/kg (**Fig. S4.4D**). When comparing the GSTpi gene expression between sexes, males have a significantly greater expression at 150 mg/kg ($p \leq 0.0001$) and 200 mg/kg ($p \leq 0.0001$) compared to females (**Fig. S4.4D**). GSTpi in the female proximal (**Fig. 4.7A**)

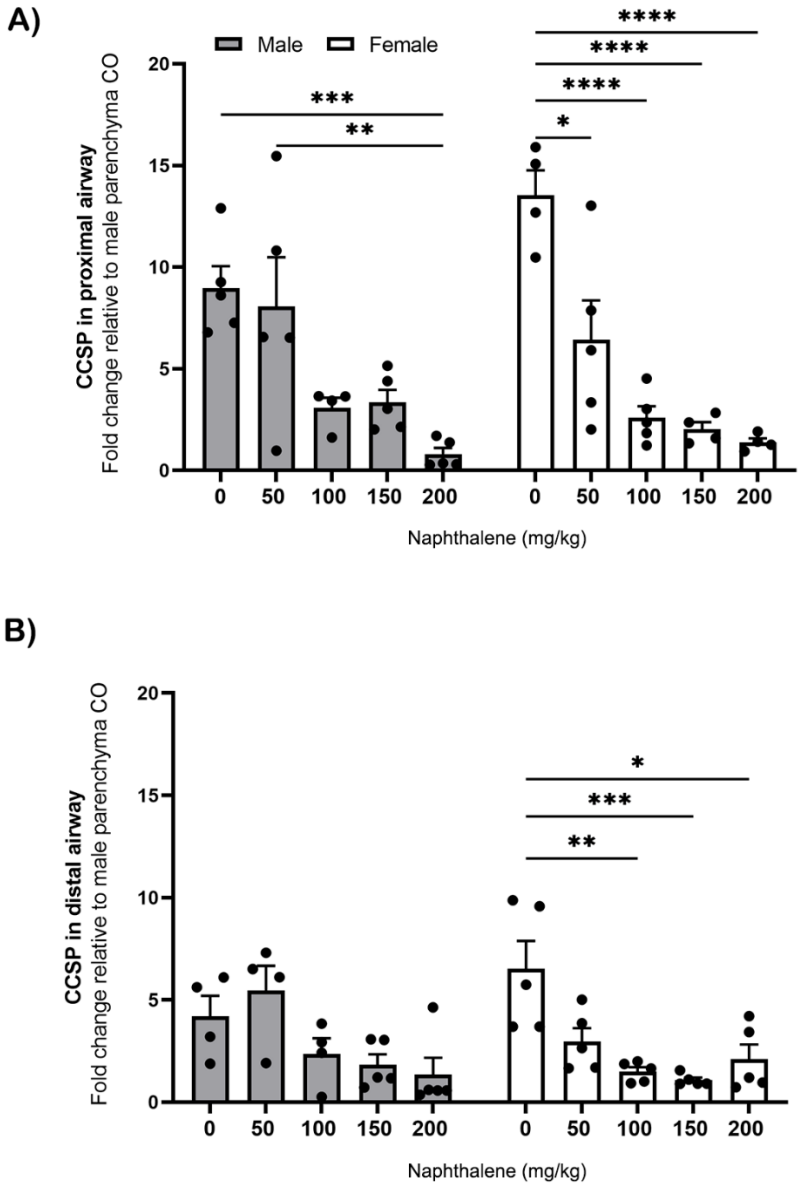


Supplemental Figure 4.3- Gene expression related to GSH production in microdissected airways from NA exposed mice. Microdissected lungs samples from male and female geriatric mice (n= 4-5) by qRT-PCR. The parenchyma, a lung region containing zero airways, was also collected. Both the proximal **[A and B]** and distal **[C and D]** airways of the lungs were analyzed for GCLM and GCLC in a NA dose response of 0 mg/kg (CO), 50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg. Values are the standard error of the mean fold change normalized to the male parenchyma region of the CO group in the lungs with Rpl13a as the housekeeping gene. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: NA, naphthalene; CO, corn oil.



Supplemental Figure 4.4- Gene expression related to GSH production and detoxification in liver.

Liver samples were collected from geriatric mice (n= 2-6) exposed to 0 mg/kg (CO), 50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg of NA for 24 hours. GCLM **[A]**, GCLC **[B]**, GSR **[C]**, and GSTpi **[D]** values are the standard error of the mean fold change normalized to the male CO; Rpl13a as the housekeeping gene. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: NA, naphthalene; CO, corn oil.



Supplemental Figure 4.5- CCSP gene expression in sham and NA exposed microdissected lungs. CCSP (Club cell secretory protein) was measured in microdissected lung samples from untreated and NA exposed geriatric mice (n=5) by qRT-PCR. Both the proximal **[A]** and distal **[B]** airways of the lungs were analyzed in a NA dose response of 0 mg/kg (CO), 50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg. Values are the standard error of the mean fold change normalized to the male parenchyma region of the CO group; Rpl13a is the housekeeping gene. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: NA, naphthalene; CO, corn oil.

and distal (**Fig. 4.7B**) airways, exposed to NA at various doses, had a significant increase ($p \leq 0.05$) at the highest NA dose of 200 mg/kg when compared to 0 mg/kg NA.

GSR expression in the male liver had no significant difference post NA exposure (**Fig. S4.4C**). The GSR expression in female liver was significantly lower at 200 mg/kg ($p \leq 0.05$) of NA compared to 100 mg/kg (**Fig. S4.4C**). GSR in the proximal (**Fig. 4.7C**) and distal airway (**Fig. 4.7D**) both had a significant sex difference ($p \leq 0.05$) at each NA dose, with males having the greatest expression of GSR at 200 mg/kg compared to females.

Histology and stereology of ET pre-treated lungs post NA exposure

To understand the effect of ET pretreatment on NA epithelial toxicity in lung of the aging mice, we examined the proximal and terminal airways using high resolution light microscopy in resin sections. When comparing the SA/CO group (control) to the ET/CO group in the larger proximal airway and distal terminal bronchioles, ET treatment shows no evidence of necrosis and looks similar to the control. The effects of 100 mg/kg of NA in the larger proximal airway and distal terminal bronchioles of male and female mice were similar in that both experience necrosis, evident as swelling and vacuolation of airway epithelial Club cells, however females had a greater distribution of cell vacuolation and some cells had already been exfoliated, indicating a more advanced stage of lung injury compared to males at the same dose (**Fig. 4.8A, S4.6A**). When we treated the mice with ET prior to the NA exposure, males had reduced Club cell necrosis in the larger proximal airway and terminal bronchioles compared to the mice not treated with ET. The larger proximal airway of the female in the ET pretreated group exposed to NA has far less vacuolated cells compared to the mice not pretreated with ET. There was no significant difference between the female ET treated and untreated exposed to NA in the terminal bronchiole (**Fig. 4.8A,**

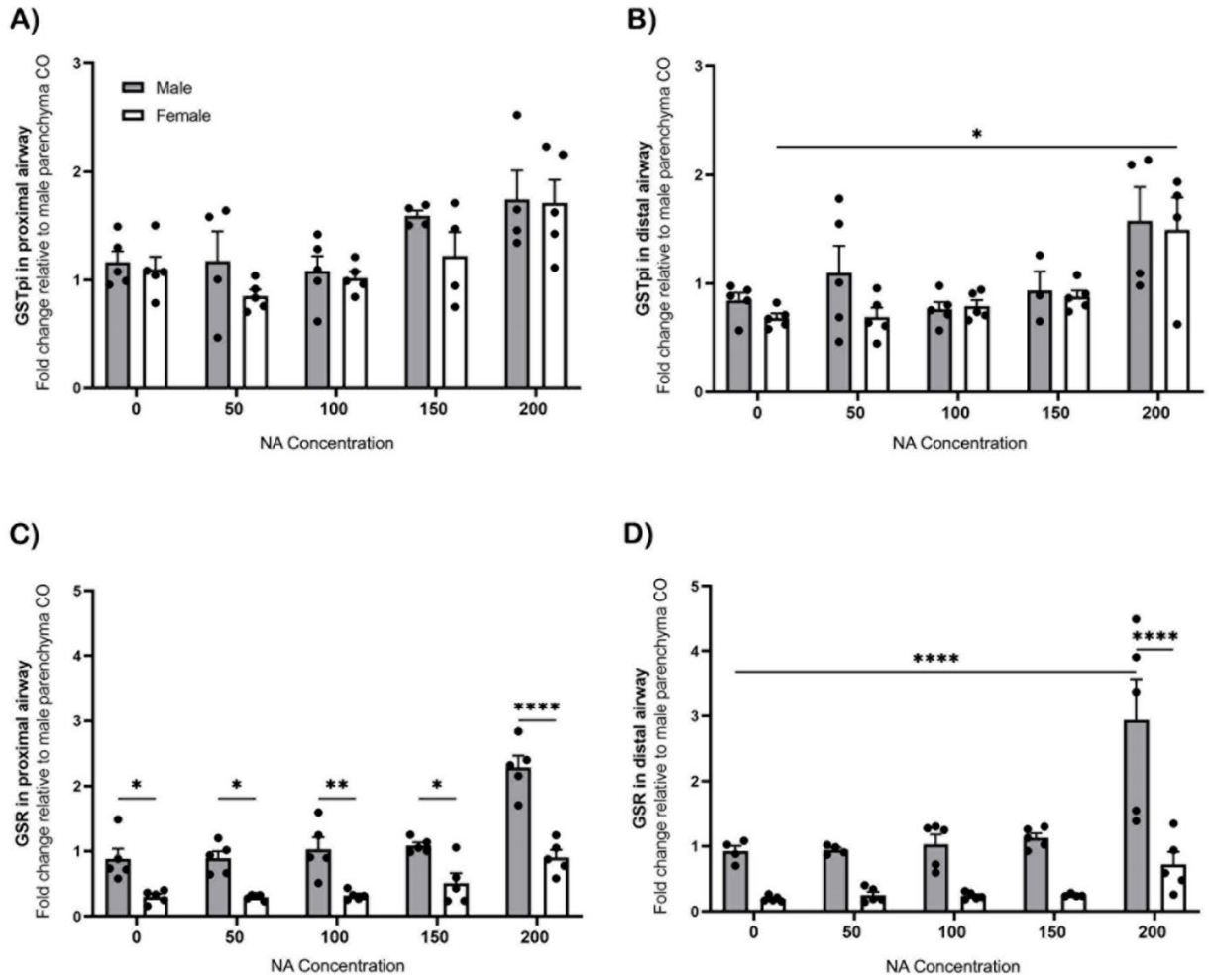
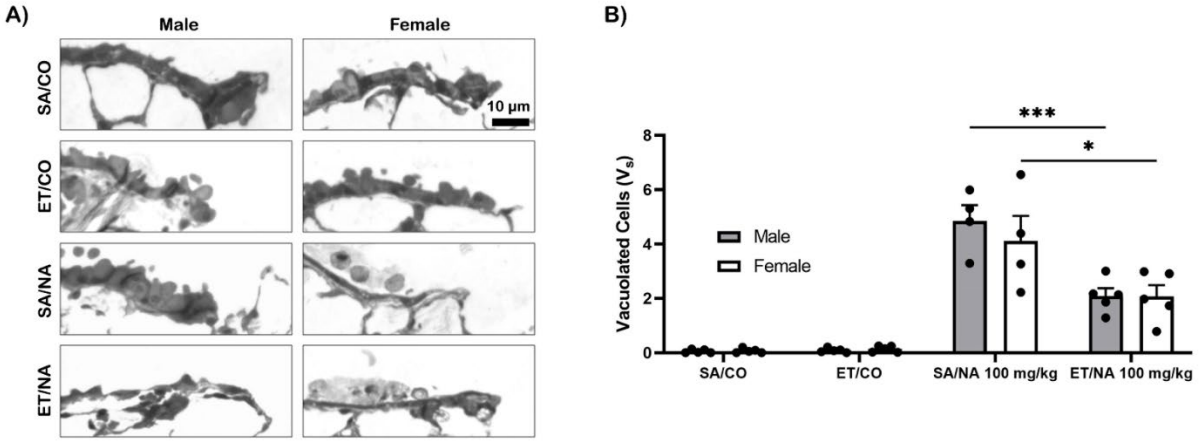


Figure 4.7- Gene expression related to GSH detoxification in NA exposed microdissected lungs. Microdissected lungs samples were collected from NA exposed male and female geriatric mice (1 to 1.5 years). GSTpi **[A,B]** and GSR **[C-D]** gene expression was determined using qRT-PCR in relation to Rpl13a as a housekeeping gene. NA dose response included 0 mg/kg (CO), 50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg. Values are standard error of the mean fold change normalized to the male parenchyma region of the CO group (n= 4-5). *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$. Abbreviations: NA, naphthalene; CO, corn oil.

S4.6A). Overall, based on histology geriatric males treated with ET and exposed to NA contained fewer vacuolated cells than in females at 24 hrs post NA exposure.

Using stereology, we measured vacuolated cells, non-vacuolated cells, and total thickness of the lung epithelium in the proximal and terminal airways of ET treated mice exposed to 100 mg/kg NA. In the proximal airway, male and female mice in the control group (treated with SA, and exposed to CO), had significantly lower vacuolated cell mass ($p \leq 0.01$) compared to the saline treated group exposed to 100 mg/kg NA (**Fig. S4.6B**). Female mice treated with ET and exposed to NA had significantly greater mass of vacuolated cells ($p \leq 0.05$) compared to female mice treated with ET and exposed to CO (**Fig. S4.6B**). When measuring non-vacuolated cells, we observed opposing results from our vacuolated measurements. Male and female mice treated with SA and exposed to CO had significantly greater ($p \leq 0.001$) non-vacuolated cells compared to mice treated with SA and exposed to NA (**Fig. S4.6C**). ET treated female mice exposed to CO has significantly greater ($p \leq 0.001$) non-vacuolated cells compared to female mice treated with ET and exposed to NA (**Fig. S4.6C**). There was no significant difference in the lung epithelium total thickness and the V_v of damaged cells (data not shown).

The terminal airway had similar vacuolated cell pattern as the proximal airway, with the control group having significantly lower ($p \leq 0.0001$) vacuolated cells compared to the saline treated mice exposed to 110 mg/kg NA (**Fig. 4.8B**). There was a significant treatment difference in vacuolated cells in both male and female terminal airways. Vacuolated cells were significantly lower in both male ($p \leq 0.001$) and female ($p \leq 0.05$) distal airways treated with ET compared to those that were treated with saline post NA exposure (**Fig. 4.8B**). The volume fraction of damaged cells, significant increased ($p \leq 0.05$) in every group that was treated with NA when compared to

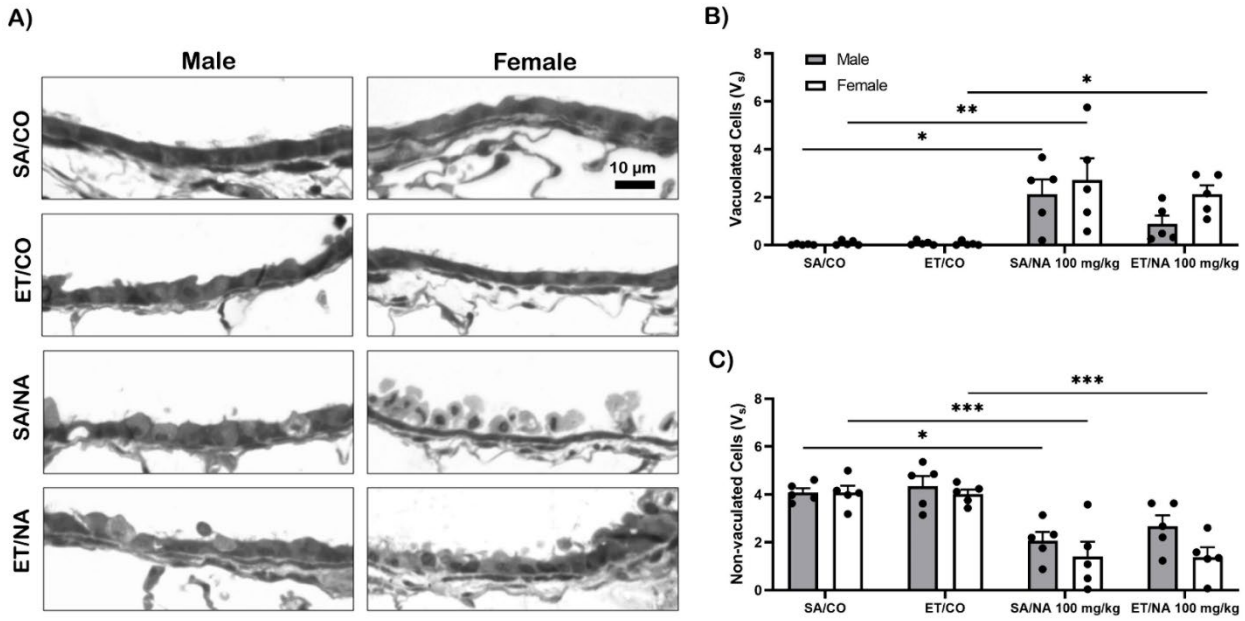


C)

Sex	Treatment	V_v of damaged cells (%)
Male	SA/CO	0.9 ± 0.5
Female	SA/CO	1.2 ± 0.5
Male	ET/CO	1.7 ± 0.8
Female	ET/CO	2.4 ± 0.9
Male	SA/NA 100 mg/kg	64.3 ± 16.1**
Female	SA/NA 100 mg/kg	76.8 ± 11.4**
Male	ET/NA 100 mg/kg	46.2 ± 4.8*
Female	ET/NA 100 mg/kg	45.0 ± 6.6*

*, $p \leq 0.05$ and **, $p \leq 0.01$, compared to 0 mg/kg NA of the corresponding age and sex (two-way ANOVA, followed by Tukey's multiple comparisons test).

Figure 4.8- Histology and stereology of ET treated geriatric terminal airway exposed to 100 mg/kg NA. Male and female geriatric mice (n= 4-5) were treated with ET prior to a 24-hour exposure period to 100 mg/kg of NA ip. The airway and terminal airways were imaged using a high-resolution light microscope at 20x, then converted to grayscale **[A]**. Stereology was conducted using the imaged lungs and the vacuolated cells **[B]**, non-vacuolated cells **[D]**, and the volume fraction (Vv) of damaged cells (presented as mean ± SD) were calculated (n= 4- 5) **[C].** Error bars represent standard error. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Abbreviations: SA, saline; ET, ergothioneine; NA, naphthalene; CO, corn oil.



Supplemental Figure 4.6- Histology and Stereology of ET treated geriatric proximal airway exposed to 100 mg/kg NA. Male and female geriatric mice (n=5) were treated with ET prior to a 24-hour exposure period to 100 mg/kg of NA ip. The airway and terminal airways were imaged using a high-resolution light microscope at 20x, then converted to grayscale [A]. Stereology was conducted using the imaged lungs and the vacuolated cells **[B]**, non-vacuolated cells **[C]** were calculated (n= 4-5). Error bars represent standard error. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Abbreviations: SA, saline; ET, ergothioneine; NA, naphthalene; CO, corn oil.

the control groups of the corresponding age and sex (**Fig. 4.8C**). Non-vacuolated cells were significantly lower ($p \leq 0.0001$) in groups exposed to NA (**Fig. 4.8D**).

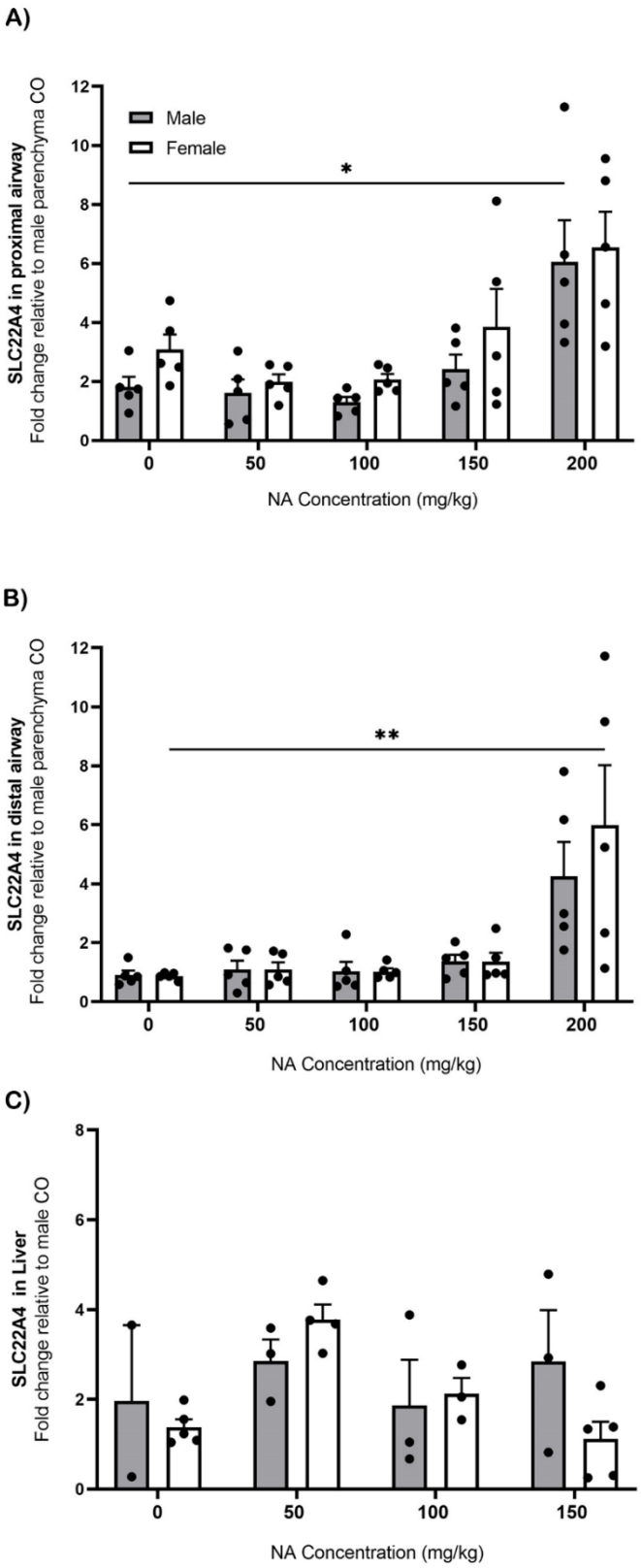
ET transporter gene expression in NA treated lung and liver

The gene expression of SLC22A4, the ET transporter, in the proximal (**Fig. 4.9A**) and distal (**Fig. 4.9B**) airway significantly increased in both males ($p \leq 0.05$) and females ($p \leq 0.01$) exposed to 200 mg/kg of NA when compared to 0 mg/kg. The female liver had maximal expression of SLC22A4 at 50 mg/kg of NA and had significantly lower ($p \leq 0.05$) expression at 150 mg/kg of NA compared to 50 mg/kg (**Fig. 4.9C**).

Gene expression of CCSP and SLC22A4, GSR, and GPx in female ET pretreated mouse lung

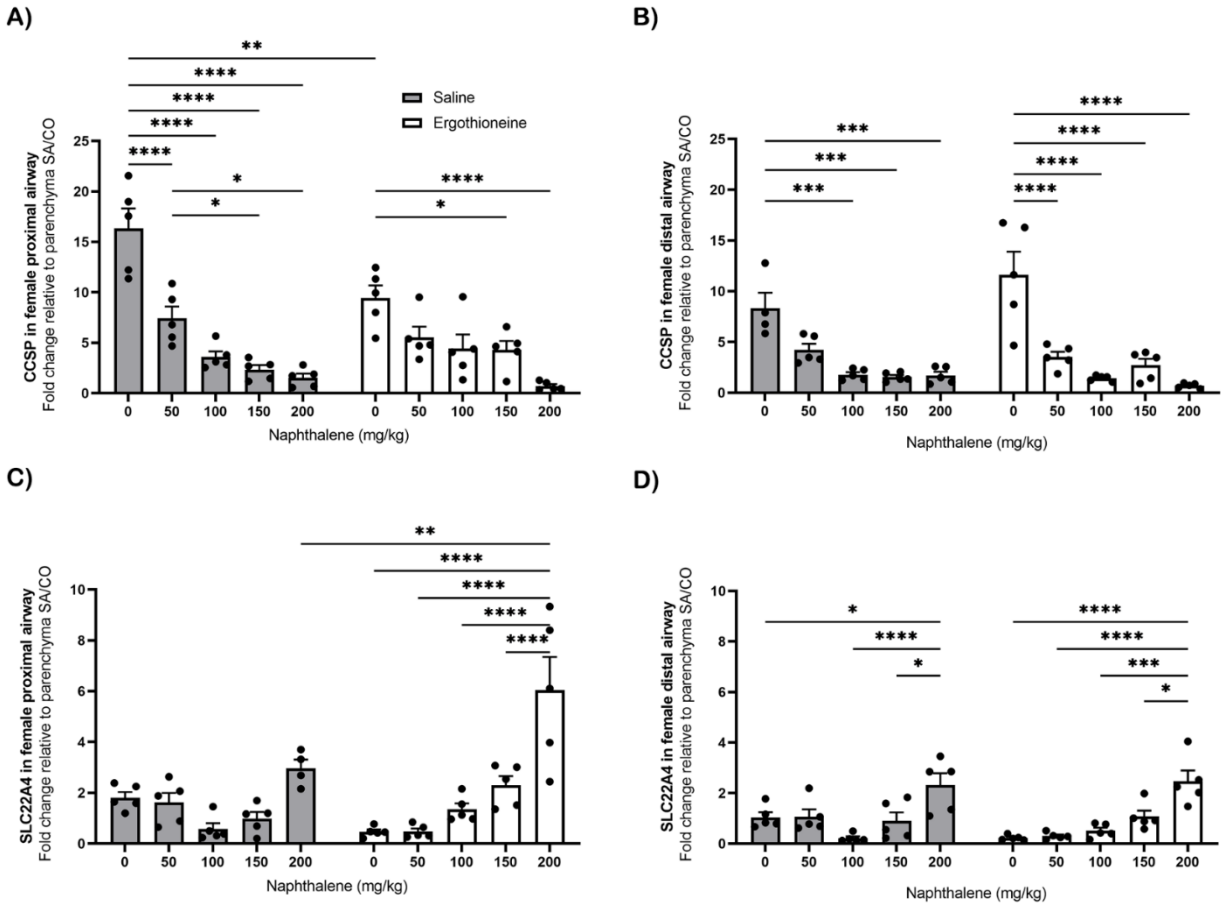
The gene expression in female geriatric mice was measured to determine if ET pretreatment had any impact on the lung post NA exposure. The gene expression of Club cell secretory proteins (CCSP) (**Fig. S4.7A, S4.7B**), ergothioneine transporter (SLC22A4) (**Fig. S4.7C, S4.7D**), glutathione S reductase (GSR) (**Fig. 4.10A, 4.10B**), and glutathione peroxidase (GPx) (**Fig. 4.10C, 4.10D**) were measured in both the proximal and distal airway of the lung. Female mice treated with SA and exposed to NA had significantly less ($p \leq 0.001$) CCSP in the proximal and distal airway as the NA concentration increases (**Fig. S4.7A, S4.7B**). When treated with ET there was significantly less CCSP at 150 mg/kg ($p \leq 0.05$) and 200 mg/kg ($p \leq 0.0001$) of NA in the proximal airway compared to 0 mg/kg NA (**Fig. S4.7A**). Similarly, mice treated with saline in the distal airway had significantly lower expression of CCSP at 100 mg/kg ($p \leq 0.001$), 150 mg/kg ($p \leq 0.001$), and 200 mg/kg ($p \leq 0.001$) compared to 0 mg/kg of NA (**Fig. S4.7B**). Mice treated with ET in the distal airway had significantly less expression of CCSP ($p \leq 0.0001$) by 2-fold as the NA concentration increased (**Fig. S4.7B**).

Figure 4.9- ET transporter changes in response to NA in geriatric mice lung and liver. SLC22A4 (ET transporter) was measured in microdissected lung **[A,B]** and liver **[C]** samples from untreated NA exposed geriatric mice (n=2-5) by qRT-PCR. Both the proximal **[A]** and distal **[B]** airways of the lungs were analyzed in a NA dose response of 0 mg/kg (CO), 50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg. Similar doses were measured in the liver **[C]**. Values are the standard error of the mean fold change normalized to the male parenchyma region of the CO group in the lungs, and male CO in the liver; Rpl13a is the housekeeping gene. *, $p \leq 0.05$; **, $p \leq 0.01$. Abbreviations: NA, naphthalene; CO, corn oil.



The female proximal airway of SLC22A4 had no significant sex differences in the untreated group when exposed to a NA dose response (**Fig. S4.7C**). On the contrary, SLC22A4 in the ET treated group had a significantly greater expression at 200 mg/kg ($p \leq 0.0001$) when compared to 0 mg/kg, 50 mg/kg, 100 mg/kg, and 150 mg/kg (**Fig. S4.7C**). When comparing SLC22A4 expression at 200 mg/kg NA in the saline and ET treated group, the mice treated with ET were significantly greater ($p \leq 0.01$) in expression than those treated with saline (**Fig. S4.7C**). The saline treated group for the distal airway had the greatest expression of SLC22A4 at 200 mg/kg NA (**Fig. S4.7D**). The expression of SLC22A4 in the distal airway at 200 mg/kg from mice treated with saline were significantly greater than 0 mg/kg ($p \leq 0.05$), 100 mg/kg ($p \leq 0.0001$), and 150 mg/kg ($p \leq 0.05$) (**Fig. S4.7D**). When compared to 200 mg/kg NA exposure in mice treated with ET, SLC22A4 was significantly less at 0 mg/kg ($p \leq 0.0001$), 50 mg/kg ($p \leq 0.0001$), 100 mg/kg ($p \leq 0.001$) and 150 mg/kg ($p \leq 0.05$) in the distal airway (**Fig. S4.7D**).

The expression of GSR in the proximal airway of female mice treated with saline had no significant differences in expression post NA exposure (**Fig. 4.10A**). However, when compared to ET treated mice exposed to NA at 200 mg/kg there was significantly less expression at 0 mg/kg ($p \leq 0.01$) of NA (**Fig. 4.10A**). The distal airway expression of GSR at 0 mg/kg NA was significantly greater ($p \leq 0.05$, 2-fold) in saline-treated mice compared to ET treated mice at 0 mg/kg NA (**Fig. 4.10B**). Mice treated with ET and exposed to NA at 200 mg/kg NA had significantly greater expression of GSR in the distal airway compared to 0 mg/kg ($p \leq 0.01$) of NA (**Fig. 4.10B**). The proximal airway expression of GPx had no significant difference between the saline treated and ET treated groups post NA exposure (**Fig. 4.10C**). The distal airway of mice treated with saline had significantly less expression of GPx at 100 mg/kg ($p \leq 0.0001$) and 150 mg/kg ($p \leq 0.01$) compared to 0 mg/kg of NA (**Fig. 4.10D**).



Supplemental Figure 4.7- Female gene expression of CCSP and SLC22A4 post ET pretreatment and NA exposure. CCSP proximal **[A]** and distal **[B]** airways, and SLC22A4 proximal **[C]** and distal airways **[D]** were determined by RT-PCR in relation to Rpl13a as the housekeeping gene. Female geriatric mice, 1 to 1.5 years of age, were treated with 70 mg/kg of ET for five consecutive days prior to a 24-hour exposure to a NA dose response via ip. Values are standard error of the mean fold change normalized to the parenchyma region of the SA/CO group (n=4-5). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: ET, ergothioneine; NA, naphthalene; SA, saline; CO, corn oil.

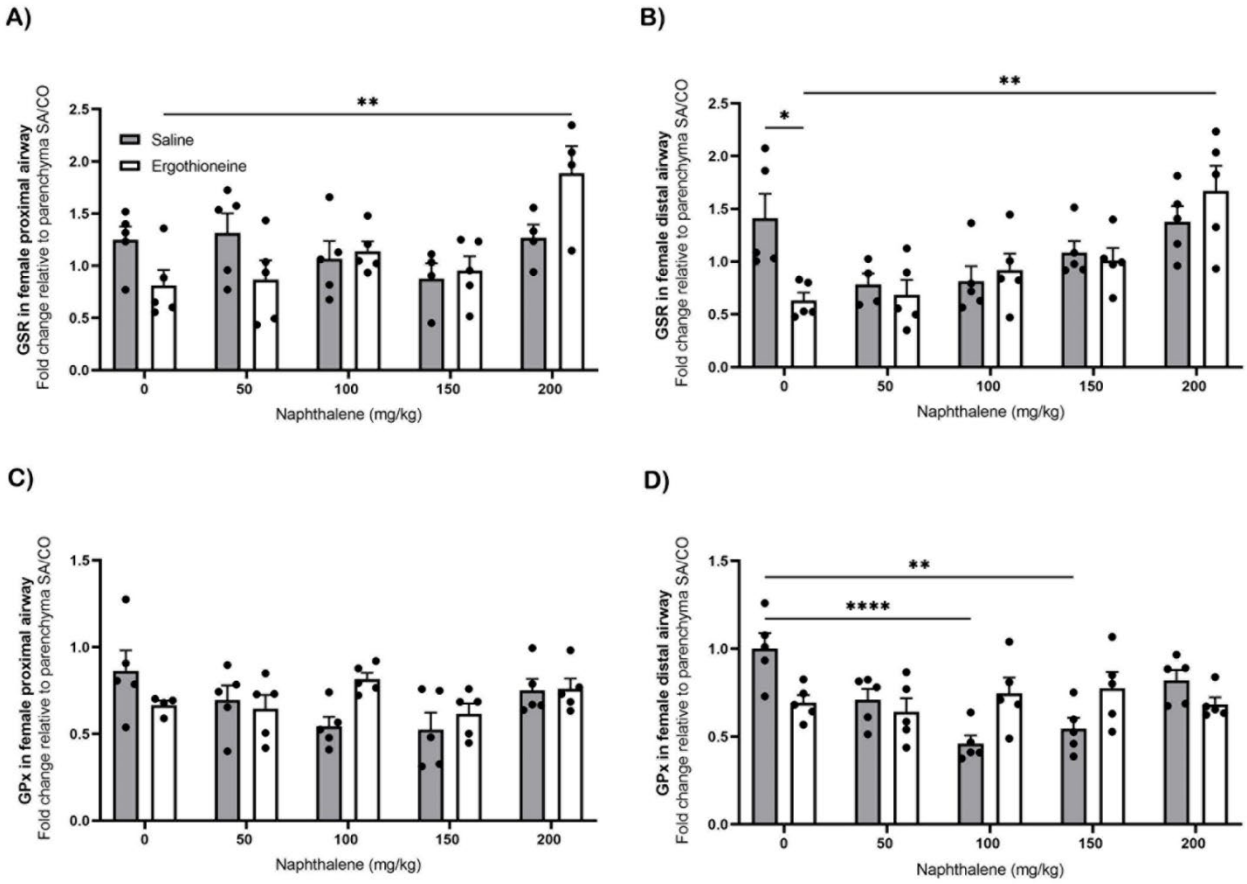


Figure 4.10- Airway gene expression post ET pretreatment and NA exposure in female mice. GSR proximal **[A]** and distal **[B]** airways, and GPx proximal **[C]** and distal airways **[D]** were determined by RT-PCR in relation to Rpl13a as the housekeeping gene. Female geriatric mice, 1 to 1.5 years of age, were treated with 70 mg/kg of ET for five consecutive days prior to a 24-hour exposure to a NA dose response via ip. Values are standard error of the mean fold change normalized to the parenchyma region of the SA/CO group (n=4-5). **, $p \leq 0.01$; ****, $p \leq 0.0001$. Abbreviations: ET, ergothioneine; NA, naphthalene; SA, saline; CO, corn oil.

Mice treated with ET in the distal airway had no significant difference post NA exposure in the expression of GPx (**Fig. 4.10D**).

DISCUSSION

This study is the first to evaluate lung region specific expression of key genes involved in detoxification in aging mice. Further it is the first to conduct a NA dose response in geriatric mice to define toxicity in the epithelium and evaluate the ability of a dietary antioxidant, ergothioneine (ET), to ameliorate oxidative stress and airway epithelial toxicity. As part of this evaluation, we determined differences in gene expression by age and lung location. The regional impacts of NA toxicity in the lung were compared using microdissected lung regions as done in historic and recent studies (Royce et al. 1996; Sutherland et al. 2012). This is especially important when measuring toxicity and cellular impacts in the lung because the response and the dose delivered can vary by location. Surprisingly we found that GSR was less abundant in the lung of females than of males in all regions in geriatric mice, and this was due to a large increase in GSR expression in geriatric male mice compared to young adult controls and geriatric females. We observed accumulation of ET in the lung, liver, and blood post oral treatment of ET, demonstrating absorption and distribution of ET to these organs in geriatric mice and that mice not treated with ET, while maintained on an ET free diet, lacked measurable ET. Importantly we were able to show spatial differences in ET transporter expression, where the transporter was expressed in both proximal and distal airways but had the greatest expression in the proximal airways. ET pretreatment was able to reduce the abundance of vacuolated cells measured by stereology in the aged male and female mouse terminal bronchioles, showing some efficacy against NA induced oxidative stress in the lungs.

GCL is the rate limiting enzyme in GSH synthesis (Lu 2009). Normal expression of GCLM in adult mice varies by sex with males having a greater expression compared to females in the intrapulmonary conducting airways, airway bifurcations, and terminal bronchioles (Sutherland et al. 2012). Our study has shown similar results in aging mice, with GCLM having a greater expression in males compared to females in both the proximal and distal airway. In GCLM knock out mice, the lung, liver, pancreas, and other cell types had a significant decrease in GSH expression compared to wildtype (Yang et al. 2002). Previous studies in GCLC knockout mice shared similar results (Dalton et al. 2000). Conversely, overexpression of GCLM and GCLC results in an increased production of GSH (Orellana et al. 2017). A limiting factor in our current study is that we did not examine the protein or enzymatic activity of GCL, however a previous rat study found that GCL activity declines with aging although paradoxically increases protein expression of GCLM and GCLC as Nrf2, a biomarker for oxidative stress, increases (Suh et al. 2004). As the oxidative stress in our study increased, we found increased gene expression of GCLM and GCLC as well. The addition of ergothioneine seems to stabilize the expression of both GLCM and GCLC. Overall, these findings add to the important role that GCL enzymes play in the production of the primary lung endogenous antioxidant GSH and the response to oxidative stress.

Glutathione reductase (GSR) is essential in the detoxification process, with the primary function to reduce oxidized GSSG, to GSH, while glutathione peroxidase will oxidize GSH to GSSG (Zalachoras et al. 2020). In this study we found lower gene expression of GSR in the proximal and distal airways of geriatric females compared to males in the control group. In addition, we saw that when treated with ET and exposed to NA there is an increased expression of GSR at 200 mg/kg in geriatric females. This data supports previous findings on the relationship between ET and GSR in the detoxification process. Prior studies have shown that GSR is capable

of reducing ET as it does for endogenous antioxidant GSH (Jenny et al. 2022). When oxidative stress was induced in adult male rats, endogenous GSH, GSSG, and GSR increased in the airways compared to the control group (Chan et al. 2013). The lungs of GSR knockout mice, a1Neu, are still capable of reducing GSH by stimulating oxidation of NADPH to GSSG, which is the alternative route for GSH generation in species lacking functional GSR. Although functional, GSR knockout mouse was not as effective in the GSH detoxification cycle compared to wildtype mice (Tipple et al. 2007). So, a lower level of GSR such as what was observed for geriatric female mice in this study would diminish regeneration of reduced antioxidant ET as well as GSH. This is quite concerning and might underpin an elevated susceptibility to oxidative stress, although ability to increase GSR was preserved at high dose challenge with NA but not at lower doses. We did examine gene expression at 24 hrs so it is also possible that the response to NA occurred earlier. However, the baseline level differences are not timing dependent and are likely the result of an aging related phenomenon. Previous studies have shown that ET can directly impact the GPx expression (Liu et al. 2023). Our study has also shown that GPx is upregulated in geriatric mice compared to adults. We have seen in this study that ET treatment in geriatric female mice stabilized the production of GPx in the presence of oxidative stress. Thus, ET may play a crucial role in relation to GSH and the detoxification cycle, however more research still needs to be done to truly understand its impact.

NA induced tissue injury and repair is characterized by a sequence of events that involve GSH loss, vacuolation of injured cells, followed by exfoliation of the injured cells into the lumen of the airway and squamation of remaining epithelia to cover the basement membrane (Van Winkle et al. 1995). Following NA exposure, we observed a depletion of CCSP, an indicator for Club cell abundance by location, as well as histological and stereological indices of NA toxicity in the

airways of animals treated with NA. However, based on stereological level of vacuolated cells detected it appears that toxicity was not different by sex in proximal airways and was greater in geriatric males than in females in the terminal bronchiole. There may be a number of contributing factors to this phenomenon, including: 1) timing of sample collection being 24 hours rather than an earlier time point, 2) vacuolated cells detached from the lumen at the 24hour time point, 3) the impact of age on the endogenous antioxidants which may impact the strength of ET treatment.

It has been previously shown that ET transporters are found in organs known to experience high levels of oxidative stress and their expression will increase in the presence of cell damage caused by free radicals (Cheah and Halliwell 2021). For example, the liver is known for its primary role to metabolize xenobiotics, a fact making it susceptible to oxidative stress, which results in a high expression of ET transporter (Tang et al. 2018). In this study we found that the liver ET transporter, SLC22A4, in male mice had no significant change in expression as the NA dose increased. Conversely, females had the highest expression of SLC22A4 at 50 mg/kg and a significant decline in expression at 150 mg/kg NA. This could be evidence of geriatric male mice metabolizing NA faster in the liver compared to females. Other known impacts of ET pretreatment includes reduced risk of stroke in rodent models (Ong et al. 2022), reduced risk of the development of mild cognitive disorders in geriatric humans (Wu et al. 2022), and it has been suggested to be a potential treatment for Coronavirus patients (Cheah and Halliwell 2020). Overall, scientists have shown that ET has potential in improving the health of both animal and human models; yet there is still more research to be done to truly understand the full functional capability of ET. Our findings emphasize the potential for ET to ameliorate toxicity by NA exposure in geriatric male mice.

Overall, this study is a first to demonstrate sex differences in the lungs of geriatric mice, understand NA toxicity in geriatric mice, and explore the impacts of ET on GSH synthesis in the lungs. Our findings are the initial step needed to understanding lung specific responses in geriatric mice including key capabilities to detoxify xenobiotics in the lung and the potential benefits of introducing a dietary antioxidant. Key gene expression differences that were found in this study exposed areas of susceptibility by age, sex and location in the lung. Further, this study found that ET was a potential pretreatment for NA toxicity with a modest effect on reduction of toxicity at some doses of NA and at some locations.

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