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FETAL ETHANOL EXPOSURE AND ZINC HOMEOSTASIS IN THE NEWBORN ALVEOLAR MACROPHAGE

By

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Doctor of Philosophy

Division of Biological and Biomedical Sciences

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Juna Viktoria Konomi

B.A., Brown University, 2006

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ABSTRACT

FETAL ETHANOL EXPOSURE AND ZINC HOMEOSTASIS IN THE NEWBORN ALVEOLAR MACROPHAGE

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Alcohol exposure increases risk of extreme premature birth (<32 weeks gestational age) by over 34 fold. One of the major complications of pre-term birth is lung immaturity which contributes up to 75% of early mortality and morbidity in the pre-term infant. Alcohol further exacerbates an already dysfunctional lung through alteration of lung development and induction of oxidative stress which results in arrested alveolarization and vascular development, increasing risk of pulmonary infections in animal models of fetal ethanol exposure. Zinc is essential for the immune system and its deficiency increases susceptibility to infection and exacerbates existing infections. Alcohol may also affect maternal-placental-fetal transfer of zinc by altering zinc transporter expression in the placental surface or by inducing an inflammatory state in the placenta that impairs zinc transport to the fetus.

Using a mouse model of chronic ethanol exposure *in utero,* we investigated whether zinc homeostasis was disturbed in the ethanol-exposed alveolar macrophage (**AM**) and whether these effects could be reversed with zinc treatments. In exploring mechanisms by which ethanol altered zinc homeostasis in the newborn AM, we focused in characterization of cytokine profiles in placental tissues from a pre-term population that was exposed to alcohol *in utero.*

In utero ethanol exposure was associated with decreased expression of zinc transporters, intracellular zinc levels, and bacterial clearance in the AM when compared to no ethanol exposure. *In vitro* zinc treatments increased expression of zinc transporters and this was concomitant with restored intracellular zinc levels and AM bacterial clearance in the ethanol-exposed pups. Additionally, fetal alcohol exposure was associated with an increase in pro-inflammatory cytokines in the placenta.

These studies suggest that zinc insufficiency is a critical component in the impaired AM immune functions associated with fetal alcohol exposure. Furthermore, it supports zinc supplements as a novel therapeutic approach for attenuating the derangements in AM bacterial clearance and risk for respiratory infections in the newborn with fetal alcohol exposure.

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DEDICATION

To my parents, Raimond and Emira

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CHAPTER 1: INTRODUCTION

In the United States, alcohol abuse affects nearly 18 million Americans and is the 3rd leading lifestyle-related cause of death [1]. Its economic costs exceed 223 billion a year [2] resulting from intentional or unintentional injuries, and morbidities and mortalities from cardiovascular, neuropsychiatric, digestive, respiratory diseases, and cancer [3], among other conditions. For women of childbearing age, 1 in 2 report drinking [4] and during pregnancy, 1 in 30 consume alcohol on either a frequent (7 or more drinks per week) or binge (5 or more drinks in one occasion) basis [5]. Alcohol exposure *in utero* is highly detrimental to the developing fetus increasing the risk for Fetal Alcohol Spectrum Disorders and other impairments [6]. Fetal Alcohol Syndrome is the most severe outcome [7-11] of Fetal Alcohol Spectrum Disorders and affects up to 2 per 1000 births in the United States [12, 13]. The majority of alcohol-exposed infants do not display the phenotypic characteristics of Fetal Alcohol Syndrome, resulting in under-diagnosis from primary care providers. Mechanisms by which alcohol impairs functions of organs and systems continue to be elucidated.

Among other outcomes, alcohol exposure *in utero* increases the risk of extreme prematurity (<32 weeks gestational age) over 34-fold [14]. Because the neonatal immune system is not fully developed at birth, the newborn lung is not equipped to fight against inhaled antigens and environmental toxins [15, 16] and premature delivery augments these impairments and further increases the susceptibility of the newborn lung to lower respiratory tract infections. Fetal alcohol exposure not only increases the risk for premature birth and further exacerbates an already dysfunctional lung [17], it independently impairs alveolar macrophage (**AM**) function [18, 19] and increases the risk for sepsis in both term [20] and pre-term infants [21].

Furthermore, alcohol abuse is associated with zinc deficiency, which may become particularly deleterious during pregnancy when nutrient demands are high [22]. Zinc is crucial for the function of highly proliferative systems, like the immune system, and its deficiency affects the innate [23, 24] and adaptive [23, 25, 26] immune responses [27-29], increases susceptibility to infection, and augments existing infections [30]. With respect to the lung, chronic ethanol ingestion decreases expression of zinc transporters and zinc levels in the AM, resulting in impairment of AM phagocytic function [31]. Zinc transporter expression and function in the newborn lung has yet to be elucidated, and how zinc transporters behave in response to *in utero* alcohol exposure is also unknown.

The experiments and analyses in this dissertation intended to aid in understanding the association between alcohol exposure during pregnancy and the adverse consequences for the vulnerable pre-term fetus, like an increased risk of respiratory infections. The dissertation was divided into experimental and clinical components. In the experimental part we examined how fetal ethanol exposure altered zinc homeostasis and zinc transporter expression in the newborn AM. In the clinical component, we examined whether *in utero* alcohol exposure of low birth weight infants resulted in inflammation of the placenta as reflected in the placental cytokine profile. The need to transition into human samples was necessary since it is difficult to obtain mouse placentas and more importantly the two species differ in placental structure and timing of development, among other differences, and results obtained from mouse placental tissues may not be directly applicable to humans. The following aims were addressed in this dissertation:

1. To establish the minimum number of weeks of chronic ethanol ingestion needed to perturb zinc homeostasis in the non-pregnant mouse and to examine if the zinc pools in the AM would be lower when ethanol ingestion was combined with the nutritional demands of pregnancy

2. To investigate whether ethanol exposure during gestation disturbed zinc homeostasis and zinc transporter expression in the newborn AMs and consequently impaired its immune functions

3. To examine whether *in vitro* zinc treatment would reverse the effects of alcohol exposure on AMs isolated from dams fed an ethanol diet during preconception and pregnancy or from pups exposed to ethanol *in utero*

4. To explore differences in cytokine profile in placenta from pre-term, low birth weight infants exposed to alcohol *in utero* with placenta from control pre-term low birth weight infants

In most studies of *in utero* ethanol exposure, the models involve initiation of ethanol ingestion at the point of breeding which does not represent the typical societal patterns where alcohol abuse and subsequent zinc depletion occurs before recognition of the pregnancy. In our model, ethanol ingestion started 3-5 weeks prior to pregnancy and continued throughout the pregnancy. We hypothesized that the combination of ethanol-induced zinc insufficiency prior to conception and the zinc requirements that occur during pregnancy would exacerbate zinc insufficiency in the newborn with fetal ethanol exposure. This would then result in decreases in expression of zinc transporter proteins, disruption of zinc homeostasis, and AM bacterial clearance in the lungs of pups. Additionally, we hypothesized that the negative effects could be restored by *in vitro* zinc treatments.

For the clinical analysis, I was fortunate to collaborate with Dr. Gauthier and her group examining fatty acid ethyl esters in placental tissue as potential biomarkers of alcohol exposure *in utero.* I focused on exploring whether the balance between pro- and anti- inflammatory cytokines in placental tissues from a vulnerable population of pre-term infants born under 1500g differed in

the presence of prenatal alcohol exposure. The subjects enrolled in the study were recruited from Emory University Hospital Midtown and Grady Memorial Hospital in Atlanta, GA. We hypothesized that placental pre-term tissues exposed to alcohol during pregnancy would display an imbalance of Th-1 and Th-2 cytokines characterized by increased levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory cytokines when compared to unexposed pre-term placental tissues.

FASD and other impairments resulting from intrauterine alcohol exposure are preventable and it is thus necessary to continue educating women on the importance of alcohol abstinence during pre-conception and throughout pregnancy. The findings of this study will be clinically relevant because zinc deficiency can be a co-teratogen with alcohol, rendering the already vulnerable newborn lung at further risk. Alternatively, dietary zinc supplementation could be an effective intervention in reducing risk and severity of lower respiratory tract infections in the highly vulnerable pre-term infant with fetal alcohol exposure. Novel strategies such as zinc supplements, in addition to other nutrients premature infants are lacking, may become particularly important in this era of antibiotic-resistant bacterial infections.

CHAPTER 2: LITERATURE REVIEW

ALCOHOL

Alcohol and zinc deficiency

Alcohol abuse is often associated with nutritional deficiencies, which could be due to a poor diet, impairment in intestinal absorption, digestion, or changes in metabolic pathways [32-34]. Chronic alcoholics obtain at least 36% of calories from alcohol, with some reporting up to 50% of calories, often leading to neglect of other nutritional components such as fats, proteins, vitamins and minerals [32]. Long-term alcohol consumption is associated with zinc deficiency, which is thought to be due to inadequate intake and increased urinary zinc losses [35, 36]. However, a study in pregnant rats showed that urinary zinc excretion did not increase with ethanol ingestion [37]. In another study, rats fed an ethanol diet (36% of calories) for 8 weeks had similar urinary and fecal excretion rates as control rats, however when ethanol was combined with a low-protein diet, urinary zinc excretion was significantly increased [38]. Additionally, alcohol may impair absorption of zinc in the small intestine, however not all studies agree [37, 39, 40]. Given the extensive derangements and damage alcohol abuse causes in the host, from depletion of nutrients to increases in oxidation state, it is not surprising that alcohol abusers have a weak immune system and a higher risk in disease development than non-alcoholics. It is well known that zinc deficiency leads to impairment of both innate and adaptive immune response and continuous alcohol exposure may aggravate these effects. Important to this study are the effects of alcohol-induced zinc insufficiency on the respiratory immune response.

Alcohol metabolism

Alcohol is absorbed through the mucosa of the gastrointestinal tract by simple diffusion. Alcohol is primarily metabolized in the liver (90%) via three pathways, 1. alcohol dehydrogenase in the cytosol is the leading route for ethanol metabolism oxidizing approximately 80% of ethanol, 2. microsomal ethanol oxidizing system in the endoplasmic reticulum and, 3. catalase in the peroxisomes [41]. All pathways result in production of acetaldehyde, which is converted to acetate in the mitochondria. The conversion of acetaldehyde to acetate is slow and in alcohol abuse there is often excess accumulation of acetaldehyde as a result, which can have various adverse effects on the system and interfere with metabolism of nutrients, particularly lipids, and contribute to liver damage [32]. Acetaldehyde is associated with damage of the colonic mucosa and liver injury [42] and has been considered as a culprit for the carcinogenic effects of alcohol in human studies [43]. Animal models also support clinical findings; rodents that inhale acetaldehyde develop respiratory tumors (reviewed in [44]), and the acetaldehyde-induced damage to hepatocytes enhances tumor proliferation [45]. Furthermore, excessive alcohol consumption leads to a range of metabolic abnormalities in hepatocytes including, lipid peroxidation, glutathione depletion, activation of hepatotoxins and carcinogens and accumulation of superoxide anions and hydroxyl radicals, all of which can explain liver carcinogenesis [46-48].

Alcohol is also metabolized by non-oxidative pathways (5-10%) resulting in formation of fatty acid ethyl esters **(FAEE)** and phosphatidylethanol. *In vitro* and *in vivo* studies have shown that when enzymes of the oxidative pathways are inhibited, alcohol metabolism by non-oxidative pathways increases [49]. FAEE concentrations formed from the breakdown of alcohol vary by tissue and cell type because of differences in FAEE synthesis and degradation, availability of free fatty acids needed to form FAEE, and FAEE carriers. FAEE synthase is widely distributed in tissues, with highest concentration in pancreas [50]. Alcohol consumption can be indirectly measured through analysis of enzyme activity and the toxic effects of alcohol on cells, or directly

through quantification of alcohol metabolites such as ethyl glucuronide, ethyl sulfate, ethyl phosphate, and fatty acid ethyl esters.

Alcohol biomarkers

Indirect biomarkers of alcohol consumption. Among the commonly measured biomarkers of alcohol exposure are liver enzymes (gamma-glutamyltransferase, alanine amino transferase, and aspartate amino transferase) which are elevated in alcohol-induced liver damage [51]. These enzymes have poor sensitivity in identifying early drinking, moderate specificity, and detection is influenced by the amount and frequency of alcohol consumed with an elimination time of 2-3 weeks [52]. Gamma-glutamyltransferase has the highest sensitivity among the liver enzymes measures [53], however it significantly correlates with alcohol consumption only in chronic alcoholics and can results in false positives from conditions such as nonalcoholic fatty liver disease, obesity, diabetes, metabolic syndrome, among others [51, 54]. Alanine and aspartate amino transferases, similarly to gamma-glutamyltransferase, are not elevated by a single heavy drinking episode and have also been used as markers for chronic alcohol use [55].

Carbohydrate-deficient transferrin (**CDT**) is an isoform of transferrin that is deficient in sialic acid groups and is readily available as a clinical diagnostic test for alcohol use disorders [54]. CDT has moderate sensitivity and specificity and is a long term marker of heavy alcohol consumption with a half-life of 14-17 days [52]. Additionally, CDT can be a good marker for abstinence monitoring since it returns to baseline levels after ethanol cessation while the aforementioned biomarkers do not decline as fast [54]. CDT can result in false positives in patients with carbohydrate-deficient glycoprotein syndrome, chronic liver disease, galactosemia, or yield no results in some patients even after excessive alcohol intake [52]. Moreover, CDT may

not be useful in women as values are less elevated than men in long-term alcohol consumption [56].

Another identifier of alcohol abuse can be the presence of macrocytosis, which is characterized by increased mean corpuscular erythrocyte volume (**MCV**) and along with alcoholic liver disease can account for more than 25% of affected cases in chronic alcoholics [57]. MCV can stay elevated for a long time after discontinuation of alcohol since the lifespan of red blood cells is 120 days, making it an insensitive marker in abstinence monitoring. Furthermore, MCV increases in other conditions such as malnutrition (vitamin B12 or folate deficiency), liver and hematological diseases, among others [54, 57].

Acetaldehyde-adducts form when acetaldehyde binds to tissue and blood macromolecules such as DNA, lipids, and protein, thus altering protein structure and causing mutagenic changes in DNA [54]. Additionally, acetaldehyde-adducts have shown to have immunogenic properties and antibodies to acetaldehyde-modified proteins have been developed [58]. A promising biomarker could be acetaldehyde-modified hemoglobin which has been shown to positively correlate with self-report drinking [59] and in the detection of alcohol abuse in women [60]. However, acetaldehyde-adducts as biomarkers of alcohol abuse have not been established and are currently used for research only [54].

A multi-marker panel may be a better approach to establish presence of alcohol consumption and additionally patterns of consumption. A study by Brinkmann and colleagues using a panel of 4 biomarkers (methanol, acetone+2-propanol, gamma-glutamyltransferase, CDT) measured in blood samples of alcoholics and non-alcoholics, reported that alcoholics could be distinguished from non-alcoholics with 93% sensitivity [61]. However, multi-marker panel examination may not always be feasible or affordable, and the need for biomarkers with high sensitivity and

specificity and ability to distinguish between levels of alcoholism and occasional drinking remains high.

Direct biomarkers of alcohol consumption. Non-oxidative biomarkers of ethanol metabolism include measurement of ethyl glucuronide (**EtG**), ethyl sulfate (**EtS**), ethyl phosphate (**EtP**), phosphatidylethanol, and fatty acid ethyl esters (**FAEE**) in fluids, tissues, and matrices such as hair and meconium. EtG is formed by the conjugation of ethanol to glucuronic acid, mediated by UPD-glucuronyl transferases, and accounts for 0.02%-0.06% of ethanol metabolism [62]. EtG has been detected in blood and its derivatives, fluids, urine, tissues and hair (reviewed in [62]), where it can be detected for up to 8h in serum [63] and up to 31.5h in urine [64] after ethanol elimination. Confounding factors of EtG levels in urine include age, cannabis use, kidney disease and alcohol consumption within a month [65]. Additionally, metabolic disorders, physical activity, or conditions with impaired enzyme activity also influence EtG concentrations [62]. Even though EtG levels appear to be a promising biomarker of alcohol consumption, establishment of better positive cutoffs for EtG is crucial as levels of EtG in urine following mouthwash use [66], hand sanitizer use [67, 68], non-alcoholic beer consumption [69] or trace amounts of ethanol consumption (1g or 3g) [70], and use of baker's yeast and brewer's yeast tablets [71], have been documented to result in false positives because they fall above the commonly-applied cutoff value of 0.1mg/L [70]. EtG has also been measured in hair and concentrations can be influenced by type of solvent used to prepare the sample and by the film that covers the hair. Studies examining the relationship between EtG hair levels and alcohol intake report mixed results [62]. EtS is formed when ethanol conjugates to sulfate, mediated by cytosolic sulfotransferases [72]. Its detection in urine follows a similar detection window as EtG [72] and like EtG it can result in false positives with consumption of non-alcoholic beer and yeast or sugar tablets [69, 71]. EtP, in contrast to EtG and EtS, has a half-life equivalent to ethanol with a narrow window of detection, making it an insensitive marker of alcohol exposure.

Phosphatidylethanol (**PEth**) is formed in cell membranes only under the presence of ethanol in a reaction catalyzed by phospholipase D [73] and has a half-life of four days in whole blood [74] and can be detected 14 days after alcohol cessation in chronic alcoholics [75]. Under normal conditions, phospholipase D hydrolyzes phosphatidyl choline into phosphatidic acid but in the presence of ethanol, PEth forms because the affinity for ethanol is >1000-fold than for water [55]. PEth does not yield false positives in sober subjects with a previous history of addiction [76] and it is not affected by liver disease or gender [54]. PEth is not detected after a single dose of alcohol intake and can be a marker for identification of chronic alcohol abuse.

FAEE are synthesized in almost all tissues and are transported in the blood bound to albumin or incorporated in the core of lipoproteins. FAEE accumulation has been connected with pancreatitis-like injury and liver damage [77], as well as damages to the nervous [78] and cardiovascular [79] systems, since these organs/systems have the highest concentration of enzymes that synthesize FAEE [80]. The extent of damage to other organs remains under investigation. Blood FAEEs appear shortly after alcohol consumption and can be detected in blood at least 24 hours after consumption when ethanol is no longer detectable [81]. It has been reported that gender differences may exist in blood FAEE concentrations, with men having higher FAEE concentration than women possibly due to enzyme activity differences [82]. Furthermore, the type of FAEE accumulation may aid in distinguishing between episodic heavy drinking (binge) and chronic alcoholism. In a study that compared blood FAEE concentration in control volunteers given alcohol in a controlled setting (episodic heavy drinkers) with chronic alcohol users admitted to an alcohol detoxification center, chronic alcohol users had significantly higher serum ethyl oleate levels compared to episodic heavy drinkers [83]. Another study showed that total plasma FAEE concentration was 3.5-fold higher in patients with a history of chronic alcohol abuse compared to patients with a history of acute alcohol abuse, while plasma FAEE of control subjects were detected in trace amounts or not detected at all [84].

Since blood samples may not always be feasible in identifying alcohol intoxication in deceased persons, FAEE concentrations in *post-mortem* liver and adipose tissues have been investigated. In individuals who were intoxicated at the time of death, FAEE have been found to accumulate in pancreas, liver, heart and brain, while in chronic alcohol users in addition to those tissues, FAEE accumulates in adipose tissue, suggesting a storage site for these compounds [85].

Alcohol and disease

Alcohol consumption is a major risk factor for disease burden and contributes to 4% of total mortality in the world [3]. In the United States, alcohol abuse affects nearly 18 million Americans and is the 3rd leading lifestyle-related cause of death [1]. Alcohol consumption can result in injury and it modifies morbidity and mortality risk of several diseases including cardiovascular, neuropsychiatric, digestive, respiratory, and cancer [3]. Alcohol-induced or -related outcomes depend on type, pattern, and amount of alcohol consumed. Alcohol abuse encompasses binge drinking (4+ for women or 5+ for men drinks / sitting), heavy drinking (1+ for women or 2+ for men, drinks / day on average), or any drinking by pregnant women or underage adolescents [5].

Global disease burden and injury resulting from alcohol is ~5% with the percentage in men being 4x higher than women [3]. Even though men consume more alcohol than women [86], women experience alcohol-related problems at lower doses of consumption than men, especially in liver cirrhosis, cardiovascular disease and brain damage [87, 88]. Additionally, excessive alcohol use is associated with an increased risk for victimization and sexual assault in women [86]. Differences in blood alcohol concentrations between men and women following an equal dose of alcohol can partly explain the gender variances in incidence of disease and mortality risks [86]. Women are on average smaller than men, have less water per kilogram of body weight, and have less gastric alcohol dehydrogenase activity, leading to higher blood alcohol concentrations when compared to

men (reviewed in [86]). Chronic alcohol use is associated with increased endotoxin levels in blood and liver which in large amounts can cause chills, fever, shock and may result in endotoxemia and acute respiratory distress syndrome [89]. Between chronic alcohol users, women have higher levels of endotoxins than men [90] and the increased risk in gut permeability observed can be partly explained by female hormones [89].

When discussing the effects of alcohol consumption, it is important to address effects of low alcohol consumption, which are thought to exert some protective effects against cardiovascular disease (**CVD**) and type II diabetes (**T2D**). The relationship between alcohol consumption and CVD follows a J-shaped curve [91], where low-to-moderate consumption of alcohol (5-10g/day) is associated with reduced risk of coronary artery disease when compared to abstainers. On the other hand, irregular heavy drinking (bingeing), heavy drinking, and the combination of low-to-moderate drinking followed by heavy drinking are associated with increased risk of hypertensive diseases [91-94]. Similar to CVD, the relationship between alcohol and T2D follows a J-shaped curve [95, 96] and is influenced by type of alcoholic beverage and by drinking pattern. For example, heavy drinking for 1-3 days increases risk for T2D but distributing the consumption of the same amount of alcohol over a week does not [97, 98]. On the other hand, the relationship between the type of alcohol consumed and T2D risk is more controversial with some studies finding a correlation and others seeing no correlation (reviewed in [99]). Even though alcohol consumption in low amounts could exert some positive effects, the detrimental effects alcohol has on disease and injury outweigh the benefits. Extensive studies in human and animal models continue to elucidate the negative consequences of chronic alcohol use and health outcomes and will be partially discussed in the succeeding paragraphs.

Alcohol and neuropsychiatric disorders As it pertains to neuropsychiatric disorders, alcohol abuse largely results in disease rather than death including depression, alcohol withdrawal syndrome, delirium and hallucinosis, seizures, and tremors [100]. Alcohol abuse has been directly

associated with depression but increased intake of alcohol can also result from environmental factors that may trigger depression, such as financial and family problems, job loss etc. A meta-analysis study found that the presence of either alcohol use disorders or major depression increased the risk of the other disorder by ~2 fold [101], with alcohol use disorders causing depression as the more plausible explanation [101, 102]. On the other hand, moderate alcohol consumption (5-15g/day) in adults (55-80y), specifically wine, may decrease risk of depression [103].

Alcohol consumption has been associated with occurrence of epilepsy and unprovoked seizures where the risk for epilepsy onset increases as the consumption of daily alcoholic drinks increases, and an average of eight drinks/day is associated with a 3.3 relative risk of epilepsy onset [104]. Causative pathways between alcohol and epilepsy occurrence continue to be explored. Delirium is another neuropsychiatric outcome observed with excessive, pro-longed alcohol consumption. Majority of alcoholics have thiamine deficiency, which has been established to cause delirium (reviewed in [105]).

Alcohol and cancer A causal, dose-dependent relationship between alcohol consumption and squamous-cell carcinoma of oral, pharynx, larynx and esophagus has been established [106-108]. Suggestions have been made that the type of alcohol consumed may have an impact on cancer risk in the upper aero-digestive system however, results have been inconsistent [108, 109]. Furthermore, inconsistent relationships have been found between alcohol and esophageal adenocarcinoma [110, 111], stomach [112-114], colorectal [115, 116], pancreatic [117, 118] and possibly lung [119, 120] cancer. There may be a dose-response to breast cancer risk and alcohol consumption [88], where with every 10g increase in alcohol consumption there is an 7.1% increased risk, irrespective of smoking status [121]. Additionally, a positive dose-response association has been established between alcohol and hepatocellular carcinoma [117, 122, 123].

Many mechanisms have been proposed on how alcohol consumption affects risk for different types of cancer. It has been suggested that variants in genes of alcohol and folate metabolism, and DNA repair may modulate risk [124]. Alcohol dehydrogenase (**ADH**), aldehyde dehydrogenases (**ALDH**) and cytochrome P-450 2E1 (**CYP2E1**) are important enzymes in alcohol metabolism. ADH has several polymorphic sites and alleles which vary in ability to metabolize alcohol (reviewed in [124]) and in cancer association risk [125-127]. Homozygous or heterozygous individuals for ALDH2*2 have build-up of acetaldehyde, hot flashes, nausea and increased heart rate [124] and are at increased risk for oral, pharyngeal, laryngeal, and esophageal cancer [128, 129], while the relationship between CYP2E1, folate metabolism, DNA repair genes, and cancer show inconsistent results [124].

Alcohol and liver Alcoholic liver damage can be directly caused by alcohol and indirectly by poor nutrition as a result of alcohol abuse and includes a range of conditions from steatosis, to steatohepatitis, fibrosis and cirrhosis, and ultimately hepatocellular carcinoma [130]. Liver cirrhosis is an advanced state of chronic liver disease where normal tissue is replaced by fibrous tissue and loss of functional liver cells is observed. In cirrhosis, the liver suffers permanent damage, leading to blockage of blood flow through the liver and prevention of normal liver metabolic processes. Not all alcohol abusers develop liver disease [131] even though the risk increases in a dose- and time- dependent manner of alcohol consumption [132]. A systematic review and meta-analysis by Rehm reported that alcohol consumption and risk of liver cirrhosis was higher in women than men for the same dose of alcohol and that risk increased in a dose-dependent manner, regardless of sex. Additionally, the risk for liver cirrhosis was higher when the end-point considered was mortality vs. morbidity [133].

Alcohol and lung The lung is also affected by alcohol consumption, contrary to previous beliefs that this organ was spared from the harmful effects of alcohol. Our group and many others have explored and established several pathways by which alcohol deranges the immune responses

in the lung and consequently renders the individual at high risk for development of acute respiratory illnesses. Alcohol abuse can independently increase risk for acute respiratory distress disorder (**ARDS**) ~4 fold [134]. Additionally, alcohol increases risk for other acute conditions such as trauma, sepsis, pneumonia, aspiration, pancreatitis, and upper GI bleeding, all of which can lead to ARDS development [135]. In a multi-center prospective study with septic shock patients, the incidence of ARDS was 70% in patients with alcohol abuse vs. 31% in patients with no alcohol abuse. Also, pneumonia was the source of sepsis in 60% of alcoholic subjects and 35% of non-alcoholics [136]. The link between alcohol abuse and pneumonia risk and occurrence has been studied [137-140] and reviewed extensively [141-143] and the mechanisms by which alcohol abuse damages the lung continue to be elucidated.

Alcohol affects the respiratory host immune response at many levels, from mouth to alveolus. Chronic alcohol use impairs salivary excretions and increases bacterial colonization in the mouth and pharynx [144]. Additionally, alcohol exposure impairs mucociliary clearance in the aerodigestive tract and adversely affects the pulmonary innate immune response through impairment of macrophage differentiation and function, decreased surfactant and chemokine production, and decreased neutrophil recruitment and further activation of adaptive immune response [145-149]. The airway epithelium in alcohol abuse is characterized by increased apoptosis of alveolar epithelial type II cells [150, 151], impaired surfactant production [152], activated matrix metalloproteinases [153], decreased liquid clearance and increased permeability [154, 155] all of which render the lung highly vulnerable to acute injury [152, 156]. So far, studied mechanisms include glutathione depletion [150, 152, 157], renin-angiotensin system [158, 159], granulocyte/macrophage colony-stimulating factor [160-162], and recently zinc [31, 163].

Glutathione (**GSH**) is synthesized in the liver and is essential for DNA and protein synthesis, detoxification of peroxides, removal of toxic substances, and control of oxidant-mediated induction of pro-inflammatory cytokines [164-166]. In the lung, GSH is concentrated in the fluid

lining the alveolar epithelial/air interface and is transported by alveolar type II cells [167]. Animal and human models show that chronic alcohol decreases GSH levels within the alveolar space and increases gluthathione disulfide resulting in oxidative stress and loss of alveolar macrophage (**AM**) and epithelial cell function [18, 152, 157, 168, 169]. Supplementation with glutathione precursors ameliorates the outcomes of alcohol abuse through reduction of apoptosis, increase of surfactant synthesis and secretion, prevention of matrix metalloproteinases activation, restoration of barrier function and protection against acute lung injury (reviewed in [135]). Another mechanism by which alcohol causes lung injury is through the renin-angiotensin system, mainly through one of its products, angiotensin II, which is increased in chronic ethanol-fed rats [158]. Additionally, alcohol modulates expression of angiotensin II receptors (type 2), which can increase cell cytotoxicity and lead to apoptosis in response to pro-inflammatory mediators such as TNFα, while inhibition of the type 2 receptor blocks TNFα induced apoptosis [159]. Furthermore, deficiency of the enzyme required to make angiotensin II is associated with less lung injury following an acute insult and treatment with an angiotensin II inactivator protects mice from lung injury [170].

In the recent years, granulocyte/macrophage colony-stimulating factor (**GM-CSF**) has gained attention as a plausible mechanism by which alcohol exerts its negative effects on the lung. GM-CSF is secreted by alveolar epithelial type II cells and is required in growth stimulation of granulocytes and macrophages [171], processes mediated by transcription factor PU.1 [172-174]. Knockout GM-CSF mice display impaired alveolar macrophage function characterized by reduced clearance, phagocytosis, decreased surfactant expression and altered receptor expression [175, 176]. Chronic ethanol ingestion decreases expression of PU.1 and GM-CSF receptors in macrophages and airway epithelium of adult rats, leading to impairment in function of those cells [160, 161]. On the other hand, intranasal treatment with recombinant GM-CSF for 3 days

improves barrier function and fluid transport [162] and restores function in the affected cells [160, 161].

Even newer is the exploration of alcohol abuse and zinc status in the lung. *In vitro* studies with rat alveolar macrophage cell lines exposed to ethanol for 4 weeks show decreased expression of zinc transporters ZIP4 and ZnT1 and of zinc storage protein metallothionein (**MT**) -I in ethanol-exposed AMs [177]. Animal studies extend these findings and show that chronic ethanol ingestion decreases intracellular zinc levels in the alveolar space and reduces gene expression of zinc transporter ZnT4 and MT -I and -II in alveolar macrophages and epithelial type II cells [31]. Furthermore, these changes are associated with impaired phagocytic function in the macrophages [31]. Zinc supplementation to rats fed an ethanol diet (36% of calories) improves intracellular zinc levels, ZnT4 and MT I gene expression, and bacterial clearance, when compared to rats fed ethanol alone [31]. Recent research links zinc to GM-CSF transcription factors PU.1 and Nrf2, which are necessary in the immune response of macrophages. *In vitro* zinc treatments restore PU.1 and Nrf2 binding capacity in AMs, improve bacterial clearance, and restore balance of redox pairs in ethanol-fed rats, suggesting mitigation of oxidative stress in the AM of alcoholic rats [163].

ZINC

Zinc sources and bioavailability

Zinc is present in all body tissues and fluids and approximately 95% is within cells [178]. Majority of zinc is in skeletal muscle (63%) and bone (20%), and in much smaller amounts in liver, lung, skin, gastrointestinal tract and other organs [179, 180]. Plasma zinc accounts for 0.1% of total body zinc content, is replenished daily, and is under tight homeostatic control. Zinc can be obtained from the diet through consumption of red meat, oysters, whole grain cereals, pulses

and legumes, which provide 25-50mg/kg of raw weight [181]. Moderate sources of zinc (10-25mg/kg) are provided from chicken, pork or meat with high fat content, and polished rice, while fruits, green leafy vegetables, fish, roots and tubers provide <10mg/kg [181, 182]. In the United States, meats provide ~50%, pulses and cereals ~30%, and dairy products ~20% of dietary zinc [183] whereas worldwide, pulses and cereals are the major sources of zinc for most people [184, 185]. Fractional zinc absorption is twice as much in a high-meat diet (non-vegetarian) [186, 187] when compared to diet based on rice and wheat flour [188].

Zinc bioavailability is affected by diet composition. Antagonists of zinc absorption include non-digestible plant ligands such as phytates, some dietary fibers, and lignin [189]. Calcium can inhibit absorption of zinc alone, depending on the form administered, or further augment phytate inhibition of zinc absorption [190, 191]. The ratios of phytate:zinc and phytate×calcium:zinc are useful indicators in the reduction of zinc absorption and are thought to aid in identifying zinc deficiency [185, 191]. Phytates are found in whole grain cereals, legumes and in small amounts in some vegetables. They have a strong affinity for binding divalent cations and once they bind to zinc they form a stable and poorly soluble compound that inhibits zinc absorption. When phytate:zinc ratios are above 6-10:1, zinc absorption declines, and at ratios above 15:1, zinc absorption is less than 20% [182]. Protein intake, however, modifies the effects of phytate on the diet and improves zinc absorption. Examples include human milk vs. cow milk (casein rich), animal protein intake [192], and legumes such as phytate-free soybeans, white, and lupin beans [192-194]. Additionally, intestinal zinc absorption can be affected by competitive interactions between zinc and other ions with similar properties [181]. For example, high ferrous iron concentrations in iron supplements [195], copper intake, and intakes of folic acid [196] or iron-folate [197] affect zinc bioavailability. The competitive interaction between zinc and iron depends on the form of iron (administration in water and fasting conditions) and dose where very

high iron:zinc ratios inhibit zinc absorption [198]. However, at realistic fortification amounts and levels found naturally in food, zinc absorption is not affected by iron or copper [182].

Zinc metabolism

Zinc is absorbed throughout the length of the small intestine, with the jejenum being the major absorption site, by carrier-mediated and non-regulated diffusional processes [199]. Zinc is transported into the portal circulation through transcellular processes carried out mainly by zinc transporters and through paracellular processes when zinc content exceeds transcellular capacity. Once in the portal circulation, absorbed zinc is primarily metabolized by the liver (30-40%) [180] and is released from the liver for delivery to other tissues, bound to α_2-macroglobulin [199] and albumin [200]. Relative distribution of circulating zinc at any given time is 57% bound to albumin, 40% bound to α_2-macroglobulin and 3% bound to other proteins or amino acids, such as cysteine and histidine [199]. Under normal conditions, zinc loss is mainly through fecal excretion (90%) which includes endogenous zinc (pancreatic and intestinal mucosal cell secretions) and unabsorbed dietary zinc, and very little is lost through urinary excretion (<10%). Zinc excretion depends on dietary intake; the higher the intake, the greater the excretion and the lower the absorption [201]. Events such as starvation and muscle catabolism increase zinc loss in urine, while activities like strenuous exercise and high temperatures increase losses through sweat.

Zinc has fixed tissue pools, which vary which vary little with reduced intakes, and a small exchangeable pool which is more vulnerable to the status of the nutrient and needs to be continuously replenished [178]. When zinc intake is decreased, the organism first reduces fecal losses [202, 203] and if the demands are not met, urinary losses also decline [204]. If these mechanisms losses cannot maintain zinc balance and there is continued insufficient zinc intake, metabolic adjustments ensue, such as release of zinc from small pools in tissues and tissue

catabolism. Since majority of zinc is in muscle tissue maintenance of zinc balance in muscle and skin are prioritized over bone, liver, and plasma [205-207]. In animal studies with fluctuating food intakes (starvation and food ingestions), during the anorexia periods muscle catabolism occurs to release protein, potassium, zinc and other components [208] and these events are mostly reversed with zinc repletion (reviewed in [178]). Since there is no readily available zinc stores that can be released in response to dietary variations, the exchangeable zinc pool, which accounts for ~10% of total zinc in the adult [209], becomes crucial and its loss leads to biochemical and clinical signs of zinc deficiency [210].

Zinc functions

Zinc is an important micronutrient that displays catalytic, structural, and regulatory functions [178, 211]. For example zinc is a catalyst for ~100 enzymes [212] such as alkaline phosphatase needed in bone formation, alcohol dehydrogenase required for alcohol metabolism, and carboxypeptidades and proteases needed for protein digestion, among other enzymes. Zinc displays a structural role in zinc finger motifs including those involved in cellular differentiation, proliferation, signal transduction, cellular adhesion or transcription [178]. Additionally, zinc maintains enzymatic structures such as in anti-oxidant enzyme Cu/Zn- superoxide dismutase [213, 214], which breaks down superoxide anion into hydrogen peroxide. Furthermore, zinc can prevent oxidation in zinc-finger transcription factors and reduce generation of free radical formation by inhibiting the binding of iron and copper to membrane and intracellular sites [215]. Zinc regulates gene expression; for example by promoting transcription of metallothionein gene through the metal response element-binding transcription factor 1 (**MTF-1**) [216]. In low zinc conditions, MTF-1 binds to zinc-sensitive inhibitor and in the presence of zinc, the inhibitor dissociates from MTF-1, allowing MTF-1 to interact with metal response elements at the promoter region of the metallothionein gene and activate its transcription. MTF-1 and zinc

regulate expression of some zinc transporters such as ZIP10 and ZnT1. In zinc excess, MTF-1 promotes expression of ZnT1 [217] and suppresses expression of ZIP10 [218] to maintain zinc balance and prevent excess zinc accumulation within the cell.

Zinc deficiency

Zinc deficiency can result from poor intake or from diseases and conditions that impair intestinal absorption or increase intestinal loss of zinc such as acrodermatitis enteropathica (**AE**), cystic fibrosis, inflammatory disease [219], sickle cell anemia [220], among others. Additionally, renal disease [221], liver disease [36, 222], diabetes [215], alcoholism [223], and stress [224] increase zinc losses through urine. Clinical manifestations of severe zinc deficiency include growth retardation, delayed sexual and bone maturation, skin lesions, alopecia, impaired appetite, and impaired immune system [225, 226]. Overt zinc deficiency is rare and mostly observed in conditions such as AE, which displays, among other manifestations, thymic atrophy and increased bacterial, viral, and fungal infections [227]. AE if left untreated results in death within a few years, and zinc supplementation rapidly improves manifestations of the disease [228]. Mild-to-moderate zinc deficiency is more common and can present itself in diverse ways since zinc is widely involved in the general metabolism.

Nutritional status plays a crucial role in the host defense response against an antigen or an environmental insult and it has been well established that zinc is essential for the functioning of the immune system. Even marginal zinc deficiency depresses the immune system response. Zinc deficiency increases susceptibility to infection and augments existing infections by affecting all cells and aspects of the immune system. Zinc deficiency results in skin lesions from damages to epidermal cells and to the linings of gastrointestinal and pulmonary tracts [183, 229]. The innate immune response during zinc deficiency is characterized by decreased neutrophil chemotaxis

[230] and phagocytosis, impaired natural killer cell activity and phagocytosis of macrophages [24, 227, 231].

Zinc deficiency causes atrophy of the thymus, the central organ for T-lymphocyte development and results in impaired T cell development and decreased cell count [25]. Zinc is a cofactor for thymic hormone, thymulin, which is secreted by thymic epithelial cells and in immature T-cells it induces differentiation markers [232], while in mature T-cells it modulates cytokine release and proliferation of CD8 and IL-2 [233]. Thymulin is dependent on plasma zinc concentration and its activity is affected even with marginal zinc changes (reviewed in [229]). In zinc deficiency, T-lymphocyte responses such as delayed hypersensitivity and cytotoxic activity are observed which are reversed with zinc supplementation [229]. Similarly, B-lymphocyte development in the bone marrow is highly affected by zinc deficiency with 50-70% decrease in precursor B-cells [25, 234] and increased apoptosis [235]. On the other hand, mature B-lymphocytes are less affected by zinc deficiency because, when compared to precursor B- and immature lymphocytes, they express higher levels of Bcl-2, a proto-oncogene that inhibits apoptosis when overexpressed [235, 236].

Zinc excess

There has been no report of zinc excess intake from food consumption and it may result from supplement use. Adverse outcomes include suppression of immune system (>300 mg of zinc/day), decrease in HDL cholesterol, reduction of copper, gastrointestinal problems (50-150 mg of zinc/day), diarrhea, loss of appetite, abdominal cramps, vomiting (225-450 mg of zinc/day) [212]. High concentrations of zinc *in vitro* can lead to inhibited cytotoxicity of natural killer cells [237], macrophage activation, mobility and phagocytosis (reviewed in [229]).

Zinc biomarkers

Since zinc is necessary for general metabolic function, it is unlikely that a single specific biomarker of zinc nutrition exists to cover all of its biological functions [178]. Indices for zinc status are debated and include measurement of zinc concentration in plasma, hair, blood cells, urinary excretion, and in physiological functioning of enzymes that require zinc. The most frequently used biomarker of zinc status is plasma or serum zinc, which is a component of the exchangeable zinc pool and is readily available for uptake depending on tissue needs. Confounders for serum zinc include diurnal variations [238-240], gender [241], age [242], food intake [243-246], starvation, pregnancy state [247, 248], steroid use [178, 184, 249], and hemolysis [250]. Additionally, plasma zinc concentrations are sensitive to decreases that occur from metabolic processes induced by infections [251], endotoxemia, carcinoma, malabsorption syndromes and chronic disease states. Plasma zinc concentrations decline rapidly in severe zinc deficiency in a dose-dependent manner – the higher the loss of body zinc, the faster the decline in plasma zinc [178, 252]. However, in low or moderate zinc deficiency, levels may remain in the "normal" range for weeks due to adjustments in excretion and absorption rates that reduce whole body zinc loss [178] despite clinical manifestation of zinc deficiency characteristics [253, 254]. Thus, plasma zinc concentration may not reflect cellular zinc status [255] since values within "normal" range may also include zinc deficiency. Even though plasma zinc is not a useful biomarker at the individual level, at the population level it is widely accepted as a suitable biomarker of zinc status [256-258], where zinc deficiency becomes a major public health problem when the prevalence of low plasma zinc levels exceeds 20%.

Other indices of zinc status include measurement of zinc in erythrocytes, leukocytes and neutrophils, and activity of zinc-dependent enzymes [203, 254, 259, 260]. Concentration of zinc in erythrocytes is 10x higher than plasma however, given that the lifespan of erythrocytes is 120

days, erythrocyte zinc does not reflect short-term changes in dietary intake or recent body zinc store changes [261]. Leukocytes contain higher amounts of zinc (25x more than erythrocytes), have a shorter lifespan than erythrocytes, and therefore could be considered more sensitive to zinc status changes than erythrocytes. However, studies are inconsistent in the usefulness of leukocyte zinc as a biomarker, possibly because of differences in cell type and mixtures of leukocytes measured [262, 263].

A promising biomarker is measurement of metallothionein (**MT**) concentration in serum, erythrocytes, and monocytes. **MT** is found in most tissues, with highest concentrations in liver, intestinal mucosa, and kidney and has a high affinity for zinc and other heavy metals, but primarily binds zinc under normal physiological conditions. MT is viewed as a "storage" zinc protein and binds 7 zinc ions at one time, three in its beta domain and four in the alpha domain. MT expression is induced by presence of zinc and its transcription is regulated by MTF-1. Furthermore, concentrations of MT in serum correspond with intake levels and reflect changes in hepatic MT levels [180, 264]. Erythrocyte MT decreases even in moderate zinc deficiency and this association becomes stronger as the period of zinc depletion is extended [265]. Measuring MT in different tissues may aid in differentiating between zinc decreases from redistribution due to infection, trauma or stress or from poor nutrition. As hepatic MT and plasma MT concentrations rise with stress or inflammation [266-268], erythrocyte MT does not [269]; whereas in the event of decreased zinc intake due poor zinc nutrition, both hepatic [270, 271] and erythrocyte MT [269] decrease. Lastly, measurement of monocyte MT mRNA is also promising, as levels may decrease even in mild zinc deficiency [272], increase with supplementation (low or high dose) [273, 274], and return to normal range once supplementation is discontinued [275]. Since there is no agreement for zinc biomarkers at the individual level and one cannot capture a range of deficiency, more than one biomarker should be used in assessing zinc status.

Zinc transporters

Uptake of zinc into the cells is regulated by zinc transporters and to date 24 mammalian zinc transporters have been identified belonging to two families, ZnT and ZIP. There are 14 mammalian ZIP (zinc-responsive transport/iron-responsive transport family; SLC39) transporters that are responsible for zinc movement into the cytoplasm from the extracellular space or from intracellular organelles. Additionally, there are 10 ZnT transporters (cation-diffusion family, SLC30) that export zinc outside of the cell or into cellular compartments [276]. The two families of zinc transporters often display opposite roles in zinc acquisition and intracellular distribution and both families are crucial in the maintenance of zinc homeostasis. Zinc transporters are involved in processes needed to maintain zinc balance such as tight regulation of absorption and reabsorption, excretion, and tissue zinc distribution [277]. Expression of zinc transporters is regulated by zinc dependent or independent pathways at the transcriptional and posttranscriptional level [278] and can be tissue specific.

ZIP family ZIP1 was first identified as a transporter in K562 erythroleukemia cells [279, 280]. Its mRNA is ubiquitously expressed in human tissues and subcellular ZIP1 protein localization varies by cell type. ZIP1 plays housekeeping roles in the cell [276] and is involved in maintenance of cytoplasmic zinc by transport from intracellular vesicles as well as from extracellular space. When zinc is limiting, ZIP1 protein localizes to the plasma membrane and in zinc replete conditions it translocates to intracellular vesicles [276, 281, 282]. ZIP1 protein is highly expressed in prostate cells at the basolateral membrane of prostate epithelia and is thought to be the major zinc uptake transporter in these cells. In fact, progression of prostate cancer is characterized by markedly decreased ZIP1 mRNA expression in prostate cancer tissue when compared to normal tissue [283], and correlates with decreased levels of zinc accumulation [284-286]. ZIP2 protein is highly expressed in prostate epithelial cells and it is downregulated in

prostate cancer as well [287, 288]. Additionally, ZIP2 is expressed in uterus [289], peripheral blood mononuclear cells and monocytes [290], cervical epithelium and is involved in zinc uptake. Regulation of ZIP2 expression by dietary zinc may be tissue and species specific. When human monocytic cell line or peripheral blood mononuclear cells are treated with TPEN (a zinc chelator) intracellular zinc decreases and mRNA ZIP2 expression increases, suggesting an attempt by the cell to restore zinc balance and also that expression of ZIP2 is regulated by zinc [290]. On the other hand, mouse ZIP2 mRNA expression is unresponsive to dietary zinc restrictions in the intestine and visceral yolk sac [280].

Similarly to ZIP1 and ZIP2, ZIP3 is involved in uptake of zinc and highest levels are found in testes [280]. ZIP3 is also expressed at the apical plasma membrane of prostate epithelial cells and is postulated to be involved in reabsorption of zinc from the prostate fluid. Protein expression of ZIP3, but not mRNA expression, responds to changes in zinc status, suggesting posttranslational regulation [282]. Additionally, ZIP3 is required for survival of mammary gland epithelial cells and its deficiency in mice results in altered structure of mammary gland, increased number of apoptotic cells, and reduced mammary gland weight [291, 292]. Homozygous knockout mouse models of ZIP1, 2 and 3 show no overt phenotypes if fed a diet adequate in zinc (reviewed in [293]), however, when dietary zinc is limited during pregnancy, embryos of ZIP1 and ZIP3 knockout mice develop more abnormalities than wild-type embryos [294].

ZIP4 is localized in the apical membrane of enterocytes and visceral endoderm cells in the embryonic visceral yolk [295] during dietary zinc deficiency [296, 297] and under normal zinc levels and repletion, ZIP4 is recycled *via* endocytosis into intracellular compartments [298, 299]. ZIP4 is responsible for zinc uptake from lumen of the gut to the enterocyte and its expression is regulated by zinc at the mRNA [280, 296, 298, 299] and the protein [298] level. Homozygous knockout of ZIP4 in mice is embryonic lethal [297]. In humans, mutations in ZIP4 are associated with acrodermatitis enteropathica (**AE**) [300, 301], a rare autosomal recessive disorder of

intestinal zinc malabsorption that displays the full range of zinc deficiency characteristics, which can be alleviated by oral zinc supplementation.

Given that oral zinc supplementation alleviates AE symptoms, other transporters must be present at the apical membrane of enterocytes involved in dietary zinc absorption from the lumen. Additionally, since not all mutations of AE map on ZIP4 protein, it was postulated that another protein was involved which was later named ZIP5 (reviewed in [293], [301]). ZIP5 is highly expressed in liver, kidney, pancreas, small intestine and colon [302] and unlike ZIP4, it is internalized and degraded in cellular compartments under zinc deprivation [299] and localizes at the basolateral surface in zinc replete conditions (reviewed in [295]). ZIP5 specifically transports zinc from the serosa to the mucosal part of enterocytes and it is suggested that it may be involved in enterocyte sensing of zinc status in the body [303, 304]. ZIP5 is regulated by dietary zinc status at the translational but not at the transcriptional level [295, 299].

ZIP6 is localized at the plasma membrane and is another zinc importer. It is highly expressed in tissues sensitive to steroid hormones such as mammary gland, placenta, and prostate [305] and its expression is stimulated by estrogen. HeLa and lung carcinoma cell lines have abundant expression of ZIP6. ZIP6 has been suggested to play a role in cancer progression [305-307] and has shown to be a reliable marker for estrogen-receptor-positive cancers and in identification of a subtype of breast cancer [308, 309]. ZIP6 is involved in the immune response, where its down-regulation after lipopolysaccharide (**LPS**) exposure is associated with decreased intracellular zinc levels and increased surface expression of MHC class II molecules in dendritic cells, which are involved in antigen presentation to T cells [310]. Exposure of dendritic cells to TPEN yields similar results as LPS exposure, while zinc supplementation or over-expression of ZIP6 inhibit LPS-induced overexpression of MHC class II molecules [310].

ZIP8 was previously named Bacillus calmette-guerin-induced gene in monocyte clone 103 (BIGM103) because the gene was induced in monocytes after exposure to Bacillus calmette-guerin cell wall skeleton [311]. ZIP8 is a zinc importer and is abundantly expressed in lung, liver, kidney, testis, brain, small intestine, and membrane fractions of RBC [312, 313]. ZIP8 mRNA expression increases during monocyte differentiation into macrophages and dendritic cells [276], upon T cell activation [314], and in the lung by inflammatory mediators such as TNFα and LPS [315] and may aid in zinc sequestration following activation of inflammatory pathway [316]. Knockdown of ZIP8 results in reduced cellular zinc content, impaired mitochondrial function in response to TNFα and increased cell death in lung epithelia [315] while induction of ZIP8 expression results in increased intracellular zinc levels and increased cell survival in presence of TNFα [315].

ZIP10 expression is regulated by MTF-1 and responds to dietary zinc supply [317, 318] . ZIP10 mRNA expression rises in invasive and metastatic breast cancer cell lines and is associated with metastasis of breast cancer to the lymph nodes. On the other hand, knockdown of ZIP10 decreases intracellular zinc levels and migratory activity of metastatic breast cancer cells [319]. Given that breast cancer is associated with zinc accumulation in breast tissue and increased expression of ZIPs 6, 7, and 10, future research should consider these transporters as possible drug targets.

ZIP13 is homologous to ZIP7 and localizes in the Golgi apparatus where it is thought to be responsible for zinc efflux from the Golgi into the cytoplasm [295]. ZIP13 loss causes dysregulation of genes involved in bone, tooth and craniofacial development (reviewed in [295]). Additionally, mutations in ZIP13 are associated with a subtype of Ehlers-Danlos Syndrome (**EDS**) [320, 321], a spectrum of connective tissue disorders caused by mutations that affect collagen synthesis and modification [322], and manifest in progressive kyphoscoliosis, hypermobility of joints, and hyperelasticity of skin combined with severe hypotonia of skeletal

muscles [323]. The subtype of EDS associated with ZIP13 loss, in addition to classical characteristics of EDS, involves skeletal dysplasia, especially in the spine and hands [324]. Previously, it was thought that the EDS subtype caused by ZIP13 mutation was a result of excessive zinc accumulation in the endoplasmic reticulum. However, it was recently suggested that this EDS subtype may result from vesicular trapping of zinc and zinc deficiency in the endoplasmic reticulum rather than overload [325]. ZIP13 is also involved in TGFβ signaling pathways, which regulate immune function, cell proliferation, epithelial to mesenchymal transition.

Lastly, ZIP14 is the closest homologue to ZIP8 and is highly expressed in liver and small intestine [326, 327]. ZIP14 mRNA expression is stimulated by LPS in mouse liver and accumulation of ZIP14 protein is observed in the plasma membrane of hepatocytes [327]. ZIP14 expression is stimulated during the acute-phase response and can lead to hypoferronemia because ZIP14, in addition to zinc and cadmium transport, has been shown to mediate transferrin-bound and non-transferrin-bound iron uptake into hepatocytes [328]. The acute-phase response is associated with hypozincemia and hypoferronemia in the host [329] possibly to reduce iron and zinc availability for the invading pathogens [330], resulting in zinc redistribution from the circulation to the liver [331].

Less research has been done on ZIP7, 9, 11, and 12. ZIP7 is ubiquitously expressed [308] and localizes in the Golgi apparatus where it transports zinc to the cytoplasm [332]. Protein expression of ZIP7 is suppressed under zinc-rich conditions however mRNA expression is not affected in a yeast mutant strain [332]. Recent research has explored the link between ZIP7 and breast cancer progression [309, 333], where ZIP7 may play an important role in zinc import into the cell, leading to activation of growth factors, and growth and invasion of cancer cells [309]. There is no information on expression, structure, regulation and roles for ZIPs 9, 11 and 12.

ZnT transporters 10 mammalian ZnT transporters have been identified. ZnT1 was the first discovered zinc transporter [334] and is ubiquitously expressed with highest expression in small intestine, kidney, and placenta [335]. ZnT1 is expressed mainly in the plasma membrane of cells where it exports zinc from enterocytes into the bloodstream [295, 336-338], aids in zinc recovery from the glomerular filtrate [336], and possibly participates in maternal zinc transfer to the fetus [217, 336]. In neuronal cell lines, ZnT1 cDNA localizes primarily to the plasma membrane with some punctuate throughout the cell [339]. Its expression is influenced by dietary zinc status with increases in response to zinc treatment or supplementation and decreases in zinc deficiency [337, 340]. Transcription of ZnT1 is regulated by MTF-1 [217], along with MT-I and ZIP10. ZnT1 protein has been detected in different molecular weights in tissues, suggesting post-translational regulation [337, 339]. Loss of function of ZnT1 is embryonic lethal in mice because maternal zinc transfer to the uterine and embryo is impaired (reviewed in [341]).

ZnT2, similar to ZnT1, is regulated by zinc status however, unlike ZnT1, ZnT2 localizes to endosomes and facilitates zinc transport from the cytoplasm into vesicles in response to high zinc conditions [342]. ZnT2 mRNA has been detected in small intestine, kidney, placenta, pancreas, testes, and mammary gland [335]. ZnT2 expression is increased in the small intestine during late gestation until weaning in neonatal pups and in the mammary gland where it is thought to play a role in zinc metabolism during lactation [343].

ZnT3 is exclusively expressed in brain and testes [344]. ZnT3 is required in loading zinc into synaptic vesicles and ZnT3 knockout mice fail to accumulate zinc in brain compartments [345]. Studies exploring outcomes in ZnT3-knockout mice show mixed results. Earlier studies report that ZnT3 knockout mice perform equally in spatial learning, memory, and sensorimotor activities and have no deficiency in fear learning [346]. However, recent research has found age-dependent deficits in learning and memory manifesting at 6 months of age in mice [347], defects

in associative fear memory [348], and complete deficits in contextual discrimination and spatial working memory [349].

ZnT4 was identified as the gene responsible for lethal milk disorder (*lm/lm*) [350] and its mutation results in a 50% decrease in zinc transport in milk [351]. ZnT4 is thought to facilitate zinc transport into secretory vesicles of exocrine glands, such as mammary gland, thus allowing zinc excretion by these glands [295]. ZnT4 mRNA is expressed ubiquitously, with highest concentrations in mammary gland [343] and ZnT4 protein is found in intracellular vesicles in several tissues including mammary gland, brain, small intestine, luminal and epithelial cells of alveolus and duct [350, 352, 353]. Regulation of ZnT4 by zinc is tissue dependent.

ZnT5 has two major splice variants [354]; splice A variant localizes to the Golgi [354] and appears to be involved in the delivery of zinc to enzymes entering the secretory pathway [355, 356] and B-splice variant has been found to be present at the apical side of CaCo-2 intestinal cell line and at the brush border membrane of human intestinal biopsies [298] where it is thought to act as an influx and efflux zinc transporter, unlike other members of the ZnT family [357]. ZnT5 mRNA is expressed ubiquitously in human tissues but is most abundant in pancreas [358], prostate, ovary and testes [359]. In insulin-containing β-cells, which contain zinc at the highest level in the body, ZnT5 localizes in the Golgi apparatus [359]. ZnT5 homozygous knockout mouse displays poor growth, lean phenotype, decreased bone density and weak muscle, and more than 60% of ZnT5-/- male mice die from bradyarrhythmias and have reduced ALP activity [359].

ZnT6 is most closely related to ZnT4 in sequence and may function in transporting zinc from the cytoplasm into the Golgi and vesicular compartments. ZnT6 mRNA is expressed abundantly in brain, liver, and small intestine, but protein has only been detected in brain and lung, suggesting regulation at the translational level with tissue specificity [360]. ZnT5 interacts with ZnT6 to form a complex that can transport zinc into the secretory pathway [361, 362]. Both transporters

are expressed in the trans-Golgi network and the ZnT5/ZnT6 complex is necessary for activation of tissue non-specific alkaline phosphatase.

ZnT7, closest in homology to ZnT1, is widely transcribed in mouse tissues including, liver, kidney, spleen, heart, brain, small intestine, and lung with highest concentrations in liver, small intestine and spleen [363]. Protein expression is limited to lung and small intestine [276]. High expression along segments of the small intestine suggests possible involvement of ZnT7 in zinc absorption. ZnT7 localizes in the Golgi and may play an important role of loading zinc into secretory or membrane-bound proteins [363]. ZnT7 knockout mice display poor growth and decreased body fat composition that cannot be corrected with zinc supplementation, suggesting that ZnT7 plays an essential role in dietary zinc absorption and regulation of body adiposity [364]. ZnT8 is expressed in insulin secreting β-cells where it facilitates zinc accumulation into intracellular vesicles from the cytoplasm [365]. Additionally, ZnT8 may be involved in providing zinc for insulin maturation and storage processes in insulin secreting β-cells (reviewed in [295]). Many genome-wide association studies linking ZnT8 to diabetes have been conducted [366, 367] and the relationship continues to be elucidated.

Finally, studies examining the function and regulation of ZnT9 and ZnT1010 are scarce. Both transporters have been associated with fetal expression, with ZnT9 discovered in embryonic lung cells [368] and ZnT10 in fetal liver and brain [369]. ZnT9 may play a role in zinc redistribution during lactation as its protein expression increases 2-fold in lactating mammary gland compared to non-lactating tissue [370]. Contrary to previous belief that ZnT10 is restricted solely to fetal expression [295], a recent publication showed decreased ZnT10 mRNA levels in frontal cortex tissues from adult patients with Alzheimer’s disease when compared to control subjects [371].

Zinc transporters in the lung

In the recent years, progress has been made on characterization of zinc transporters in the lung and it has been established that ZIP1, ZnT1, ZnT4 and ZnT7 mRNA and protein are expressed [214]. In human epithelial cell line, under normal conditions 8/10 ZnT members and 11/14 ZIP members are consistently expressed [315] and TNFα stimulation results in marginal changes of some transporters. ZIP8 is not detected in lung under normal conditions but its expression increases during host innate response against stress, LPS challenge or TNFα stimulation, resulting in increased intracellular zinc and cell survival in presence of TNFα, while inhibition of ZIP8 leads to decreased intracellular zinc, impaired mitochondrial function, and cell viability loss [315]. ZIP8 regulation does not appear to depend on zinc status. Similar to ZIP8, ZIP14 mRNA expression is up-regulated in response to LPS challenge and inflammatory stimuli such as IL-6, LPS+IFNγ and TNFα, in murine and human macrophages from the lung and spleen [372]. Additionally, ZIP14 mRNA expression is stimulated by NF-κB inhibitors, blockers of calcium recapture, immunosuppressive and anti-inflammatory drugs, and TPEN, while NF-κB, DNA-binding protein inhibitors (mithramycin A), and extracellular calcium-chelating agents inhibit ZIP14 expression [372]. Among the ZnTs, ZnT4 is the most studied in the lung. ZnT4 mRNA expresses in apical and basal surfaces of the epithelium [373], in alveolar epithelial type II cells, and macrophages [31]. In an allergen-induced airway inflammation, ZnT4 expression of mRNA and protein decrease and are associated with decreased levels of intracellular zinc [373-375]. In animal models of chronic alcohol exposure, mRNA expression of ZnT4 decreases in alveolar epithelial cells and macrophages along with decreased intracellular zinc [31].

MATERNAL OUTCOMES

Zinc requirements during pregnancy

During pregnancy, RDAs for many vitamins and minerals increase and suboptimal nutrition can compromise pregnancy outcomes and enhance teratogenicity of insults such as alcohol [376]. Proper zinc nutrition during pregnancy is essential for fetal development, growth, and immune function [377]. The amount of zinc required for pregnancy changes slightly depending on age group [212]. RDA for zinc in healthy, well-nourished, pregnant female rises ~27% when compared to non-pregnant counterpart [212] and the zinc requirement for pregnant women in developing countries may be greater than 43% when compared to RDA for non-pregnant, non-lactating women [378, 379]. Moreover, zinc requirements may be as much as 50% in vegetarians [212] whose diet is rich in phytates and grains and bioavailability of zinc is low.

Zinc deficiency and supplementation in pregnancy outcomes

It is estimated that zinc deficiency contributes to 0.4 million maternal and child deaths every year [380]. In developing countries, maternal zinc concentrations could be lower because of decreased intake (consumption of cereal rich diet instead of animal based foods) or because of chronic infections that reduce plasma zinc concentrations [215], ultimately resulting in lower feto-placental zinc transfer [381]. Maternal zinc deficiency during pregnancy exerts negative effects on both mother and fetus and may have long-lasting effects postnatally. Zinc-deficient animals have increased incidence of prolonged labor, hemorrhage, obstructed labor, and placental abruption [382-386]. Additionally, induced marginal zinc deficiency during pregnancy is associated with a higher incidence of stillbirths, abortions and delivery complications in rodents [387]. Timing and duration of zinc deficiency in the periconceptional period also influence embryo development. In rodents, severe zinc deficiency prior to pregnancy results in disruption

of estrous cycle and no mating may occur [215]. Furthermore, zinc deficiency in gestational days 0-10 results in 22% fetal malformation, while zinc deficiency in gestation days 0-12 increases the number of abnormal fetuses to 56% [388]. Human studies do not report clear correlations between zinc deficiency and pregnancy complications or maternal outcomes such as hypertension, amnionitis, and post-partum infections [389]. Additionally, studies examining the correlation between maternal plasma zinc levels and fetal outcomes such as premature birth and low birth weight yield contradicting results [389, 390].

Even though poor zinc availability during pregnancy has been established to have negative outcomes for both mother and fetus, human supplementation studies do not show consistent results or strong associations. In a systematic review by Chaffee and King using data from 16 trials, maternal zinc supplementation was associated with a reduced risk of pre-term birth by 14% [381] possibly due to decreased incidence or severity of maternal infections or alteration of circulating hormones that affect the onset of labor [386]. The findings by Chaffee and King support previous findings by Mahomed and colleagues [391, 392].

Zinc biomarkers in pregnancy

Some of the zinc biomarkers used during pregnancy include measurement of zinc concentrations in plasma or serum, leukocytes, erythrocytes, amniotic fluid, red blood cell metallothionein, and assessment of dietary intake [393-397]. Low maternal serum zinc or leukocyte zinc concentrations have been associated with prolonged labor, pre-term delivery, hemorrhage, small for gestational age babies, intrauterine growth retardation, low birth weight, and hypertension [381]. However, plasma zinc is affected by pregnancy state and it is not a good biomarker of zinc status during pregnancy. Plasma zinc concentrations decline throughout pregnancy [398] and at term are 35% lower than non-pregnant women [389], possibly due to hemodilution, decreases in

levels of zinc binding protein, hormonal changes during pregnancy [399], and active transport of zinc from the mother to the fetus [389, 400]. Red blood cell metallothionein has been assessed in pregnancy in several randomized controlled trials and trends toward increase in late pregnancy when compared to non-pregnant women with similar zinc intakes but does not correlate with other indices measured, except for erythrocyte zinc [401, 402]. Dietary zinc intake, often obtained through questionnaires, is another method used to assess zinc status in pregnant women. Caulfield *et al.* using the estimation that pregnant women consume on average 9.6 ± 1.2 mg Zn/d [403] reported that 82% of pregnant women worldwide may have inadequate intakes of zinc during pregnancy [404].

Zinc transporters in pregnancy

In mammals, the placenta is essential for maintenance of pregnancy and zinc is a crucial micronutrient needed not only for placental development but also for fetal growth. The rates of fetal zinc accumulation increase substantially in the last trimester [405] emphasizing the important role zinc transporters play in the maternal-placental-fetal interface. In murine placenta, mRNA expression of some ZnT transporters is observed with ZnTs 1-6 showing no changes and ZnT7 increasing with pregnancy progression [406]. Other animal studies demonstrate protein localization of ZnT1 and ZnT4 in placental regions where fetal and maternal blood circulate and nutrient exchange occurs, indicating involvement of these transporters in zinc transfer to the fetus [343]. Null mutations of ZnT1 result in embryonic lethality [217].

Of importance to the mammary gland is ZnT4 whose mRNA and protein expression increase during lactation [343, 350]. ZnT4 null mutation (*lm/lm)* [350, 407] in mice results in milk with 35% less zinc, decreased mammary gland size, and decreased milk secretion [408] that can be corrected upon supplementation or by fostering pup to a normal dam [341]. Milk from mice with

the *lm* mutation is not completely void of zinc [295] suggesting that other transporters may be involved in zinc transport into breast milk [353]. In fact, in cultured mammary cells and mouse lactating mammary gland, a greater than 2-fold increase is observed in protein expression of 6 ZIP transporters (ZIP3, ZIP5, ZIP7, ZIP8, ZIP10, ZIP11) and ZnT transporters (ZnT2, ZnT4, ZnT9), from which ZIP5, ZIP8 and ZIP10 are thought to be involved in zinc acquisition from maternal circulation [370]. With exposure to physiologically high levels of zinc, ZnT2 protein redistributes from the plasma membrane to intracellular vesicles of mammary gland epithelial cells, possibly contributing to sequestration of excess zinc from the cytoplasm [342, 409, 410]. Mutations in ZnT2 result in > 90% decrease in zinc secretion into the breast milk, leading to transient neonatal zinc deficiency [411].

Alcohol and pregnancy

Alcohol consumption during pregnancy leads to a range of growth and developmental disabilities classified as Fetal Alcohol Spectrum Disorders (**FASD**) [9, 10, 412] and increases the risk for premature delivery [14, 413] as well as the perinatal morbidity and mortality associated with prematurity [414, 415]. Combined with the physiological changes in pregnancy, the increased nutrient demands can be further worsened by malnutrition, inadequate or lack of prenatal care, or by diseases or conditions that cause malabsorption. Alcohol-related zinc insufficiency or deficiency could be due to a combination of inhibition of absorption and poor diet [33, 41]. Given that 1 in 2 women of childbearing age report alcohol drinking and that nearly 50% of pregnancies are unplanned, alcohol consumption during pregnancy can be detrimental to maternal zinc supplies and to the developing fetus. In human studies, an inverse relationship has been shown between maternal plasma zinc and expression of fetal alcohol syndrome [416] and in animal models, the teratogenicity of ethanol during pregnancy is amplified when combined with a zinc deficient diet [417, 418], possibly due to induction of maternal metallothionein and redistribution of zinc in maternal tissues, resulting in reduction of zinc transfer to the fetus [419, 420].

Alternatively, in metallothionein-knockout mice the negative effects of ethanol are reduced because zinc is not sequestered by maternal tissue and is made available for transfer to the fetus [421]. Additionally, zinc supplementation combined with alcohol exposure during pregnancy may protect against physical abnormalities, cognitive and spatial impairments associated with fetal alcohol exposure, reduce the number of stillbirths, and decrease the risk for postnatal mortality in pups exposed to ethanol during gestation [422-424]. Fetal outcomes will be discussed in detail later.

Alcohol biomarkers during pregnancy

Body water content increases significantly in pregnancy when compared to non-pregnancy state [425], therefore measuring blood alcohol content in pregnant women is not an appropriate indicator of alcohol consumption, which may be diluted as a result of increased body water and may not accurately reflect amount consumed. To date, there is not a laboratory test screening that can accurately identify and quantify prenatal alcohol exposure and clinicians have to still rely on maternal self-report even though it does not accurately depict consumption [426] or patterns of consumption due to difficulty to recall and because of the social stigma associated with drinking [427, 428]. In a study assessing agreement between maternal self-reported drinking and quantification of ethanol metabolites, no agreement was found between reported alcohol intake and FAEE concentrations in meconium (first stool of the newborn) [429]. Detection of alcohol in the breath, skin, urine, blood, and cord blood are inexpensive tests and can be used to detect acute alcohol exposure but because alcohol is rapidly eliminated from the body, these tests are not informative [426]. Similarly, acetaldehyde, a metabolite of alcohol, is removed quickly from the body and is insensitive to recurrent alcohol consumption.

FAEE as markers of prenatal alcohol exposure have gained momentum in the past decades and researchers continue to examine their correlation to alcohol consumption and patterns in different tissues and matrices such as meconium and hair. Measurement of FAEE in blood may not be useful for identification of prenatal alcohol exposure since it has a half-life of 24h [82]. The half-life of FAEE in human tissues such as placenta, hair, cord blood, and meconium is still unknown [426]. In mouse placental tissue, the half-life of FAEE was estimated to be 50-80h [430] and in adipose tissue of rabbits it has been reported to be 16h [431], however human placenta is different than these species and more similar to baboons and macaque [432]. Our group has recently investigated placental FAEEs as potential predictive biomarker of prenatal alcohol exposure and has proposed cutoffs for detection of alcohol-exposed infants in a population of premature newborns born <1500g using a combination of fatty acid ethyl esters (Gauthier 2014; manuscript under review).

FAEE synthase, one of the enzymes responsible for FAEE synthesis, can also provide useful information in regards to FAEE accumulation. In pregnant mouse models of acute ethanol exposure in late gestation, FAEE synthase activity is high in liver, placenta and heart with FAEE accumulation observed in adult, fetal, and placental mouse tissues [430]. In contrast, saline treated animals display complete absence of FAEE in placental tissues suggesting that formation of FAEE occurs only in the presence of ethanol [430]. Looking at specific fatty acids, ethanol-exposed mouse placentas accumulate higher concentrations of ethyl stearate, while the liver and heart accumulate ethyl oleate when compared to non-exposed placentas [430]. On the other hand, animal fetal tissues accumulate ethyl palmitate, ethyl stearate, and ethyl oleate [430]. FAEE synthase activity has been observed in human placenta as well and in the absence of alcohol no FAEE are formed [430]. Studies measuring differences in FAEE synthase activity in pregnant women are scarce [426]. Phosphatidylethanol, a product formed in the non-oxidative pathway of alcohol metabolism, may be a promising biomarker since it can be detected long after alcohol

cessation. Liver enzymes, which are more useful for detection of liver disease rather than alcohol exposure, may not be suitable biomarkers in identifying prenatal alcohol exposure.

FETAL OUTCOMES

Alcohol exposure *in utero*

As discussed above, 1 in 30 pregnant women consume alcohol on either a frequent (7 or more drinks per week) or binge (5 or more drinks in one occasion) basis [5]. Prenatal alcohol exposure leads to a range of growth and developmental disabilities classified as Fetal Alcohol Spectrum Disorders (**FASD**) [9, 10] that are estimated to affect 2-5% of births in the United States [433]. Among mothers who consume alcohol, there is a higher risk that the baby will be born premature, hospitalized in the neonatal unit, and diagnosed with FASD when compared to those who do not consume alcohol [434]. The majority of infants exposed to alcohol *in utero* do not display the phenotypic characteristics associated with Fetal Alcohol Syndrome (**FAS**) [435] and often are undetected by primary care providers. FAS is the most severe outcome of FASD [7-11] and ranges from 0.5-2 per 1000 live births in the U.S. [12, 13], where Northern Plains American Indians have the highest postulated FAS prevalence at 8.5 per 1000 live births [436]. In the U.S., the annual cost for FAS is estimated at $3.6 billion/year with a lifetime cost of $2.9 million/individual [6]. Infants with FAS are hospitalized an average of 30 days during their first year of life, while control infants are hospitalized 4.4 days [437]. The two leading causes of hospitalization for infants with FAS and FASD are otitis media and pneumonia [437].

Infants chronically exposed to alcohol *in utero* have a >34-fold higher risk of extreme prematurity (<32 weeks gestational age) than non-exposed infants [14] increasing the risk of the perinatal morbidity and mortality associated with prematurity [414, 415], however it is unknown what percentage of pre-term births is attributed to alcohol consumption during pregnancy. The

etiology of pre-term labor is complex and in most cases a precise mechanism on its initiation cannot be established [438]. Pre-term birth affects 12% of total births in the U.S. [439] and is the leading cause of perinatal morbidity and mortality in developed countries. In developing countries, the percentage of pre-term births is thought to be substantially higher. One of the major complications of pre-term birth is lung immaturity which contributes up to 75% of early mortality and morbidity in the pre-term infant [440].

Lung development

Lung development is divided into five stages: embryonic, pseudoglandular, canalicular, saccular and alveolar, starting at gestational day 26 in humans (day 9.5 in mouse) and ending in early childhood [440]. The last stage – the alveolar period – can additionally be split into two stages, alveolarization (36 weeks-2 years postnatally) and microvascular maturation (birth-3 years postnatally) [441]. Pre-term infants born at the end of canalicular period (weeks 16-26) are capable of surviving because their lungs are equipped with complete conducting airways and a vascular bed to permit minimal gas exchange [441]. During the saccular stage (weeks 24-38) saccules, alveolar ducts, and air sacs are formed, and surfactant is produced. During alveolarization, secondary crests form, which are essential for proper formation of mature alveoli, where the gas exchange occurs. However, these secondary crests are still thick, contain a capillary bilayer and are still not fully efficient in gas exchange. During microvascular maturation (birth-3 years of age), the intraalveolar septa that contain the capillary bilayer thin into a monolayer with mature blood vessels that increases gas-exchange surface area [441].

Human and mouse lung differ from each other in two aspects, the lung lobe pattern and time frame of lung development. The human lung has two lobes in the left and three lobes in the right, while the mouse lung has one lobe deriving from the left bronchus and four from the right. At

birth, the full-term human lungs are mostly complete in development with partial alveolarization and microvascular maturation, which continues postnatally, while the mouse lungs are at the saccular stage at birth and are more primitive in function than human full-term lungs. The mouse lungs provide a suitable model in studying the premature human lungs, which, depending on gestational age of the infant, is similar to lungs of a full-term mouse pup.

Fetal alcohol exposure and lung immune response

The respiratory tract presents a route of exposure to environmental toxins or aeroallergens and can modulate immune maturation. After inhalation of foreign organisms, the response of the lung is coordinated by activation of the pulmonary epithelium and alveolar macrophages, which release cytokines and chemokines to recruit additional inflammatory cells to the airspace [442]. At birth, the newborn lung is not fully developed and its defense systems are immature [15, 16] and the newborn alveolar macrophage (**AM**), which initiates the first immune response of the lung against foreign particles [443] is not as well equipped as adult macrophages for immune functions such as chemotaxis, phagocytosis, and bacterial killing [15, 444]. Pre-term delivery augments these impairments [445] and further increases the susceptibility of the newborn lung to respiratory syncytial virus which can cause lower respiratory tract infections (**LRTI**). LRTI are further associated with an increased risk of respiratory morbidities such as pneumonia and bronchiolitis [446], resulting in more frequent hospitalizations and longer and more severe episodes of infections than term infants, and even death. Additionally, severe LRTI in infancy is thought to be associated with wheezing [447-449] and asthma [450-452] in childhood and in adulthood by mechanisms that are still being explored. Fetal alcohol exposure not only increases the risk for premature birth, it further exacerbates an already dysfunctional lung through alteration of lung development [453, 454] and induction of oxidative stress which results in arrested alveolarization and vascular development [455]. This additional impairment of AM function [17,

456, 457] increases the risk of pulmonary infections [19] in animal models of fetal alcohol exposure. The AM impairments observed in animal models are supported by clinical studies where alcohol exposure *in utero* increases risk of infection in both pre-term [21] and term [20] infants.

Zinc deficiency and neonatal outcomes

A balanced nutrition during pregnancy is necessary for a successful pregnancy and extensive studies have addressed the importance of nutrition in proper fetal growth and development. In general, the fetus adapts for survival taking most nutrients it needs, regardless of maternal nutrition status and stores, except in severe nutrient deficiencies when maternal stores are already depleted. Chronic alcohol consumption, as previously mentioned, affects the status of many nutrients directly through inhibition of absorption from the small intestine and indirectly through poor diet and alcohol-induced conditions that affect the liver and pancreas. Therefore, chronic alcoholics that are pregnant are at great risk for complications and adverse fetal outcomes because the demands for many nutrients are higher during pregnancy and the maternal stores may be inadequate for the extra demands [22]. During the 2^{nd} and 3^{rd} trimesters, there is fetal accumulation of fat, iron, calcium and phosphorus and the shorter the gestational age at birth, the higher the nutrient deficiencies will be. Deficiencies in protein and energy in the pre-term infant accumulate fast since parenteral and enteral nutrition do not match placental nutrient delivery [458, 459]. Cord blood of pre-term infants is characterized by decreased total protein and fat, ferritin, iron, selenium, calcium, magnesium and zinc [458]. Complete nutrition is crucial for proper fetal development and in human populations, deficiency of one nutrient is often associated with deficiencies in other nutrients, however the overall vitamin and mineral requirements for proper birth and development, aside from zinc, are out of the scope of this study and will not be discussed extensively.

Zinc is the most widely studied microelement in infant feeding because it is an essential cofactor in approximately 300 enzyme-dependent processes involved in immunity, growth, cell differentiation, and metabolism [215, 460]. In studies of global disease burden for 2010, one of the key risk factors for death in early infancy was serious bacterial infections with links to zinc insufficiency [461-464]. Another leading risk factor was alcohol use [461], which is also a known risk factor for zinc insufficiency. Alcohol-related zinc deficiency is exacerbated and becomes deleterious during pregnancy when nutrient demands are high [22, 376]. Furthermore, a poor diet results in a maternal-fetal environment in which the teratogenicity of insults such as alcohol become amplified and the risk of FASD and prematurity is increased [376, 416].

Zinc nutriture has been linked with the development of the nervous system because zinc is critical for zinc-finger proteins required for neurotransmission, zinc-dependent neurotransmitters [465, 466], and productions of thyroid hormones [467]. Offspring of rhesus monkeys deprived of zinc during the third trimester have alterations in structure and migration of neuronal cells, and as a result, are not as active and explore less in early weaning period when compared to control offspring [468]. Additionally, marginal or moderate zinc deficiency in rhesus monkeys has adverse outcomes on fetal activity pattern, leads to lower growth rate and impairments in development of motor and reflex patterns in the first year of life, and also in altered behavior patterns during infancy and adolescence [469-472]. Epidemiological studies indicate a correlation between zinc deficiency and congenital malformations, which can be ameliorated with oral zinc supplementation [400, 473, 474]. However, controlled clinical studies exploring a causal relationship between zinc deficiency and occurrence of malformations such as neural tube defects are few and often report conflicting results [475, 476].

Additionally, the link between maternal zinc status and fetal growth and development parameters such as birth weight, prematurity, head circumference, crown-heel length yield contradictory results with some studies finding an association, and others not [395, 477-479]. In rhesus

monkeys, maternal zinc deficiency during pregnancy is associated with increased occurrence of still-births, abortions, delivery complications, and lower birth weight [387]. Furthermore, marginal zinc deprivation during pregnancy in rhesus monkeys leads to growth retardation at birth or at one month of age [471] and lower body weight during juvenile period [480] that continues into adolescence [470]. In humans, perinatal zinc deficiency is not well studied, however many supplementation studies have been conducted and will be discussed in further sections.

Zinc deficiency and neonatal immunity

It is well established that zinc deficiency suppresses the immune system and lack of zinc can be especially harmful during gestation, where even marginal perturbations in maternal zinc status and in the maternal immune system can have profound effects for development and specifically for the fetal immune system [229, 481], which starts to develop *in utero* and continues to mature through infancy and childhood. Environmental factors such as malnutrition, infection, or alcohol can have enormous consequences for the fetal immune system particularly if exposure occurs during critical periods when precursor cells are forming (4-7 weeks gestation for myeloid-derived cells such as macrophages) [482]. Prenatal zinc deficiency is associated with depression of the innate immune response characterized by decreased chemotaxis and phagocytosis of neutrophils [483]. Moreover, gestational zinc deficiency in animal models leads to reduced growth of spleen, thymus and lung, at a greater extent than other organs such as heart, kidney, liver, or brain, while cross-fostering zinc deprived progeny to control dams improves growth of most organs, including lung [484].

Zinc deprivation leads to decreased gamma-globulin production, depressed mitogenesis of peripheral blood monocytes, and reduced neutrophil function [485]. Not all studies of gestational

zinc deficiency report similar patterns in reduction of immunoglobulin production. In some studies, IgM is decreased by prenatal zinc deficiency, but not IgA [485], while others report decreases in IgA, IgM and IgG2a that persist into adulthood [24, 28, 229, 481]. During development of fetal immunity, certain B-lymphocytes produce antibodies of IgA and IgM isotypes that recognize bacterial polysaccharides and other common pathogens [229] and are thought to provide an early line of defense for the naïve neonatal immune system. Consequently, suppression of IgA and IgM may lead to an altered antibody response against bacteria that can continue for generations [229]. Another study reported that even though IgM numbers did not differ between adult mice whose mothers had been moderately zinc deprived, the repertoire of the antigens the antibodies recognized was altered [229]. Also, zinc deficiency may affect placental transfer of antibodies during last trimester of pregnancy, which unlike natural antibodies, recognize a greater diversity of antigens with high specificity [229].

Zinc supplementation and neonatal outcomes

Randomized controlled trials studying the effects of prenatal zinc supplementation on birth weight do not always agree. In one systematic review, 13/17 studies found no association, 3/17 reported a positive association, and 1/17 negative association between prenatal supplementation and birth weight [486]. Others report no significant associations between prenatal zinc supplementation and head circumference, birth weight and length [381, 391, 400]. Additionally, fetal growth does not consistently show improvement with zinc supplementation in human studies [381, 391, 400].

In a randomized controlled trial in Bangladeshi women, zinc supplementation during pregnancy did not improve growth and neurobehavioral development at 13 month of age [487]. However, the intervention did not involve an adequate diet to meet lactation and infant needs and it cannot

be concluded that zinc supplementation was not beneficial when the overall nutritional status of the infant was poor. In contrast, a randomized controlled trial in Peruvian women concluded that prenatal zinc supplementation at 28 weeks gestation to zinc deficient pregnant women improved fetal neurobehavioral development *in utero* [488, 489], suggesting that timing of supplementation and when neurobehavioral development is measured may yield different results. Other studies have found no association between zinc supplementation and visual or auditory scores, sequential memory, or gross motor scale scores at 4-5 years of age [490, 491]. In the aforementioned randomized controlled trial in Bangladeshi women [487], zinc supplementation to zinc deficient mothers decreased risk of diarrhea in low birth weight infants but not in normal weight infants [492]. Other studies support that prenatal zinc supplementation may reduce diarrhea incidence in infants 6-13 months of age [492-494] suggesting that, *in utero*, neonatal immunity development may be favored over fetal growth [381].

Alcohol, zinc and FASD

The outcomes of fetal alcohol exposure can differ in each infant since differences in amount, pattern, duration of alcohol exposure, ethnicity, and diet may differ in each pregnant woman consuming alcohol. Alcohol affects the status of many nutrients such as folate, cobalamin, iron, vitamin A, and zinc, all of which are needed for proper fetal brain development and overall growth, and the teratogenic effects of alcohol *in utero* are more severe when the diet is poor [376]. Zinc deficiency during embryonic development leads to abnormal nucleic acid metabolism and protein synthesis, impaired cell migration and signaling, and increased oxidative stress, among other derangements [376]. Animal models show an increase in apoptotic cells in zinc-deficient embryos exposed to ethanol during gestation [376]. A link between FASD and fetal zinc deficiency is supported by animal and human studies [376, 417, 418]. Additionally, an inverse relationship between maternal plasma zinc and FAS expression has been reported [416].

One of the mechanisms by which alcohol exposure *in utero* results in fetal toxicity is hypothesized to be mediated through the induction of the acute-phase response which triggers maternal hepatic metallothionein synthesis leading to maternal liver zinc sequestration and decreased feto-placental zinc transfer [419, 420]. In animal models, ethanol-treated dams with normal metallothionein expression (**MT+/+**) have higher liver zinc sequestration and decreased plasma zinc when compared to metallothionein-null (**MT -/-**) ethanol-treated dams [421]. Consequently, MT +/+ ethanol-treated dams transfer less zinc to their fetus when compared to saline groups (MT +/+ and MT -/-) or ethanol-fed MT -/- [421]. Offspring exposed to ethanol *in utero* born to MT -/- dams display significantly less external abnormalities than offspring exposed to ethanol *in utero* form MT +/+ dams [495].

Zinc supplementation combined with alcohol exposure during pregnancy may protect against physical abnormalities, cognitive and spatial impairments associated with fetal alcohol exposure, reduce the number of stillbirths, and decrease the risk for postnatal mortality in offspring exposed to ethanol during gestation [422-424]. In mouse models, even a subcutaneous injection of zinc mid-gestation in conjunction with ethanol administration protects from external abnormalities and the negative pregnancy outcomes seen in ethanol-fed mice with no zinc treatment [496]. However, even though this finding is exciting, it is hardly applicable in a clinical setting.

Animal models of alcohol exposure

In most non-pregnant animal adult models of chronic ethanol ingestion where blood alcohol level (**BAC**) reaches 0.08% [18] (legal intoxication level in the USA), ethanol comprises 36% of calories, which is comparable to the alcohol calories consumed by otherwise healthy alcoholics. Ethanol can be administered in the diet [31, 457] or water [497, 498], by injection [453, 499, 500], or abomasal cannulation [501], mostly in rodent [17, 456, 457, 502] and sheep [501, 503,

504] models. Many animal models of fetal alcohol exposure have been established, which range from partial [453, 499, 501, 503, 505] to full ethanol exposure throughout gestation [457] with most studies initiating fetal alcohol exposure after conception [17, 502]. In order to maintain the pregnancy, ethanol-derived calories constitute 25% rather than 36% of total calories consumed, resulting in a blood alcohol concentration of 0.05 ± 0.01% [17]. Our laboratory has shown that this level of ethanol ingestion during gestation results in compromised pulmonary immune function in the newborn pups without pregnancy loss or distress [19, 457].

Our model of fetal alcohol exposure

Alcohol consumption pre-conception is strongly associated with drinking during pregnancy [506]. In most studies of *in utero* ethanol exposure, the models involve initiation of ethanol ingestion at the point of breeding. However, this model does not recapitulate the zinc insufficiency that occurs with chronic ethanol ingestion prior to conception. Women who abuse alcohol prior to pregnancy could have decreased zinc homeostasis at the time of conception when compared to women who do not consume alcohol. Continued alcohol consumption during pregnancy may then exacerbate maternal zinc insufficiency. In order to develop a better understanding of the effects of alcohol-induced zinc insufficiency prior to conception, we established a mouse model where chronic ethanol ingestion by the dams started prior to conception and continued for the duration of pregnancy.

Newborn biomarkers of fetal alcohol exposure

Since newborns exposed to alcohol *in utero* have a high risk of being born pre-term and experiencing post-natal complications, it is important to develop markers that are non-invasive yet have high sensitivity and specificity. In the recent years, measurement of FAEE in meconium has been validated in humans as a sensitive biomarker of heavy alcohol exposure in second and

third trimesters of pregnancy [427]. Maternal FAEEs do not cross the placenta but alcohol can easily cross the placenta and FAEE synthesized in neonatal matrices represent fetal production [507]. FAEE accumulate in tissues and fluids [508] and meconium, which begins to form in the fetal intestine with the swallowing of amniotic fluid at 15 weeks [508] and accumulates FAEE, providing a wide window of detection for prenatal alcohol exposure [426]. Meconium FAEEs have also been detected in infants from abstaining mothers however, concentrations are much lower than exposed infants and high sensitivity and specificity of detection can be obtained when excluding certain FAEEs (lauric and myristic acid ethyl esters) that are present at measurable amounts in non-exposed infants and after adjusting the positive cutoff value to 2nmol total FAEE/mg [428]. The link between meconium FAEE and *in utero* alcohol exposure continues to be elucidated and since pregnant women that consume alcohol during pregnancy are not likely to admit to drinking, the positive predictive value between the self-report and biomarker being tested is low [429]. Differences such as uneven accumulation of FAEE in meconium, genetic variations in alcohol metabolism, and illnesses or conditions that may affect FAEE, may all contribute to the differences observed between prenatal alcohol exposure and meconium FAEE concentrations [426]. On the other hand, meconium FAEEs have shown to be useful biomarkers in identifying infants not exposed to alcohol with a negative predictive value as high as 99% between self-report and FAEE [509].

In animal studies of late ethanol exposure, total meconium FAEE (palmitate, linoleate, oleate, and stearate) is significantly higher in ethanol-exposed pre-term fetuses when compared to controls, at a much lower positive cutoff value than in human studies and a higher specificity and sensitivity of fetal ethanol exposure detection, further supporting that maternal self-report is a poor indicator of alcohol consumption in pregnancy [508]. Ethyl oleate, linoleate, linolenate, palmitate and sterate are among the most used fatty acid ethyl esters [427, 428, 430, 509-512]. High concentrations of FAEE in meconium of ethanol-exposed fetuses correspond with other

derangements often observed in ethanol-exposed animals such as collagen deposition in the lung and decreased number of nephrons in the kidney [508]. Additionally, meconium FAEE levels have been associated with decreased mental and psychomotor development at 6, 12, and 24 months of age [511]. To date, most human studies examining FAEE as biomarkers of alcohol exposure *in utero* are done in full-term infants [507, 509, 513] while limited research has focused on the premature infant.

Additional neonatal biomarkers include measurement of other ethanol metabolites in meconium such as EtG and EtS. The methods for EtG and EtS measurements in meconium have been recently validated [514, 515] and studies show that EtG in meconium can identify true cases of prenatal alcohol exposure while EtS has less sensitivity [516, 517]. Further studies are needed to establish EtG as a marker for heavy alcohol exposure *in utero*.

INFLAMMATION AND PREGNANCY

Placental development and the effect of alcohol

The placenta acts primarily to protect and transport nutrients and oxygen to the fetus [518]. Human placenta starts to develop at day 6 or 7 post-conception, but the fetal-placental-maternal circulation is not established until the 10^{th} week of pregnancy [518]. When compared to animal models, the human placenta resembles certain primate species such as macaque and baboons [432, 519] and differs substantially from mouse placenta in mode of implantation, presence of a prominent yolk sac placenta in the mouse, placental hormones, gestational period (19 days in mouse vs. 270 days in humans), and pup development at birth [519]. However, mouse and human placentas share similar cell types and genes that control placental development [519, 520] and some anatomical similarities and analogous functions have been found between these two species [521].

Nutrient transport across the placenta can be through passive or facilitated diffusion, active transport, or pinocytosis, and the amount and speed of transport of different compounds is dependent on compound characteristics, such as weight, charge, and lipid-solubility and also on composition of the maternal-fetal circulation, including surface area of the exchange membrane, maternal blood flow, and blood pressure in fetal capillaries [518]. Additionally, it has been established that placenta can metabolize a variety of active molecules as it contains several cytochrome P450s that are responsible for detoxification of drugs and toxins [522]. It is well recognized that alcohol passes through the placenta and affects fetal growth, development, and the fetal immune system [420, 523]. Placenta can metabolize alcohol *via* oxidative pathways, however its cytosol enzyme activity for acetaldehyde is nearly 100-fold less than the liver [524]. Placenta also metabolizes alcohol through non-oxidative pathways where ethanol conjugates with free fatty acids. In animal models, chronic ethanol exposure compromises placental circulation and nutrient exchange by preventing expansion of maternal uterine blood vessels and by impairing placentation and placental development [525]. Additionally, alcohol exposure induces oxidative stress, reduces placental weight and birth weight, and increases risk for small-for-gestational age [524].

The decrease in zinc availability in the newborn alveolar macrophage can be a result of complications on the maternal side, placental interface, or neonatal end. Additionally, alcohol may affect maternal-placental-fetal transfer by altering zinc transporter expression in the placental surface or by inducing an inflammatory state in the placenta that impairs zinc movement to the fetus. In this study we focused in characterization of cytokine profiles in human placental tissues from a pre-term population exposed to alcohol *in utero.*

Cytokines and pregnancy

A successful pregnancy is characterized by a balance of pro- and anti- inflammatory cytokines which play an integral role in implantation, placental growth and development, and pregnancy maintenance [526]. Cytokines are mainly, but not exclusively, immunoregulatory proteins produced by immune cells in response to an external stimulus and they affect immune cell differentiation and function [526]. The placenta and its membranes produce a variety of cytokines throughout gestation. During the pre-implantation period, the conceptus secretes an abundant variety of cytokines, hormones, and other unknown molecules [527] while upon maternal recognition of pregnancy, there is a shift to a predominantly T-helper 2 (**Th2**)- cells profile [528] mediated in part by secretion of IL-10 by decidual leukocytes, which inhibits proliferation and secretion of T-helper 1 (**Th1**), and production of IL-4 by human cytotrophoblasts, which promotes Th2 differentiation [527]. In early pregnancy, human chorionic gonadotropin and human placental lactogen are important polypeptides indicative of trophoblast differentiation and their production is stimulated by IL-1β, IL-6, TNFα and granulocyte-macrophage colony-stimulating factor, respectively [529]. On the other hand, labor is characterized by an increased release of pro-inflammatory cytokines and a reduction in anti-inflammatory cytokines, to facilitate inflammatory processes associated with parturition [529].

Maintaining the Th1/Th2 ratio is of outmost importance as disruptions in this balance lead to maternal and fetal complications, such as pre-term labor, preeclampsia and intrauterine growth restriction. In extraplacental membranes of pre-term deliveries, increases in IL-1β, IL-6 and IL-8 compared to term deliveries have been reported [530], while IL-4 abundance in fetal membranes and uterine lining does not differ from term deliveries [531]. Preeclampsia is characterized by edema, hypertension, proteinuria and an increased placental production of pro-inflammatory cytokines such as IL-1β, IL-2, TNFα, and superoxide production, and decreases in placental IL-10 and IL-8 production (reviewed in [529]). Additionally, maternal conditions such as obesity

and gestational diabetes are associated with dysregulation of metabolic, vascular and cytokine pathways and result in increased local production of pro-inflammatory cytokines such as IL-6 and TNFα [532].

Alcohol and cytokines during pregnancy

Exposure to high levels of alcohol can disrupt cytokine functions and delay onset of humoral and cell-mediated immunity [533]. In pregnancy, exposure to alcohol can increase pro-inflammatory cytokine expression in first trimester human-derived trophoblast cell line leading to fetal immune dysfunction which is further exacerbated by addition of lipopolysaccharides [534]. Additionally, chronic alcohol pregnant users and their infants have significantly increased cytokine concentrations of IL-1β, IL-6 and TNFα in blood and cord blood, respectively, while moderate alcohol consumption does not significantly alter cytokine levels [533].

Placental transfer of nutrients largely impacts fetal growth and has been linked with lifelong health outcomes for the fetus [535], who relies heavily on the placenta for nutrient transfer, including zinc. Maternal alcohol consumption impairs placental transport of nutrients (reviewed in [524]). In mouse models, acute ethanol exposure causes placental malfunction by increasing permeability, decreasing thickness of barrier, and interfering with placental barrier interaction, suggesting a route for alcohol-induced decrease of nutrient transfer [536]. In human trophoblast cell lines or in a single cotyledon human term placental models, ethanol exposure for 48h and 4h, respectively, did not reduce zinc transfer or uptake [537] however, given the short exposure time in this study it cannot be ruled out that chronic alcohol exposure may impair placental zinc transfer and/or possible differences with a pre-term model. Alcohol may impair placental zinc transfer *via* direct alteration of zinc transporter expression or by inducing an inflammatory state in the placenta which then leads to stimulation of certain zinc transporters and inhibition of others.

CHAPTER 3: ZINC INSUFFICIENCY MEDIATES ETHANOL-INDUCED ALVEOLAR MACROPHAGE DYSFUNCTION IN THE PREGNANT FEMALE MOUSE

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Abstract

Aims: 1) Establish the minimum number of weeks of chronic ethanol ingestion needed to perturb zinc homeostasis, 2) Examine zinc pool status in the alveolar macrophages (AMs) when ethanol ingestion is combined with pregnancy, 3) Investigate whether *in vitro* zinc treatment reverses the effects of ethanol ingestion on the AM.

Methods: C57BL/6 female mice were fed a liquid diet (± 25% ethanol derived calories) during preconception and pregnancy. The control group was pair-fed to the ethanol group. In the isolated AMs, we measured intracellular AM zinc levels, zinc transporter expression, and phagocytic index. Some cells were treated prior to analysis with or without zinc acetate.

Results: AM zinc pools were decreased within 3 weeks of ethanol ingestion. After ethanol ingestion prior to and during pregnancy, zinc transporter expression, intracellular zinc levels, and bacterial clearance were decreased in the AMs when compared to controls. *In vitro* zinc treatment reversed these effects of ethanol.

Conclusion: Ethanol ingestion prior to and during pregnancy perturbed AM zinc balance resulting in impaired bacterial clearance, but these effects were ameliorated by *in vitro* zinc treatments.

Introduction

Alcohol consumption during pregnancy leads to a range of growth and developmental disabilities classified as Fetal Alcohol Spectrum Disorders [9, 10, 412], increases risk for premature delivery [14, 413], and the perinatal morbidity and mortality associated with prematurity [414, 415]. Proper zinc nutrition during pregnancy is essential for fetal development, growth, and immune function [538] and zinc requirements in pregnancy increase ~30% when compared to recommended daily allowance for non-pregnant, non-lactating women [539]. Zinc is an important micronutrient that displays catalytic, structural, and regulatory functions [178, 211] and is particularly crucial for the function of highly proliferative systems such as the immune system. Zinc balance is maintained through tight regulation of absorption and reabsorption, excretion, and distribution to tissues, processes that are carried out primarily by zinc transporters [277]. Zinc transporters are classified into two families that often display opposite roles in regulation of zinc homeostasis. Zip (SLC39) family transporters are responsible in zinc movement into the cytoplasm from the extracellular space or intracellular organelles. In contrast, the ZnT (SLC30) family transporters move zinc from the cytoplasm into cellular compartments or outside the cell [276]. Because there is no readily available zinc stores that can be released in response to dietary variations, the exchangeable zinc pool, which accounts for ~10% of total zinc in the adult [226], becomes crucial and its loss leads to manifestation of biochemical and clinical signs of zinc deficiency [210].

Overt zinc deficiency is rare and mostly observed in conditions such as acrodermatitis enteropathica, which displays, among other manifestations, thymic atrophy and increased bacterial, viral, and fungal infections [227]. Mild-to-moderate zinc deficiency is more common and can present itself in diverse ways since zinc is widely involved in the general metabolism. [41]. Marginal zinc deficiency results in depression of the immune response, even if clinical manifestations such as skin lesions and alopecia, among others, are not observed. Animal studies

show that zinc levels in the alveolar space decrease even when ethanol ingestion is combined with a well-balanced diet [31]. Given that 1 in 2 women of childbearing age report routine alcohol abuse [4], zinc insufficiency could be initiated prior to pregnancy. Since nearly 50% of pregnancies are unplanned [502], this zinc insufficiency could become exacerbated if alcohol consumption is coupled with pregnancy, leading to further compromised maternal zinc stores and zinc availability to the developing fetus.

In most non-pregnant animal adult models of chronic ethanol ingestion where blood alcohol concentrations reach 0.08% [18] (legal intoxication levels in the USA), ethanol comprises 36% of calories, which is comparable to the alcohol calories consumed by otherwise healthy alcoholics. Ethanol can be administered in the diet [31, 457] or water [497, 498], by injection [453], or abomasal cannulation [501, 505], mostly in rodent [17, 456, 457] and sheep [501, 503, 504] models. Many animal models of fetal alcohol exposure have been established, which range from partial [453, 501, 503, 505] to full ethanol exposure throughout gestation [457] with most studies initiating fetal alcohol exposure after conception. In order to maintain pregnancy, ethanol-derived calories constitute 25% rather than 36% of total calories consumed, resulting in blood alcohol concentrations of 0.05 ± 0.01% [17]. Our laboratory has shown that this level of ethanol ingestion during gestation results in compromised pulmonary immune function in the newborn pups, without loss or distress of pregnancy [19, 457] but the mechanisms of altered immune functions are unknown.

Current models initiating fetal alcohol exposure after conception do not represent the typical societal patterns where alcohol abuse occurs before recognition of the pregnancy. In order to develop a better understanding of the effects of alcohol prior to conception, we established a mouse model where chronic ethanol ingestion by the dams started prior to conception and continued for the duration of pregnancy. The goal was to establish the minimum number of weeks of chronic ethanol ingestion needed to perturb zinc homeostasis. Since chronic ethanol

ingestion perturbs zinc homeostasis in the lung and increases the risk of respiratory infections [31], we chose the lung and the AM as the particular site for monitoring effects on zinc homeostasis. Additionally, we wanted to examine if the zinc pools in the AMs would be lower when alcohol consumption was combined with the demands of pregnancy and whether *in vitro* zinc treatment would reverse the effects of alcohol exposure on the AM.

Materials and Methods

Mouse model of ethanol ingestion prior to and during pregnancy. Female C57BL/6 mice shipped from the vendor (Charles River, Burlington, MA) were allowed to acclimate in the Emory Pediatrics facilities for a week. After acclimatization, the mice were introduced to the liquid diet for an additional week and then randomized to receive an isocaloric liquid diet ± 25% ethanol derived calories. For the ethanol group, the ethanol content of the diet was ramped from 0% (7 days) to 12.5% (3 days) and 25% (2 days) where they were maintained throughout the study. The control group was pair-fed to the ethanol group and 25% of the calories came from maltose-dextrin. The nutritional content of the liquid diet used is especially designed for experimentation in pregnant rodents, including adequate dietary zinc (Bioserv, Frenchtown, NJ). First, we needed to establish the time period of ethanol ingestion at 25% of total calories that would result in zinc insufficiency in the alveolar space. Non-pregnant mice were assigned to different time point groups, 2 weeks, 3 weeks and 6 weeks. Some mice were fed their assigned diet (± ethanol) and then maintained on the appropriate diet throughout mating and pregnancy. Food consumption was recorded daily and the liquid food was changed every day. The assigned experimental liquid diet was the only access to food and water. Mice were weighed during the diet acclimation period to ensure no weight loss occurred and once a week upon the start of the experiment. Once pregnancy occurred, the mice were not disturbed except for food and cage changes and weights during days 8-20 of pregnancy (mouse equivalent to 2nd and 3rd trimester)

were not recorded. All animals were used with protocols reviewed and approved by the Emory University Institutional Animal Care Committee in accordance with NIH Guidelines (*Guide for the Care and Use of Laboratory Animals*).

Alveolar macrophage (AM) isolation. After anesthesia with intraperitoneal pentobarbital sodium, the trachea was identified and cannulated with a 19-G catheter. The lungs were serially lavaged with 1.5 mL sterile saline (5X) to obtain samples of the alveolar lining fluid. The initial lavage from each mouse was centrifuged (1200 rpm for 8 min), and the supernatant [designated bronchoalveolar lavage fluid (**BAL**)] was saved for further analysis. The subsequent lavages from each mouse were pooled and similarly centrifuged. The cell pellet obtained from the initial and subsequent lavages was resuspended in RPMI 1640 1X media with 10% fetal bovine serum and antibiotics. Cell viability and count were determined with Trypan Blue stain (0.4%; Life Technologies, Grand Island, NY). The cells obtained from the lavage were predominantly AMs (>94%). Cells were incubated overnight in a 10% CO_2 incubator at 37^{o}C. Some wells of the isolated AMs were treated with 25 μM (final concentration) sterile zinc acetate and were incubated overnight, similar to the cells that received no zinc treatment.

Zinc levels in AMs. Intracellular zinc levels in the isolated AMs were measured using a membrane permeable zinc specific dye, FluoZin-3AM (Invitrogen, Carlsbad, CA). FluoZin-3AM has a high affinity for zinc (K_d ~ 15 nM) and minimal interfering calcium sensitivity. FluoZin-3 is suitable for detection of zinc in the 1-100 nM range and is the most sensitive and zinc-specific of the different FluoZin fluorescent dyes. This dye detects small changes in intracellular zinc and has previously been shown to be an effective strategy to measure zinc pools in monocytes and macrophages [31, 540]. AMs were incubated with FluoZin-3AM (45 min; 37^{o}C) and then incubated in medium free of the dye to allow for complete de-esterification of the intracellular acetoxymethyl esters of the fluorophore. Cells were then washed and fixed with 3.7%

paraformaldehyde and fluorescence was quantified using fluorescent microscopy (Olympus Corp, Melville, NY) and ImagePro Plus software for Windows. Data are presented as mean relative fluorescent unit (**RFU**) / field ± S.E.M.

Zinc transporter protein expression. After isolation and fixation with 3.7% paraformaldehyde, immunostaining was used to evaluate protein expression of the zinc transporters Zip1, ZnT1 and ZnT4. AMs were incubated with the primary antibody in a 1:100 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h, washed (3X; PBS; 5 min) and then treated with the secondary antibody (anti-goat IgG) (1:200 dilution; 45 min). Zinc transporter expression was quantified using fluorescent microscopy and ImagePro Plus for Windows. Data are presented as mean RFU / field ± S.E.M.

AM phagocytic index. Isolated AMs were incubated overnight, after which, pHrodo *Staphylococcus aureus* bioparticles conjugates (Life Technologies, Grand Island, NY) were added to the wells for an additional 4 h incubation. Cells were then washed with PBS and fixed with 3.7% paraformaldehyde. Fluorescence of phagocytosed TRITC-*S.aureus* was quantified by fluorescent microscopy (Olympus Corp, Melville, NY) and ImagePro Plus for Windows. Background fluorescence of unstained macrophages was used to account for autofluorescence. The phagocytic index was calculated as previously described [541] with the percentage of cells positive for internalized fluorescence × mean RFU per field, as tallied from at least 10 experimental fields per set. Values are expressed as PI mean RFU / field ± S.E.M.

Statistical analysis. GraphPad software (LaJolla, CA) and IBM SPSS Statistics (Armonk, NY) were used for generation of graphs and statistical analysis, respectively. ANOVA was used to detect overall differences between groups and *post-hoc* analysis was conducted (Tukey's) for group comparisons. Where data was not normally distributed, non-parametric tests were done

followed by pair-wise comparisons. A $p < 0.05$ was deemed as statistically significant. Data are presented as mean ± S.E.M where each mouse represents an *n* of 1.

Results

Study demographics

Chronic ethanol ingestion by dams: Published data from our laboratory group demonstrated in diverse animal models that a lower ethanol content (25% vs. 36%) is safely tolerated by pregnant dams with minimal negative pregnancy outcomes. Similarly, in this study the liquid diet was well tolerated by the dams at all exposure period: 2-6 weeks preconception, during mating, and throughout pregnancy, without significant loss of pregnancy or distress. Mice that were not pregnant differed significantly in diet intake when compared to pregnant mice ($p = 0.016$ vs. pregnant control, $p = 0.003$ vs. pregnant ethanol) (**Table 3-1**). There were no statistical differences between pregnant control and ethanol groups ($p = 0.817$) and between non-pregnant control and ethanol groups ($p = 1.00$) in diet consumption. Additionally, there were no statistical differences in initial ($p = 0.52$) and final weight ($p = 0.054$) between groups (pregnant and non-pregnant) or within groups (control and ethanol). The period required to establish pregnancy was ~2.5 weeks resulting in female mice fed the appropriate liquid diet for 3 weeks prior to conception, 2.5 weeks for mating and 3 weeks of pregnancy yielding a total of 8.5 weeks. There were no significant differences between control and ethanol-fed groups on diet exposure duration or in the number of successful pregnancies (**Table 3-1**). Additionally, there were no significant differences in litter viability or number of abnormal fetuses.

Macrophage profile: In the BAL fluid, the isolated cells from control and ethanol-fed dams were predominantly AM (≥ 94%). Total cell counts in pregnant groups (control and ethanol) were lower only when compared to non-pregnant ethanol group, however there were no significant

differences in cell viability between not pregnant and pregnant groups or within those groups, control vs. ethanol (**Table 3-2**).

Chronic ethanol ingestion prior to pregnancy by the dam decreased zinc availability in the AMs and bacterial clearance. Our first goal was to establish a model where dams, fed a diet containing 25% of calories derived from ethanol, entered pregnancy with zinc insufficiency in the AM pool. In previous studies with adult male rats, chronic ethanol ingestion (36% of calories) for 6 weeks decreases zinc pools in the alveolar lining fluid and the AM phagocytic index [31]. In the current study, zinc pools of AMs from non-pregnant ethanol group were not significantly lower than the control group after two weeks of ethanol ingestion (**Figure 3-1A**). However, ethanol ingestion for 3 and 6 weeks resulted in significant decreases in the intracellular zinc pools of AMs when the ethanol non-pregnant group was compared to the control non-pregnant group (**Figure 3-1B, C**). Additionally, three weeks of ethanol ingestion was associated with a trend towards decreased AM bacterial clearance but did not reach statistical significance (p = 0.06) (**Figure 3-2A**). For the non-pregnant group, continued ethanol ingestion for 6 weeks significantly decreased bacterial clearance when compared to the control non-pregnant group (**Figure 3-2B**). There were no significant differences between control or ethanol groups of different exposure lengths in the variables measured and groups were pooled for further analysis.

Chronic ethanol ingestion decreased zinc availability and AM immune function in pregnant mice that ingested alcohol prior to and during pregnancy. We next determined if the zinc insufficiency associated with drinking prior to pregnancy was exacerbated if the dam was maintained on the ethanol diet throughout pregnancy. AM zinc pools in ethanol + pregnancy group had significantly lower intracellular zinc levels when compared to pair-fed control + pregnancy group (**Figure 3-3**). AM intracellular zinc pools of control + pregnancy group had, on average, slightly higher, but not significant, increases in intracellular zinc levels when compared

to the control non-pregnant group (**Figure 3-3**). Similarly, bacterial clearance in pregnant mice fed an ethanol diet prior to and during pregnancy was decreased by 54% from control pregnant group (**Figure 3-4**). There were no significant differences between control groups, CTRL-P vs. CTRL-NP, or between ethanol groups, EtOH-P vs. EtOH-NP, in AM intracellular zinc levels or phagocytic index (**Figure 3-3 and 3-4**).

In vitro zinc treatments reversed ethanol-induced effects on the zinc pool and bacterial clearance in the AMs from the pregnant dams. In a subgroup of pregnant mice, we examined whether changes in the zinc pool and bacterial clearance were related to the availability of zinc to the AM. In an adult male rat model, the impaired AM bacterial clearance associated with chronic ethanol ingestion could be improved by zinc supplements [31]. In the current subgroup, chronic ethanol ingestion prior to and during pregnancy resulted in a 60% decrease in AM phagocytic functions but this was reversed to control levels by *in vitro* zinc treatments (**Figure 3-5A**). Additionally, *in vitro* zinc treatments restored the intracellular zinc pool in the AMs from ethanol-fed dams to control values without zinc treatment (**Figure 3-5B**). No statistical significant differences were observed between control groups.

Chronic ethanol ingestion is associated with decreased expression of zinc transporters and in vitro zinc treatments reversed ethanol-induced effects on the zinc transporters in the AMs from the pregnant dams. Since zinc pools were decreased, we next examined whether this was due to altered expression of the zinc transporters. For Zip1, one of the transporters that imports zinc into the cell, chronic ethanol ingestion prior to and throughout the pregnancy significantly decreased AM protein expression ($p < 0.001$) when compared to controls (**Figure 3-6A**). Thus, the reduction in zinc levels was congruent with decreased Zip1 protein expression. We also examined the zinc transporter ZnT protein family, which also contribute to the cytoplasmic zinc balance by exporting cytoplasmic zinc out of the cell or by sequestrating cytoplasmic zinc into intracellular compartments [276]. When zinc levels are high, these zinc exporters are up-regulated in order to

increase export and thereby prevent intracellular zinc toxicity. In the current study, AMs isolated from ethanol-fed dams had an ~75% decrease in the protein expression of both ZnT1 (**Figure 3-6B**) and ZnT4 (**Figure 3-6C**) when compared to all other groups. This is in agreement with the aforementioned study in rats which saw a 80% decrease in ZnT4 mRNA expression in AMs of ethanol-fed rats (36% of calories) for 6 weeks [31].

As expected, *in vitro* zinc treatments of the AMs from ethanol-fed dams restored Zip1 protein expression when compared to the ethanol group without zinc treatments (**Figure 3-6A**). Likewise, *in vitro* zinc treatments of AMs from ethanol-fed dams normalized the expressions of ZnT1 and ZnT4 (**Figure 3-6**). Taken together, these results suggest that chronic ethanol ingestion prior to conception and during pregnancy decreased protein expression of Zip1 leading to decreased zinc influx into the AM. Furthermore, expression of zinc efflux proteins, ZnT1 and ZnT4, were also decreased which would prevent zinc transport out of the cell or into subcellular compartments. This further supports the concept that the ethanol-induced decreases in these transporters were related to decreased zinc availability.

Discussion

Most animal models of fetal ethanol exposure rely on initiation of ethanol ingestion after conception. However, this is unlikely to represent the typical exposure where alcohol abuse occurs before recognition of pregnancy. Therefore, we established a fetal ethanol model where chronic ethanol ingestion by the dams started prior to conception and continued for the duration of pregnancy. Our model of fetal alcohol exposure aimed to mimic societal patterns, where women who routinely abuse alcohol have a zinc pool composition that is most likely decreased compared to women who do not drink prior to pregnancy. In this study, we demonstrated that chronic ethanol ingestion prior to pregnancy (≥ 3 weeks) decreased zinc pools and AM immune

function of the dams when compared to the control group. Continued ethanol ingestion during pregnancy was associated with perturbations in zinc homeostasis in the AM despite adequate zinc and other nutrients in the diet.

Zinc is a cofactor in approximately 300 enzyme-dependent processes involved in immunity, growth, cell differentiation, and metabolism [178, 542] and it is essential for proper fetal growth and development. Zinc homeostasis is tightly regulated through intestinal absorption, fecal excretion, renal reabsorption and mobilization of zinc pools, carried out *via* trans-cellular processes primarily by zinc transporters [277]. Zinc transporters are categorized into two classes, the Zip (SLC39) family transporters that move zinc into the cytoplasm from the extracellular space or intracellular organelles, and the ZnT (SLC30) family transporters that transport zinc from the cytoplasm into cellular compartments or outside the cell [211, 276].

During pregnancy, nutrient demands increase and zinc requirements rise 27% from RDA of non-pregnant, non-lactating women [539], however zinc intake requirements are often higher in women who follow a non-meat based diet [181] and women in developing countries [543]. Alcohol consumption leads to nutrient deficiency through a combination of a poor diet and through inhibition of nutrient absorption [34, 41], which could have deleterious consequences during periods of increased nutrient demands. An animal-based study showed that ethanol ingestion (36% of calories) decreases zinc levels in the alveolar space, zinc transporter mRNA expression and phagocytic index in AMs from rats despite a well-balanced diet [31]. Our studies demonstrating that the AMs from mice fed 25% ethanol-derived calories had decreased phagocytic function (54% of control) verify this earlier observation. Furthermore, the current study demonstrated that dams with chronic ethanol ingestion during pregnancy, while being fed a well-balanced diet designed to meet pregnancy requirements, still displayed reductions in zinc pools and zinc transporter expression when compared to unexposed dams. However, when

pregnant groups were compared with non-pregnant groups in each exposure, there were no statistical significant differences observed in intracellular zinc levels. This could be partly explained by differences in diet intake. All groups, irrespective of pregnancy state, consumed the same type of diet designed for pregnant rodents however, pregnant groups were, on average, consuming more than non-pregnant groups. Control groups were only pair-fed to the ethanol-fed groups within a pregnancy state. Alternatively, the increased zinc demands due to pregnancy were minimal when compared to the effects of ethanol.

In summary, this study suggests that chronic ethanol abuse in adult female mice decreased protein expression of zinc transporter, Zip1, which in turn, decreased intracellular zinc availability and led to impaired bacterial clearance by AMs. In an effort to preserve zinc within the cell, AMs decreased expressions of ZnT transporters, which export zinc away from cytoplasm. Additionally, *in vitro* zinc treatments normalized the negative effects of chronic ethanol ingestion on zinc transporter protein expression, zinc levels and phagocytic function. In this study, we focused on AMs and it is unclear if other immune cells are similarly affected and further studies are warranted. In the United States, 1 in 2 women of childbearing age (18-44) consume alcohol, and 15 percent report binge drinking among those who drink in this group [4]. Nearly 50% of all pregnancies are unplanned [502] and alcohol consumption pre-conception is strongly associated with drinking during pregnancy [506]. Therefore, many women may begin their pregnancies with zinc insufficiencies, thereby increasing the likelihood that the newborn will also have zinc insufficiency. Additional studies are needed to determine if these negative effects of chronic ethanol ingestion on the maternal zinc pools extend to the newborn.

Table 3- 1. Study Demographics

	Control	Ethanol	*p*
DIET CONSUMED (ml/day)			**<0.001**
Not pregnant	11.6 ± 0.5*, †	11.6 ± 0.5*, †	
Pregnant	16.2 ± 0.9	17.2 ± 0.97	
Total weeks on diet			
Not pregnant	8.3 ± 0.74	8.6 ± 0.67	
Pregnant	8.7 ± 0.4	8.5 ± 0.4	
WEIGHT (g)			
Initial dam weight			**0.52**
Not pregnant	21.8 ± 0.47	21.8 ± 0.48	
Pregnant	20.8 ± 0.57	20.9 ± 0.62	
Final dam weight			**0.054**
Not pregnant	23.0 ± 0.66	24.6 ± 0.76	
Pregnant	25.2 ± 0.59	25.8 ± 0.54	
PREGNANCY			
Weeks on diet pre-conception	5.7 ± 0.4	5.5 ± 0.4	0.657
Successful pregnancies (%)	76 ± 8.7	73.7 ± 10.4	0.864
Litter viability (%)	82 ± 5.6	93 ± 4.4	0.085

Values represent mean ± S.E.M.

*p = 0.003 vs. pregnant ethanol

†p = 0.016 vs. pregnant control

Table 3-2. Macrophage characteristics

	Control	Ethanol	*p*
Total cell count (cells/ml)			**0.003**
Not pregnant	5.94e5 ± 1e5	7.24e5 ± 1.4e5	
Pregnant	4.52e5 ± 6.9e4*	3.63e5 ± 3.7e4**	
Cell viability (%)			**0.326**
Not pregnant	70 ± 6.0	67 ± 4.8	
Pregnant	66 ± 5.3	76 ± 3.3	
Values represent mean ± S.E.M.			
*$p < 0.05$ vs. not pregnant ethanol			
** $p < 0.01$ vs. not pregnant ethanol			

Figure legends

Figure 3-1. Chronic ethanol ingestion for 3 weeks, or more, prior to conception decreased intracellular zinc in the adult AM.

Intracellular zinc concentrations in the AM were determined using the zinc-specific, cell-permeable fluorescent dye, FluoZin-3AM. Bar heights represent mean RFU/field ± S.E.M. from at least 5 dams/group. CTRL = control and EtOH = ethanol. Intracellular zinc levels in AMs from mice exposed to ethanol in diet for **(A)** 2 weeks, $p = 0.09$ **(B)** 3 weeks, *denotes $p = 0.025$ **(C)** 6 weeks, **denotes $p = 0.009$.

Figure 3-2. Chronic ethanol ingestion for 3 weeks or more prior to conception decreased phagocytic function in the adult AM.

Isolated AMs from experimental groups were incubated with inactivated TRITC-labeled *Staphylococcus aureus* for 4 h. Internalization of TRITC-labeled *S. aureus* was determined using fluorescent analysis. Phagocytic index (**PI**) was calculated as the percentage of cells with internalized fluorescence × the mean RFU per field. Bar heights represent PI (mean RFU/field) ± S.E.M. from at least 5 dams/group. CTRL = control and ETOH = ethanol. Phagocytic index of AMs from mice exposed to ethanol in diet for **(A)** 3 weeks, $p = 0.06$ **(B)** 6 weeks, **denotes $p = 0.004$

Figure 3-3. Intracellular AM zinc in non-pregnant and pregnant mice.

Intracellular zinc concentrations in the AM were determined as previously described. Bar heights represent mean RFU/field ± S.E.M. from at least 5 dams/group. CTRL-NP = non-pregnant control, EtOH-NP = non-pregnant ethanol, CTRL-P = pregnant control and ETOH-P = pregnant ethanol. Non-pregnant mice that ingested ethanol in diet for 2-6 weeks had significantly lower AM zinc levels when compared to non-pregnant and pregnant controls. **denotes $p < 0.01$ for CTRL-NP vs. EtOH-NP, ***denotes $p < 0.001$ for CTRL-P vs. EtOH-NP. Pregnant mice that

ingested ethanol prior to and during pregnancy had significantly lower intracellular AM zinc levels when compared to pregnant controls only. ***denotes $p < 0.001$ for CTRL-P vs. EtOH-P. There were no significant differences between control groups, CTRL-NP vs. CTRL-P or between ethanol groups, EtOH-NP vs. EtOH-P.

Figure 3-4. Phagocytic index in non-pregnant and pregnant mice.

Isolated AMs from experimental groups were incubated with inactivated TRITC-labeled *Staphylococcus aureus* for 4 h. Internalization of TRITC-labeled *S. aureus* was determined using fluorescent analysis. Phagocytic index (**PI**) was calculated as the percentage of cells with internalized fluorescence × the mean RFU per field. Bar heights represent PI (mean RFU/field) ± S.E.M. from at least 5 dams/group. CTRL-NP = non-pregnant control, EtOH-NP = non-pregnant ethanol, CTRL-P = pregnant control and ETOH-P = pregnant ethanol. Bacterial clearance was lower in AMs isolated from ethanol mice (EtOH-NP and EtOH-P) when compared to control groups. *denotes $p < 0.05$ vs. CTRL-NP, ***denotes $p < 0.001$ vs. CTRL-P. There were no significant differences between control groups CTRL-NP vs. CTRL-P, or between ethanol groups, EtOH-NP vs. EtOH-P.

Figure 3-5. Zinc treatments restore AM intracellular zinc and bacterial clearance in mice fed an ethanol diet prior to and during gestation.

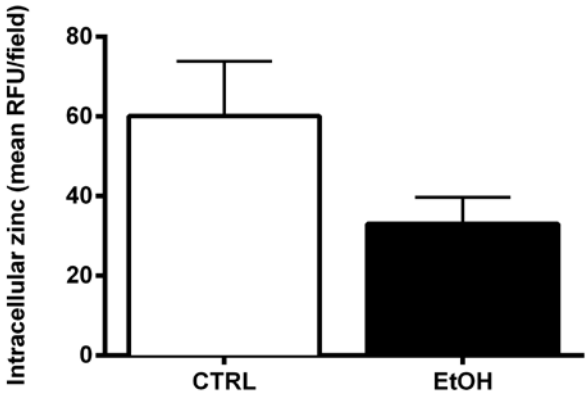
Freshly isolated AMs from dams were incubated overnight in media with some containing 25μM zinc acetate. At the end of this incubation, AMs were treated with FluoZin-3AM, a cell-permeable, zinc specific dye. Additionally, inactivated TRITC-labeled *Staphylococcus aureus* was added to the cells and incubated for another 4 h. Internalization of TRITC-labeled *S. aureus* was determined using fluorescent analysis. Phagocytic index (**PI**) was calculated as the percentage of cells with internalized fluorescence × the mean RFU per field. Bar heights represent mean RFU/ field ± S.E.M. CTRL-P = pregnant control group; CTRL-P + Zn = AMs

from pregnant control group treated *in vitro* with zinc acetate; EtOH-P = pregnant ethanol group; and EtOH-P + Zn = AMs from pregnant ethanol group treated *in vitro* with zinc acetate **(A)** Phagocytic index *** denotes $p < 0.001$ for CTRL-P, CTRL-P + Zn and EtOH-P + Zn vs. EtOH-P. No significant differences were observed between CTRL-P, CTRL-P + Zn and EtOH-P + Zn groups ($p > 0.05$). **(B)** Intracellular AM zinc, *denotes $p < 0.05$ for EtOH-P + Zn vs. EtOH **denotes $p < 0.01$ for CTRL-P vs. EtOH-P, ***denotes $p < 0.001$ for CTRL-P + Zn vs. EtOH-P. †denotes $p < 0.05$ for CTRL-P + Zn vs. EtOH-P + Zn. No significant differences were observed between CTRL-P vs. CTRL-P + Zn, or between CTRL-P vs. EtOH-P + Zn ($p > 0.05$).

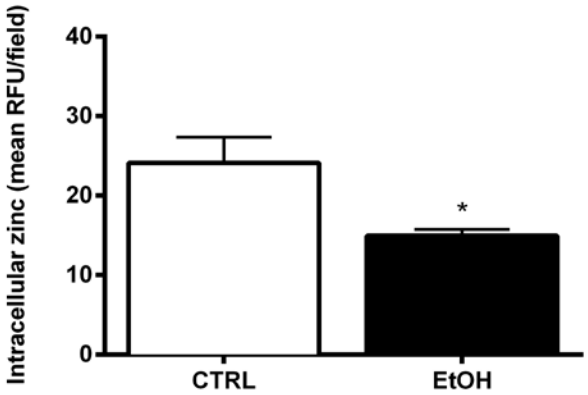
Figure 3-6. Chronic ethanol ingestion prior to and during pregnancy decreased protein expression of zinc transporters in the pregnant dam but was restored by zinc treatment. After delivery, protein expressions of the zinc transporters Zip1 (**A**), ZnT1 (**B**), and ZnT4 (**C**) were determined in the AMs from the dams *via* immunostaining and quantified using fluorescent microscope and ImagePro Plus analysis. Data were expressed relative to the control AMs and bar heights represent mean RFU ± S.E.M. from at least 5 different dams. CTRL-P = pregnant control group; CTRL-P + Zn = AMs from pregnant control group treated *in vitro* with zinc acetate; EtOH-P = pregnant ethanol group; and EtOH-P + Zn = AMs from pregnant ethanol group treated *in vitro* with zinc acetate. For Zip1 (**A**), ***denotes $p < 0.001$ for CTRL-P, CTRL-P + Zn vs. EtOH-P, **denotes $p < 0.01$ for EtOH-P + Zn vs. EtOH-P, and * denotes $p < 0.05$ for CTRL-P, CTRL-P + Zn vs. EtOH-P + Zn. No significant differences were observed between control groups ($p > 0.05$). For ZnT1 (**B**), **denotes $p = 0.001$ for CTRL-P + Zn, EtOH-P + Zn vs. EtOH-P, ***denotes $p < 0.001$ for CTRL-P vs. EtOH-P. No significant differences were observed between CTRL-P, CTRL-P + Zn and EtOH-P + Zn ($p > 0.05$). For ZnT4 (**C**), ***denotes $p < 0.001$ for CTRL-P, CTRL-P + Zn, and EtOH-P + Zn vs. ETOH-P. No significant differences were observed between CTRL-P, CTRL-P + Zn and EtOH-P + Zn ($p > 0.05$).

Figure 3-1. Intracellular zinc in AMs from non-pregnant mice

A. 2 weeks



B. 3 weeks



C. 6 weeks

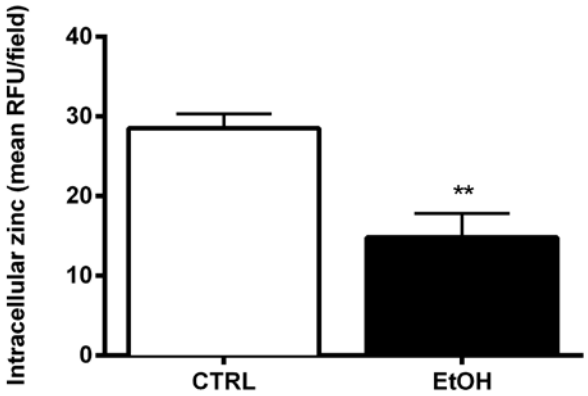
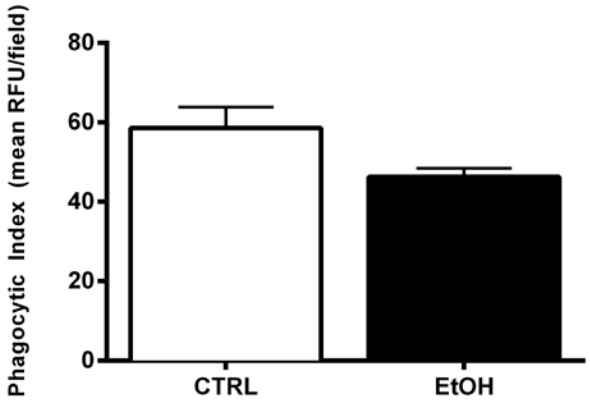


Figure 3-2. Phagocytic index of AMs from non-pregnant mice

A. 3 weeks



B. 6 weeks

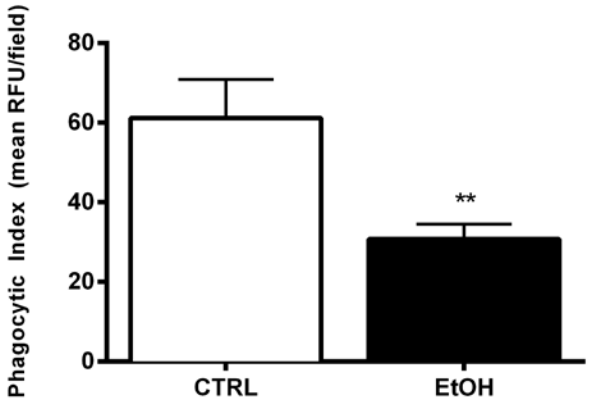


Figure 3-3. Intracellular zinc levels in pregnant and non-pregnant mice

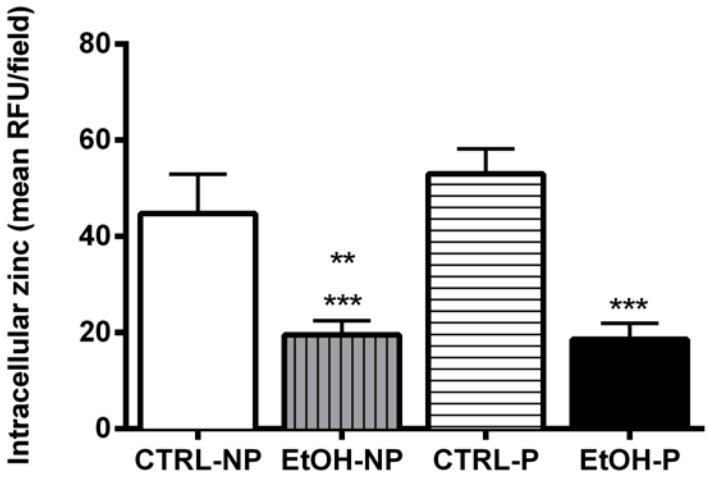


Figure 3-4. Phagocytic index in pregnant and non-pregnant mice

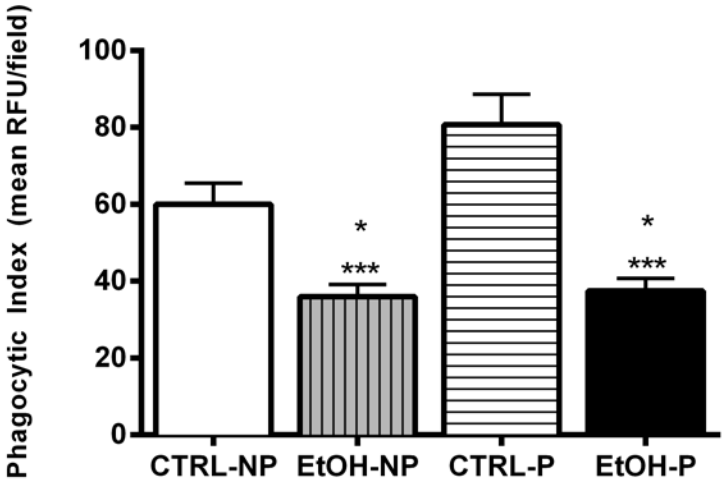
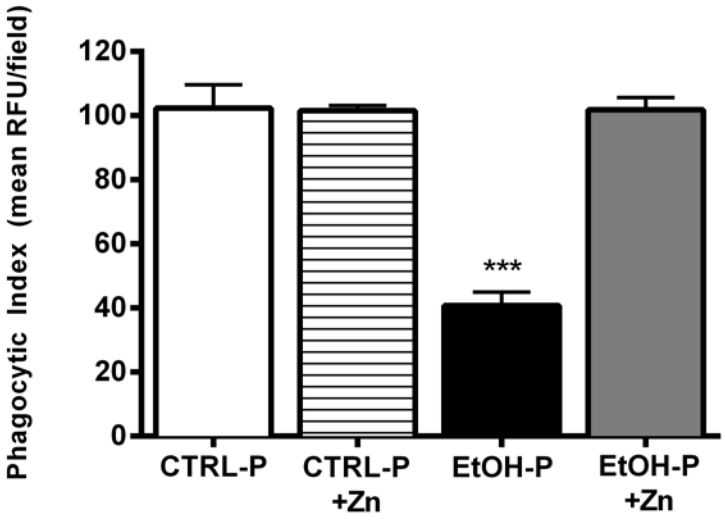


Figure 3-5. *In vitro* zinc treatments improve phagocytic function and intracellular zinc levels in AMs of pregnant mice that ingested ethanol prior to and during pregnancy

A. Phagocytic index



B. Intracellular zinc

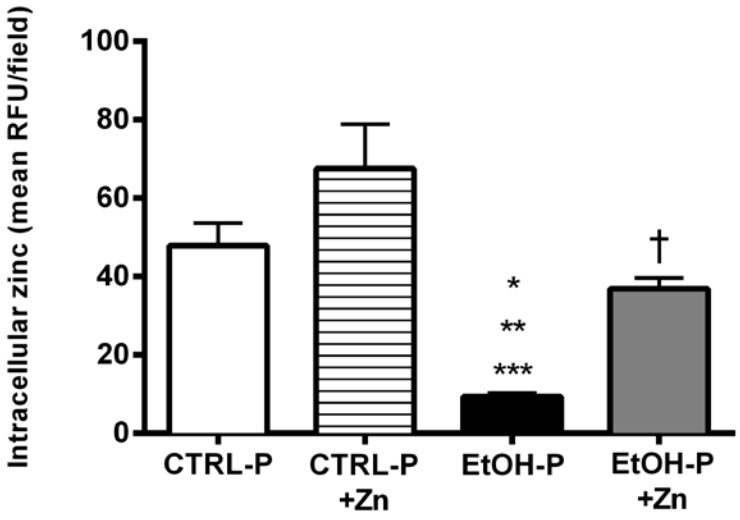
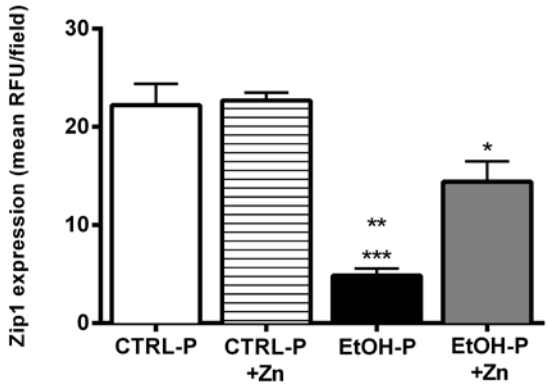
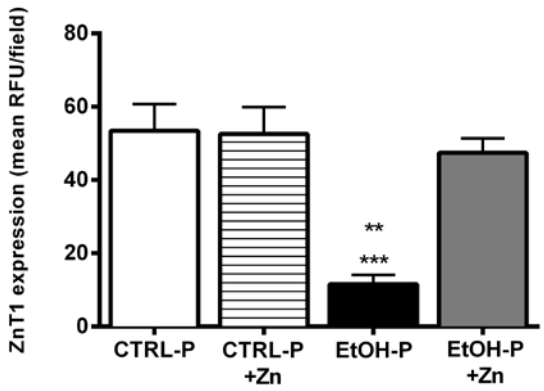


Figure 3-6. *In vitro* zinc treatments improve zinc transporter expression in AMs of pregnant mice that ingested ethanol prior to and during pregnancy

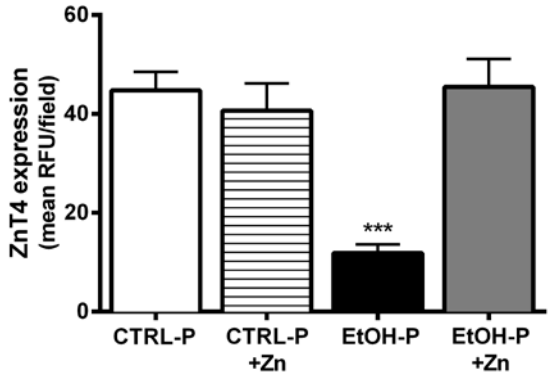
A. ZIP1



B. ZnT1



C. ZnT4



CHAPTER 4: ZINC INSUFFICIENCY MEDIATES THE FETAL ALVEOLAR MACROPHAGE DYSFUNCTION ASSOCIATED WITH FETAL ETHANOL EXPOSURE

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Abstract

Background: Our laboratory has previously established that prenatal alcohol exposure impairs viability, differentiation and function of the newborn alveolar macrophage. Alveolar macrophages (**AM**) orchestrate the primary immune defense response in the lung against inhaled antigens, microorganisms and environmental toxins. With fetal ethanol exposure, the phagocytic and microbicidal AM functions are suppressed and the risk of infections is increased *via* pathways that are not well explored. In adults, alcohol abuse results in zinc deficiency, which could be detrimental to the developing fetus if maternal zinc stores are inadequate. Zinc is essential for the immune system and its deficiency increases susceptibility to infection and exacerbates existing infections.

Methods: C57BL/6 female mice were randomized to a liquid diet (± 25% ethanol derived calories) for three weeks before conception and then throughout pregnancy. The control group received 25% of their calories as maltose-dextrin and was pair-fed to the ethanol group. At delivery, AMs from pups were isolated and treated with or without zinc acetate. After an overnight incubation, intracellular zinc levels, zinc transporter expression and phagocytic index, were measured using fluorescence microscopy.

Results: In the AMs from newborn pups, *in utero* ethanol exposure was associated with decreased expression of zinc transporters, intracellular zinc levels, and bacterial clearance when compared to unexposed pups. *In vitro* zinc treatments increased expression of zinc transporters and this was concomitant with restored intracellular zinc levels and AM bacterial clearance.

Conclusion: These studies suggest that zinc insufficiency is a critical component in the impaired AM immune functions associated with fetal alcohol exposure. Future studies are warranted to explore the effects of *in vivo* zinc supplementation - prenatally to the dam or postnatally to the pup - on the ethanol-exposed newborn AM.

Introduction

Prenatal alcohol exposure leads to a range of growth and developmental disabilities classified as Fetal Alcohol Spectrum Disorders [9, 10], which affect 2-5% of births in the United States [433]. The majority of infants who are exposed to alcohol *in utero* do not display the phenotypic characteristics associated with Fetal Alcohol Syndrome [435] and as a result, exposure often goes undetected by primary care providers. Infants chronically exposed to alcohol *in utero* have a >34-fold higher risk of extreme prematurity (<32 weeks gestational age) than non-exposed infants [14], increasing the risk of the perinatal morbidity and mortality associated with prematurity [414, 415].

At birth, the newborn lung is not fully developed and its defense systems are immature [15]. The newborn alveolar macrophage (**AM**), which initiates the first immune response of the lung against foreign particles [443], is not as well equipped as adult AMs for immune functions such as chemotaxis, phagocytosis, and bacterial killing [15]. Pre-term delivery augments these impairments [445] and further increases the susceptibility of the newborn lung to respiratory syncytial virus, which can cause lower respiratory tract infections. Fetal alcohol exposure not only increases the risk for premature birth, it further exacerbates an already dysfunctional lung [17], independently impairs AM function [17, 456, 457], and increases risk of infection [19] in animal models of fetal alcohol exposure. These findings are further supported by clinical studies that show that alcohol exposure *in utero* leads to an increased risk of infection in both pre-term [21] and term [20] infants.

Zinc is the most widely studied microelement in infant feeding because it is an essential cofactor in approximately 300 enzyme-dependent processes involved in immunity, growth, cell differentiation, and metabolism [215, 460]. In studies of global disease burden for 2010, one of the key risk factors for death in early infancy was serious bacterial infections with links to zinc insufficiency [461, 463]. Another leading risk factor was alcohol use [461], which is also a

known risk factor for zinc insufficiency. Alcohol-related zinc deficiency is exacerbated and becomes deleterious during pregnancy when nutrient demands are high [22, 376]. Furthermore, a poor diet results in a maternal-fetal environment in which the teratogenicity of insults such as alcohol become amplified and the risk of fetal alcohol spectrum disorders is increased [376]. The impact of maternal alcohol consumption and the resulting fetal zinc insufficiency in derangements of newborn AM immune functions remain to be explored.

Zinc homeostasis is regulated by zinc transporters which are categorized into two families, the Zip (SLC39) family transporters that import zinc from the extracellular space or intracellular organelles, and the ZnT (SLC30) family transporters that transport zinc away the cytoplasm into cellular compartments or outside the cell [211, 276, 295]. Because there are no readily available zinc stores that can be released in response to dietary variations, the exchangeable zinc pool, which accounts for ~10% of total zinc in the adult [209], becomes crucial and its loss leads to manifestation of biochemical and clinical signs of zinc deficiency [210].

In an adult rat model, chronic ethanol ingestion decreased AM zinc levels, zinc transporter expression, and impaired bacterial clearance but these events were reversed by zinc supplements [31]. Neither zinc transporter expression and function in the newborn lung nor the response of zinc transporters to *in utero* alcohol exposure have been explored. In most studies of *in utero* ethanol exposure, the models involve initiation of ethanol ingestion at the point of breeding. However, this model does not recapitulate the zinc insufficiency that occurs with chronic ethanol ingestion prior to conception. We speculated that the combination of alcohol-induced zinc insufficiency prior to conception and the zinc requirements that occur during pregnancy would exacerbate zinc insufficiency in the newborn with fetal ethanol exposure. Specifically, we hypothesized that fetal ethanol exposure would decrease expression of zinc transporter proteins, disrupt zinc homeostasis, and AM bacterial clearance in the lungs of pups, but these negative

effects could be restored by *in vitro* zinc treatments. In this study, we used a mouse model with three weeks of chronic ethanol ingestion prior to pregnancy with continued ethanol ingestion throughout the pregnancy. In this model, zinc availability, zinc transporter expression, and bacterial clearance in the newborn AMs was depressed when compared to the appropriate controls. However, *in vitro* zinc treatments restored zinc levels, zinc transporters, and bacterial clearance in the AMs isolated from newborn pups.

Materials and Methods

Mouse model of in utero ethanol exposure prior to and during development. Our model of fetal alcohol exposure is based on a continuous presentation of ethanol in a liquid diet (BioServ, Frenchtown, NJ), especially prepared for experimentation in pregnant mice. Female C57BL/6 mice shipped from the vendor (Charles River, Burlington, MA) were allowed to acclimate in the Emory Pediatrics facilities for a week. After acclimatization, the mice were introduced to the liquid diet for an additional week and then randomized to receive an isocaloric liquid diet ± 25% ethanol derived calories. For female mice assigned to the ethanol group, the ethanol content of the diet was ramped from 0% to 12.5%, and then 25% of ethanol-derived calories over a 1 week timeframe. The control group was pair-fed to the ethanol group with 25% of the calories from maltose-dextrin. Mice remained on their assigned diet for at least three weeks prior to conception, during mating, and throughout pregnancy. Food consumption was recorded daily and the liquid food was changed every day. The assigned experimental liquid diet was the only access to food. Mice were weighed once a week prior to pregnancy. Pregnant mice were not disturbed except for food and cage changes and weights during days 8-20 of pregnancy (mouse equivalent to 2nd and 3rd trimester) were not recorded. At delivery, pups were checked for physical abnormalities and weighed. All animals were used with protocols reviewed and approved by the Emory University

Institutional Animal Care Committee in accordance with NIH Guidelines (*Guide for the Care and Use of Laboratory Animals*).

Alveolar macrophage isolation. After delivery and anesthesia with pentobarbital sodium intraperitoneally, the trachea of the pup was identified under a dissecting microscope and cannulated with a 27-G catheter. The lungs were serially lavaged with 40 µl sterile saline (5X) to obtain samples of the alveolar lining fluid. The initial lavage from each pup in a litter was pooled and centrifuged (402 *g* for 8 min), and the supernatant [designated bronchoalveolar lavage fluid (**BAL**)] was saved for further analysis. The subsequent lavages from each pup within a litter were pooled and similarly centrifuged. The cell pellet obtained from the initial and subsequent lavages was resuspended in RPMI 1640 1X media with 2% fetal bovine serum and antibiotics and pooled. Cell viability and count were determined with Trypan Blue stain (0.4%; Life Technologies, Grand Island, NY). The cells obtained from the lavage were predominantly AMs (>95%). Cells were incubated overnight in a 10% CO_2 incubator at 37 ^{o}C. Some wells of the isolated pup AMs were treated with 25 μM (final concentration) sterile zinc acetate and were incubated overnight, similar to the cells that received no zinc treatment.

Zinc levels in AMs. Intracellular zinc levels in the isolated pup AMs were measured using a membrane permeable zinc specific dye, FluoZin-3AM (Invitrogen, Carlsbad, CA). FluoZin-3AM has a high affinity for zinc (K_d ~ 15 nM) and minimal interfering calcium sensitivity. FluoZin-3 is suitable for detection of zinc in the 1-100 nM range and is the most sensitive and zinc-specific of the different FluoZin fluorescent dyes. The cell permeable acetoxymethyl ester form of the fluorophore is useful for detecting low concentrations and small changes of intracellular zinc. This dye has previously been shown to be an effective strategy to measure intracellular zinc pools in monocytes and macrophages [31, 540]. After isolation, the AMs were incubated overnight and treated with FluoZin-3AM for 45 min at 37^{o}C. This was followed by a 30 min incubation with

medium free of the fluorophore to allow for complete de-esterification of the intracellular acetoxymethyl esters. Cells were then washed and fixed with 3.7% paraformaldehyde and fluorescence was quantified using fluorescent microscopy (Olympus Corp, Melville, NY) and ImagePro Plus software for Windows. Data are presented as mean relative fluorescence units (**RFU**) per field ± S.E.M.

Zinc transporter protein expression. After isolation and fixation with 3.7% paraformaldehyde, the AMs were evaluated for protein expression of the zinc transporters Zip1, ZnT1 and ZnT4 *via* immunostaining. Cells were incubated with the primary antibody in a 1:100 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h. Slides were washed three times with phosphate buffered saline over 5 min and the secondary antibody (anti-goat IgG) was added in 1:200 dilution and further incubated for 45 min. Zinc transporter expression was quantified using fluorescent microscopy and ImagePro Plus for Windows. Data are presented as mean RFU/field ± S.E.M.

AM phagocytic index. AMs isolated from pups were incubated overnight for 16 h, after which, pHrodo *Staphylococcus aureus* bioparticles conjugates (Life Technologies, Grand Island, NY) were added to the wells followed by an additional 4 h incubation. Cells were then washed with phosphate buffered saline and fixed with 3.7% paraformaldehyde. Fluorescence of phagocytosed TRITC-*S.aureus* was quantified by fluorescent microscopy (Olympus Corp, Melville, NY) and ImagePro Plus for Windows. Background fluorescence of unstained AMs was used to account for autofluorescence. The phagocytic index was calculated as previously described [541] with the percentage of cells positive for internalized fluorescence × mean relative fluorescent unit per field, as tallied from at least 10 experimental fields per set. Values are expressed as mean PI RFU/field ± S.E.M.

Statistical analysis. GraphPad software (LaJolla, CA) and IBM SPSS Statistics (Armonk, NY) were used for graph generation and statistical analysis, respectively. ANOVA was used to detect overall differences between groups and *post-hoc* analysis was conducted (Tukey's) for group comparisons. A $p < 0.05$ was deemed as statistically significant. Data are presented as mean ± S.E.M where each litter represents an *n* of 1.

Results

Chronic ethanol ingestion by dams: The liquid diet was well tolerated by the dams prior to conception, during mating, and throughout pregnancy, without significant loss of pregnancy or distress. As outlined in **Table 4-1**, there were no statistical differences between the control and ethanol groups in food consumption before and during pregnancy, the number of successful pregnancies, or body weights of the dams before and after pregnancy. For the pups, there were no statistical differences between the two groups on the number of pups per litter, litter viability, or pup body weight (**Table 4-1**). Fetal alcohol abnormalities were observed in some pups from one ethanol litter and no abnormalities were observed in the control group.

Fetal ethanol exposure decreased zinc availability in the AMs of the newborn pups but these effects were reversed by in vitro zinc treatments. In our model of fetal alcohol exposure, dams fed an ethanol diet for at least 3 weeks entered pregnancy with insufficient zinc pools in the AM when compared to non-pregnant control group (unpublished data), despite *ad lib* access to a liquid diet designed especially to meet the nutritional needs of pregnant rodents. In the pup AMs, the intracellular zinc pools were decreased ~70% with fetal ethanol exposure (**Figure 4-1**) when compared to unexposed pups. For the AMs with *in utero* ethanol exposure, *in vitro* zinc treatments restored zinc pools to control levels (**Figure 4-1**). For the control group, *in vitro* zinc treatment did no alter the AM zinc pools.

Fetal ethanol exposure decreased protein expression of AM zinc transporters but in vitro zinc treatments ameliorated these effects. Since zinc pools were decreased, we next examined whether this was due to altered expression of the zinc transporters. Chronic ethanol exposure *in utero* was associated with significantly decreased protein expression of Zip1, one of the transporters that imports zinc into the cell, when compared to controls (**Figure 4-2**). The reduction in zinc import supported the observed decrease in intracellular zinc levels. Additionally, we examined transporters of the ZnT family, which contribute to cytoplasmic zinc balance by exporting zinc out of the cell or into intracellular compartments. [276]. AMs isolated from pups exposed to ethanol *in utero* had ≥ 60% decrease in protein expression of ZnT1 and ZnT4 when compared to all other groups (**Figures 4-3 and 4-4**). These results suggested that decreased zinc availability in the AM associated with fetal ethanol exposure correlated with decreased protein expression of the zinc transporters Zip1, ZnT1, and ZnT4. Additionally, *in vitro* zinc treatments to the newborn AMs restored zinc pools and protein expression of Zip1, ZnT1, and ZnT4 (**Figures 4-2 – 4-4**), further supporting the concept that the reductions observed in protein expression of zinc transporters were related to the ethanol-induced decrease in fetal lung zinc pools.

Ethanol-induced decreases in zinc availability was associated with decreased AM bacterial clearance but was reversed with in vitro zinc treatments. In earlier studies, we demonstrated an association between fetal ethanol exposure and impaired AM phagocytic function [17, 456, 457]. In an adult male rat model, the impaired AM bacterial clearance associated with chronic ethanol ingestion could be reversed by zinc supplements [31]. In the current study, ethanol exposure *in utero* was associated with decreases in phagocytosis, which were improved by *in vitro* zinc treatments (**Figure 4-5**), suggesting that the impaired bacterial clearance associated with fetal ethanol exposure was secondary to the effects of ethanol on zinc availability. The decreased AM phagocytic index associated with fetal ethanol exposure was due to decreases in the number of

cells that internalized bacteria and the overall mean fluorescence per cell when compared to all other groups. With *in vitro* zinc treatments of the *in utero* ethanol-exposed AMs, the improvement was seen in the overall fluorescence per field and in the percent of cells that internalized bacteria. For the control group, *in vitro* zinc treatment did not affect phagocytosis (**Figure 4-5C**).

Discussion

It is well established that *in utero* alcohol exposure has negative outcomes for the developing fetus. Previously, it was believed that alcohol did not affect the lung but growing evidence shows that the developing lung is also vulnerable to alcohol-induced toxicity [17, 453, 456, 457, 503, 505]. Extensive research from our laboratory group has shown that the newborn lung exposed to alcohol *in utero* has fewer and less mature AMs, decreased pools of the major AM antioxidant glutathione, and impaired AM phagocytic function. The mechanisms by which alcohol impairs the newborn AM are under continued investigation. In an experimental adult rat model, six weeks of chronic ethanol ingestion decreased AM zinc levels, expressions of zinc transporters, and impaired bacterial clearance [31]. The purpose of this study was to investigate whether the impaired AM bacterial clearance associated with fetal ethanol exposure is also related to decreased zinc availability.

Most animal models of fetal ethanol exposure rely on initiation of ethanol ingestion after conception. However, this is unlikely to represent the typical exposure where alcohol abuse occurs before recognition of pregnancy. Therefore, we established a fetal ethanol model where chronic ethanol ingestion by the dams started prior to conception and continued for the duration of pregnancy. Our model of fetal alcohol exposure aimed to mimic societal patterns, where women who routinely abuse alcohol have a zinc pool composition that is most likely different

than that of women who do not drink prior to pregnancy. In the current study, the goal was to determine if fetal zinc pools and zinc transporter expression were similarly altered with fetal ethanol exposure or be maintained. In theses studies, fetal ethanol exposure decreased Zip1 protein expression in the AMs of the newborn pup and this was accompanied by corresponding decreases in the zinc pool. The observed decreases in the protein expressions of ZnT1 and ZnT4 in the AMs of the ethanol-exposed pup would attenuate zinc efflux and preserve the remaining cytosolic and subcellular zinc pools. The central role of zinc availability was further demonstrated by the capacity of *in vitro* zinc treatments to restore expressions of the zinc transporters, zinc pools, as well as bacterial clearance in the AMs of ethanol-exposed pups to control values. These results suggest that *in utero* ethanol exposure impaired AM phagocytosis in part by decreasing zinc availability, presumably because of zinc insufficiency in the pregnant dam with ethanol ingestion despite adequate zinc in the diet.

In the United States, it is estimated that only ~ 60% of pregnant women meet zinc requirements during pregnancy [544] and mild to moderate zinc deficiency is not restricted to women of low socioeconomic status. Although the zinc deficiency during pregnancy may be mild to moderate, it is sufficient to cause deleterious short- and long- term effects on the neonatal immune system which starts to develop *in utero* and continues to mature through infancy and childhood. Studies from animal models show that gestational zinc deficiency leads to reduced lymphoid organ size, decreased neutrophil function, and suppression of immunoglobulin production, such as IgA and IgM, that persists into adulthood [24, 28, 460, 481]. Persistent suppression of IgA and IgM has serious ramifications since these molecules are critical to immune function because of their interactions with bacterial polysaccharides and other common pathogens. IgA and IgM provide an early line of defense for the naïve neonatal immune system and continuous suppression may lead to an altered antibody response against bacteria, even later in life [229].

Alcohol-related zinc insufficiency or deficiency could be from a combination of absorption inhibition and poor diet [34, 41]. However, alcohol decreases zinc levels and zinc transporter mRNA expression in AMs from rats, despite a well-balanced diet [31]. The current study further demonstrated that pups exposed to ethanol *in utero* displayed reductions in zinc pools, zinc transporter expression and AM function when compared to unexposed pups suggesting that maternal zinc supplies were insufficient to meet fetal requirements. With alcohol abuse, zinc transfer to the fetus may be compromised due to altered zinc transporter expression in the maternal-placental-fetal interface. Given that placental development requires zinc, improper development of the placenta could lead to impaired fetal acquisition of maternal antibodies that are crucial for neonatal immunity in the first six months of life [229].

In summary, this study suggests that fetal alcohol exposure decreased protein expression of zinc transporter, Zip1, in the newborn alveolar macrophage. This, in turn, decreased intracellular zinc availability and led to impaired bacterial clearance by AMs. In an effort to preserve zinc within the cell, AMs decreased expressions of ZnT transporters, which export zinc away from cytoplasm. A casual role for decreased zinc availability in the ethanol-induced suppression of neonatal AM functions was further demonstrated by the ability of *in vitro* zinc treatments to restore zinc transporter protein expression, zinc levels and phagocytic function of AMs to control levels. In this study, we focused on AMs and it is unclear if other immune cells are similarly affected. It is also unclear if these effects of fetal ethanol exposure on zinc homeostasis or AM bacterial clearance improve with maturation. Future studies can investigate whether zinc insufficiency is corrected postnatally when the ethanol- exposed pup is cross-fostered to a control dam. Additionally, *in vivo* zinc supplementation needs to be explored. Realistically, pregnant women consuming alcohol during pregnancy are less likely to take prenatal supplements or eat healthy, thus it is important to explore if postnatal zinc supplementation to the pup can reverse the

negative effects of alcohol on the newborn lung. Dietary zinc supplementation could be an effective intervention in reducing risk and severity of lower respiratory tract infections in the highly vulnerable pre-term infant with fetal alcohol exposure. Novel strategies such as zinc supplements may become particularly important in this era of antibiotic-resistant bacterial infections.

Figure Legend

Figure 4-1. Fetal ethanol exposure decreased intracellular zinc levels in the AMs from newborn pups but was restored by zinc treatment. After birth, AMs from the pups (± fetal ethanol exposure) were isolated by BAL and cultured overnight. During that incubation, some AMs were treated with 25 μM zinc acetate. After 16 h of the incubation, FluoZin-3AM was added to the incubation. RFUs were quantified by computerized analysis and expressed relative to the values for the control group. Bar heights represent the mean RFU/field ± S.E.M. from at least 6 different litters. CTRL = control group; CTRL + Zn = control AMs treated *in vitro* with zinc acetate; EtOH = ethanol group; and EtOH + Zn = ethanol AMs treated *in vitro* with zinc acetate. (**A**) *** denotes $p = 0.001$ for CTRL, CTRL + Zn, and EtOH + Zn vs. the EtOH group. No significant differences were observed between CTRL, CTRL + Zn and EtOH + Zn groups ($p > 0.05$). (**B**) Fluorescence microscopy images of cells taken at 40x magnification.

Figure 4-2. Fetal ethanol exposure decreased Zip1 protein expression in the AMs from newborn pups but expression was restored by zinc treatment. After birth, AMs from the pups (± fetal ethanol exposure) were isolated by BAL and cultured overnight. Protein expression of zinc transporters in AMs was determined *via* immunostaining and quantified using fluorescent microscope and ImagePro Plus analysis. Bar heights represent mean RFU/field ± S.E.M. from at least 5 different litters per group. CTRL = control group; CTRL + Zn = control AMs treated *in vitro* with zinc acetate; EtOH = ethanol group; and EtOH + Zn = ethanol AMs treated *in vitro* with zinc acetate. (**A**) *** denotes $p < 0.001$ CTRL, CTRL + Zn vs. EtOH, ** denotes $p = 0.001$ EtOH + Zn vs. EtOH. No significant differences were observed between CTRL, CTRL + Zn and EtOH + Zn ($p > 0.05$). (**B**) Fluorescence microscopy images of cells taken at 40x magnification.

Figure 4-3. Fetal ethanol exposure decreased ZnT1 protein expression in the AMs from newborn pups but was restored by zinc treatment. Protein expression of zinc transporter,

ZnT1 in AMs was determined *via* immunostaining and quantified using fluorescent microscope and ImagePro Plus analysis. Bar heights represent mean RFU/field ± S.E.M. from at least 5 different litters per group. CTRL = control group; CTRL + Zn = control AMs treated *in vitro* with zinc acetate; EtOH = ethanol group; and EtOH + Zn = ethanol AMs treated *in vitro* with zinc acetate. (**A**) *** denotes $p < 0.001$ CTRL, CTRL + Zn, EtOH + Zn vs. EtOH. * denotes $p < 0.05$ CTRL + Zn vs. EtOH + Zn. No significant differences were observed between control groups and CTRL vs. EtOH + Zn ($p > 0.05$). (**B**) Fluorescence microscopy images of cells taken at 40x magnification.

Figure 4-4. Fetal ethanol exposure decreased ZnT4 protein expression in the AMs from newborn pups but expression was restored by zinc treatment. Protein expression of zinc transporter ZnT4 was determined and quantified similar to other transporters. Bar heights represent mean RFU/field ± S.E.M. from at least 5 different litters per group. CTRL = control group; CTRL + Zn = control AMs treated *in vitro* with zinc acetate; EtOH = ethanol group; and EtOH + Zn = ethanol AMs treated *in vitro* with zinc acetate. (**A**) ** denotes $p < 0.01$ for CTRL, CTRL + Zn, and EtOH + Zn vs. EtOH. No significant differences were observed between CTRL, CTRL + Zn and EtOH + Zn ($p > 0.05$). **(B)** Fluorescence microscopy images of cells taken at 40x magnification.

Figure 4-5. The phagocytic index in the AMs from the newborn pups was decreased by ethanol but was improved by zinc treatment. Inactivated TRITC-labeled *Staphylococcus aureus* was added to the cells and incubated for another 4 h. Internalization of TRITC-labeled *S. aureus* was determined using fluorescent analysis. Phagocytic index (**PI**) was calculated as the percentage of cells with internalized fluorescence × the mean RFU per field. Bar heights represent mean PI relative to the control ± S.E.M. from at least 5 separate litters. CTRL = control group; CTRL + Zn = control AMs treated *in vitro* with zinc acetate; EtOH = ethanol group; and EtOH + Zn = ethanol AMs treated *in vitro* with zinc acetate. (**A**) *** denotes $p < 0.001$ for

CTRL, CTRL + Zn, and EtOH + Zn vs. EtOH and ** denotes $p < 0.01$ for CTRL and CTRL + Zn vs. EtOH + Zn. No significant differences were observed between CTRL, CTRL + Zn ($p > 0.05$). (**B**) Fluorescence microscopy images of cells taken at 40x magnification. (**C**) Summary of bacterial clearance by AMs. * denotes $p < 0.05$ CTRL + Zn vs. EtOH + Zn in mean RFU/field category, ** denotes $p < 0.01$ CTRL, CTRL + Zn vs. EtOH + Zn in phagocytic index, *** denotes $p < 0.001$ CTRL, CTRL + Zn, EtOH + Zn vs. EtOH in mean RFU/field, percent positive cells, and phagocytic index categories.

Table 4-1. Fetal data

	Control	Ethanol	*p*
DAMS			
Diet consumed (ml/day)			
Prior to mating	10.1 ± 0.1	10.3 ± 0.1	0.218
Prior to pregnancy	12.0 ± 0.2	11.4 ± 0.4	0.212
Mating + 3 wks	11.0 ± 0.4	10.8 ± 0.3	0.685
During pregnancy	18.0 ± 1.1	19.0 ± 0.5	0.433
Dam weight (g)			
Initial weight	19.5 ± 0.2	19.0 ± 0.3	0.138
Prior to pregnancy	20.5 ± 0.4	21.2 ± 0.5	0.272
After delivery	25.9 ± 0.4	25.1 ± 0.3	0.101
PUPS			
Successful pregnancies	85.7%	87.5%	
Total pups per litter	8.7 ± 0.5	7.4 ± 0.5	0.08
Live only	7.3 ± 0.8	6.6 ± 0.8	0.493
Litter viability (%)	84 ± 1%	90 ± 1%	0.591
Average pup weight (g)	1.3 ± 0.06	1.3 ± 0.04	0.521
Live only	1.3 ± 0.04	1.3 ± 0.04	1
Number of abnormal fetuses*	0	3	
Litters with abnormal fetuses	0	1	
Values represent mean ± S.E.M.			
***Dead pups in controls were fully developed, mom delivered past due date**			
Dead pups in ethanol group came from one litter and had the following abnormalities (frequency):			
Cleft palate (1)			
One eye (1)			
Eyes didn't migrate (1)			
Very small size (2)			

Figure 4-1. *In vitro* zinc treatments restored intracellular zinc levels in AMs of pups exposed to ethanol *in utero*

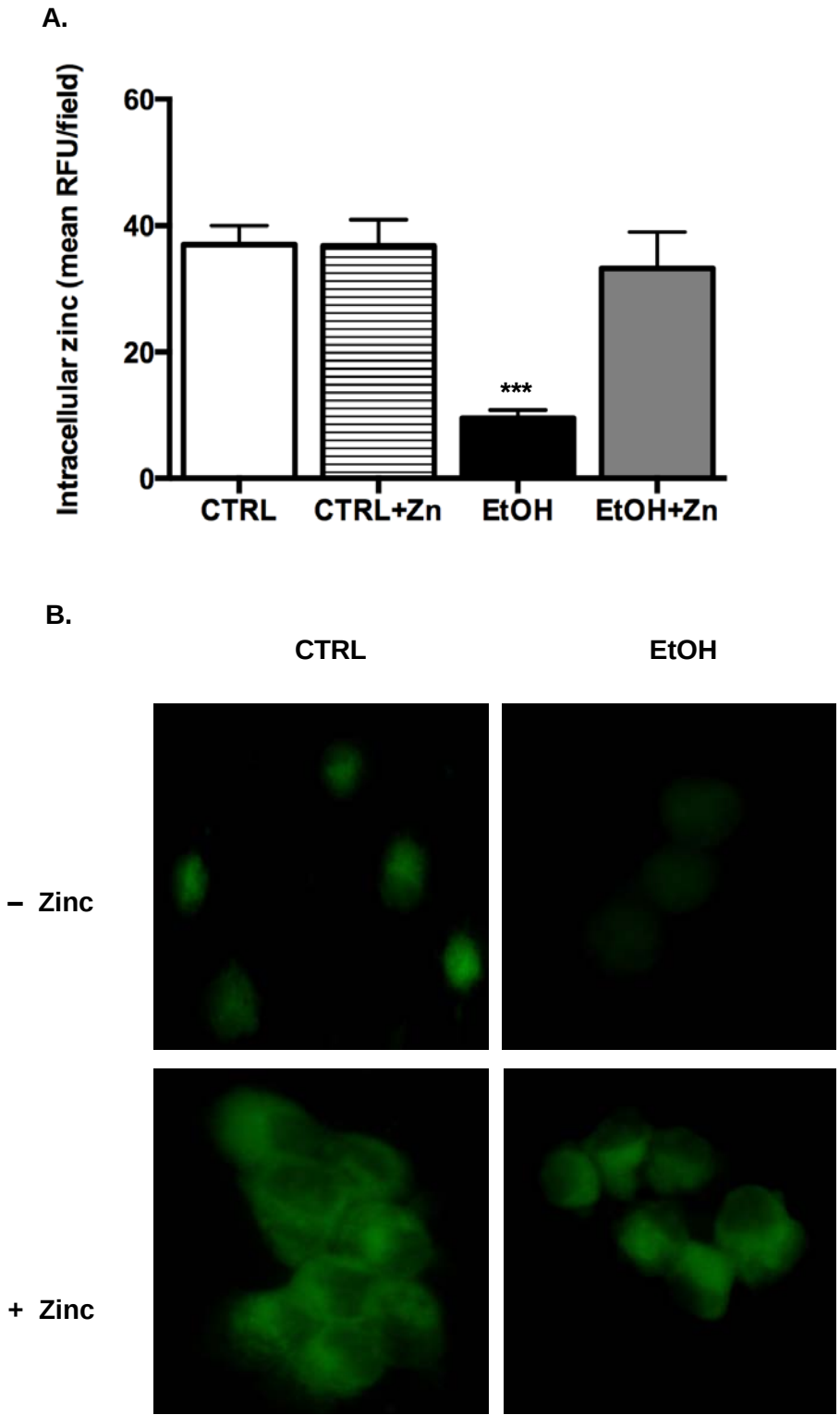


Figure 4-2. *In vitro* zinc treatments improved ZIP1 protein expression in AMs of ethanol-exposed pups

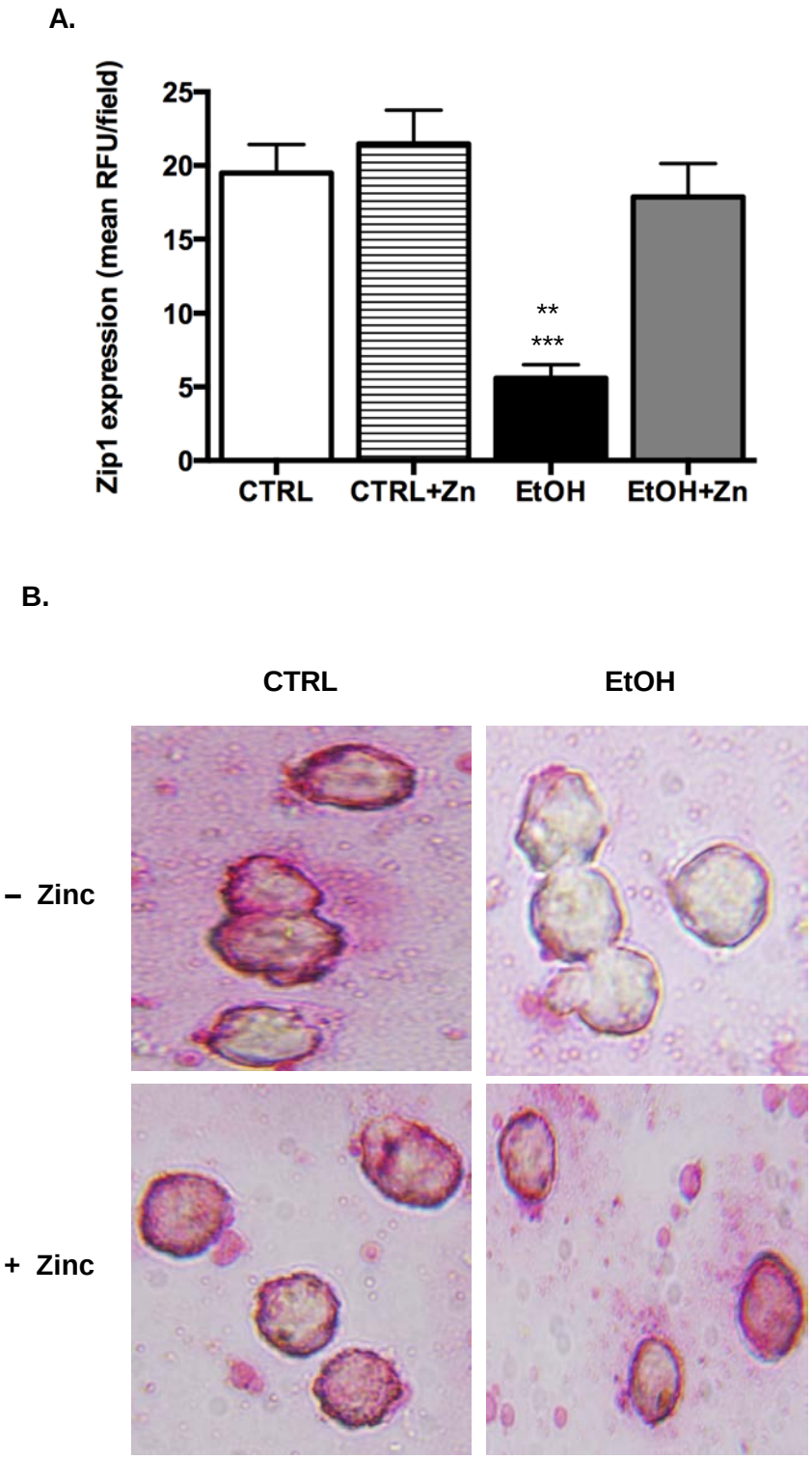


Figure 4-3. *In vitro* zinc treatments improved ZnT1 protein expression in AMs of ethanol-exposed pups

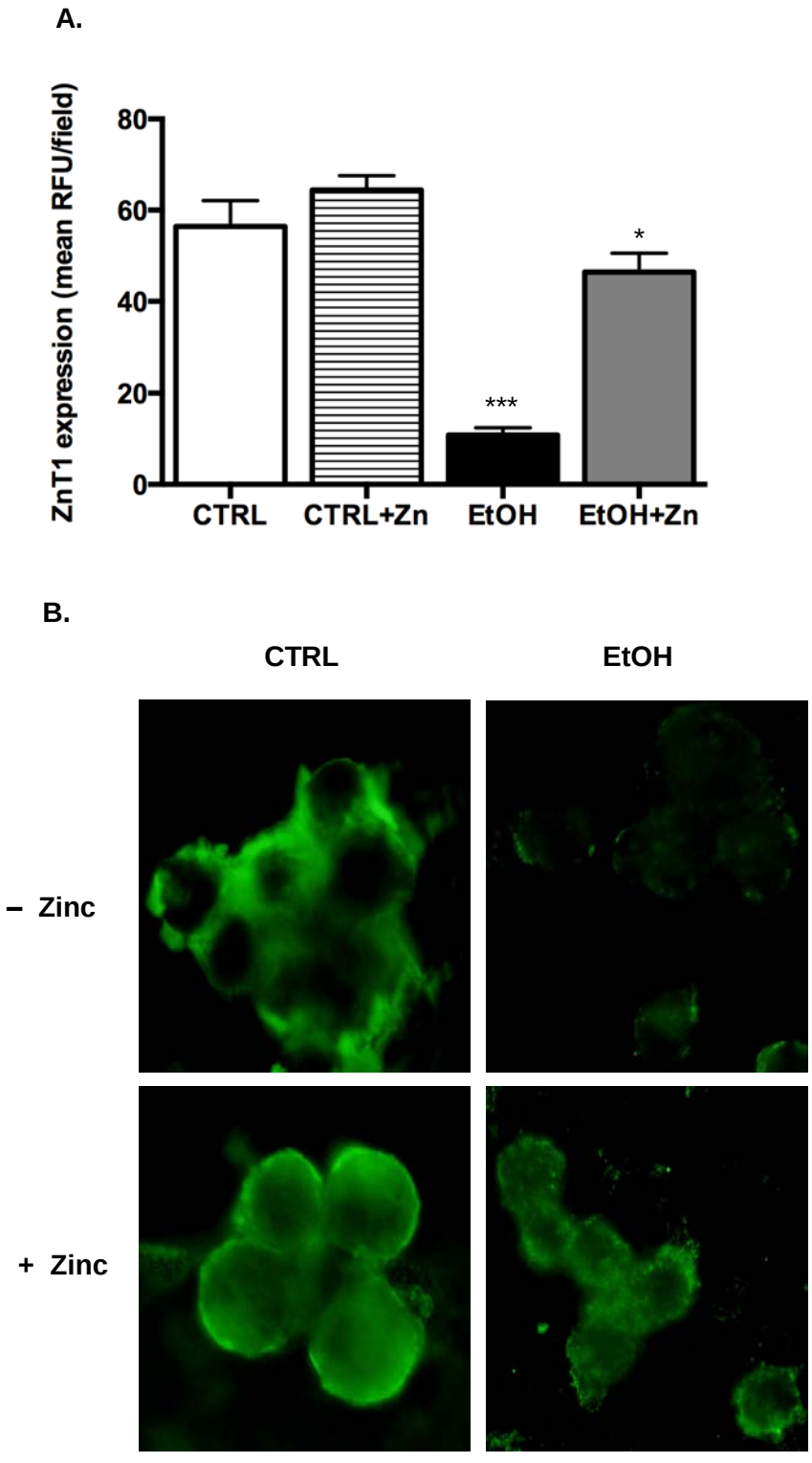
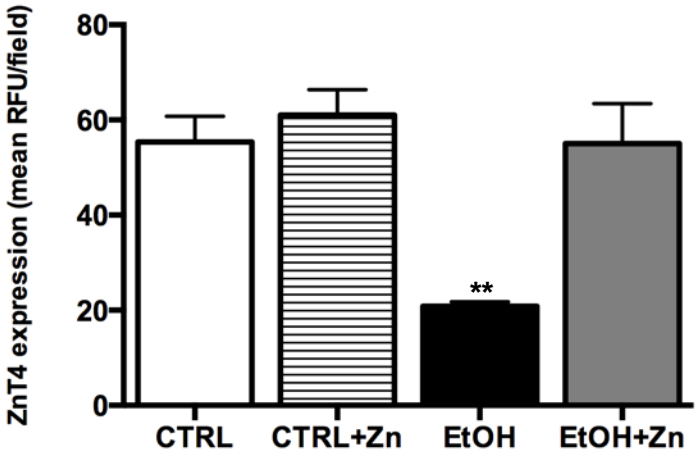


Figure 4-4. *In vitro* zinc treatments restored ZnT4 protein expression in AMs of ethanol-exposed pups

A.



B.

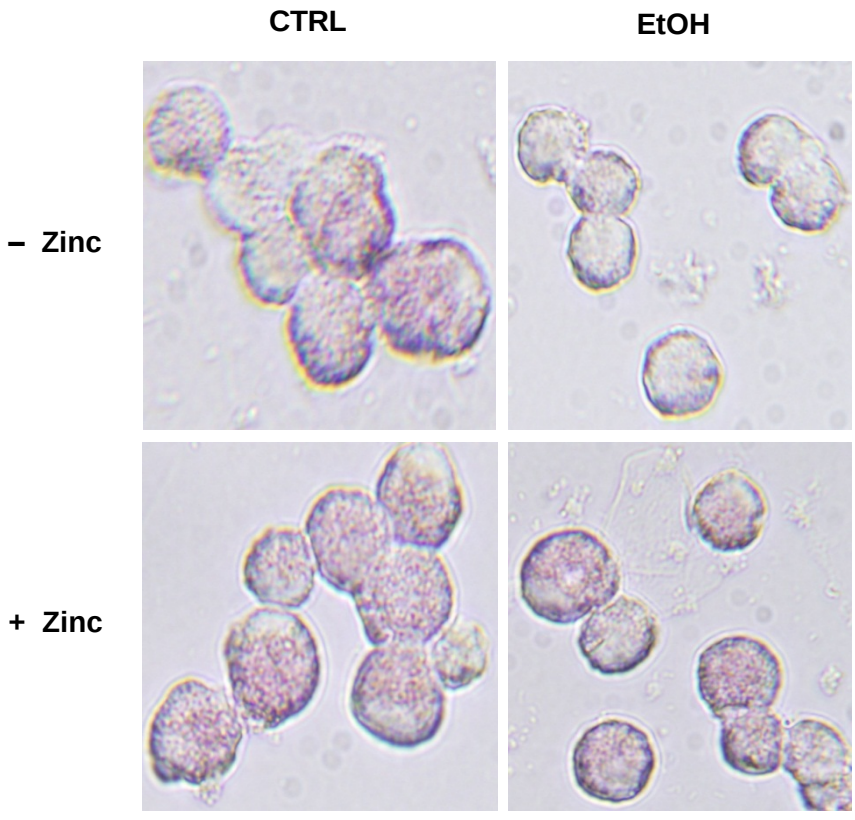
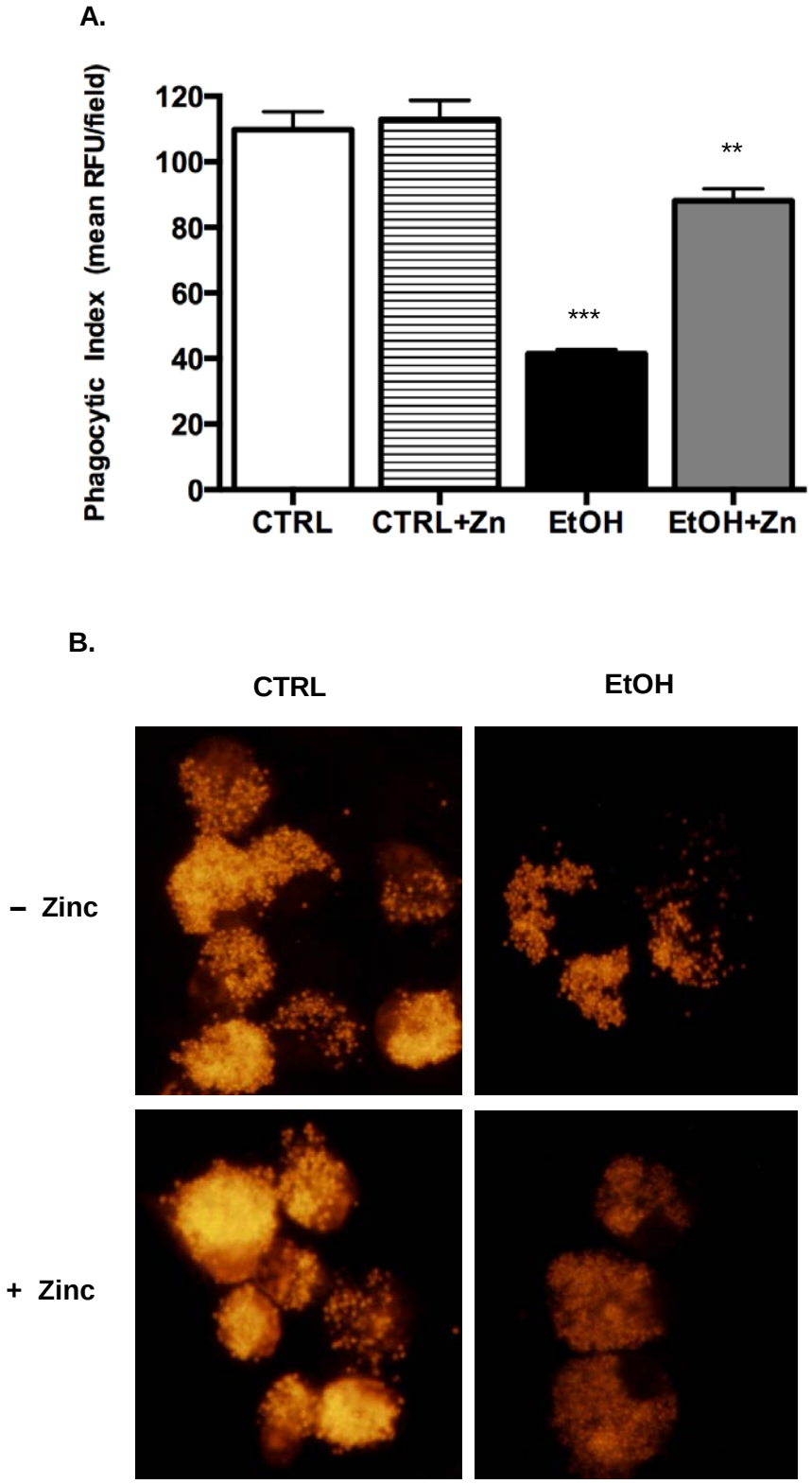


Figure 4-5. *In vitro* zinc treatments improve bacterial clearance in AMs of pups exposed to ethanol *in utero*



C.

	CTRL	CTRL+Zn	EtOH	EtOH+Zn	*p*
Mean RFU /field	110.7	114	52.5 ***	92.4 *	<0.001
% of positive cells	99.3	98.9	83.0 ***	97.4	<0.001
Phagocytic index	109.9	112.7	43.2 ***	89.7 **	<0.001
RFU = relative fluorescence units					
Phagocytic index = mean RFU/field × % of positive cells					
Values represent mean from at least 5 litters/group					
ANOVA was performed for multiple comparisons followed by post-hoc tests (Tukey's) for pairwise comparisons					
*** *p* < 0.05 CTRL + Zn vs. EtOH + Zn**					
**** *p* < 0.01 CTRL, CTRL + Zn vs. EtOH + Zn**					
******p* < 0.001 CTRL, CTRL + Zn, EtOH + Zn vs. EtOH**					

CHAPTER 5: FETAL ALCOHOL EXPOSURE IS ASSOCIATED WITH INCREASED CYTOKINE PRODUCTION IN THE PLACENTA OF THE PREMATURE NEWBORN

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Abstract

Background: High levels of alcohol exposure can disrupt cytokine functions and delay immune responses. Maternal self-report of alcohol consumption during pregnancy is unreliable. Recently, placental FAEEs have been investigated as a potential negative predictive biomarker of prenatal alcohol exposure in a population of premature newborns born <1500g. In the current study, we investigated whether the balance between pro- and anti- inflammatory placental cytokines in this population differed in the presence of prenatal alcohol exposure.

Methods: This cross-sectional study evaluated 86 placentas from 80 women after premature delivery. Subjects were interviewed for alcohol intake using an extensive standardized questionnaire. After informed consent, placental FAEEs were quantified via GC/MS. Cytokine concentrations were quantified using a cytokine multiplex kit and samples were classified as exposed and not exposed to alcohol by placental FAEE combination of oleic, linoleate, linolenate (**OLL**) and stearate.

Results: There was low agreement between maternal self-report and placental FAEE in classification of subjects as exposed or not exposed to alcohol *in utero*. Using OLL + Stearate as a biomarker of fetal alcohol exposure, placental tissue pro-inflammatory cytokine levels were higher in pre-term placentas with exposure (OLL+ Stearate above cutoff) when compared to unexposed placentas. Additionally, anti-inflammatory cytokines IL-2 and IL-10 were significantly increased in exposed placentas while no significant differences were observed in IL-4 levels.

Conclusions: Fetal alcohol exposure was associated with an increase in pro-inflammatory cytokines in the placenta. Whether fetal alcohol exposure leads to a fetal inflammatory response or exacerbates that associated with pre-term delivery remains to be determined.

Introduction

Prenatal alcohol exposure leads to a range of growth and developmental disabilities classified as Fetal Alcohol Spectrum Disorders [9, 10], which affect 2-5% of births in the United States [433]. The majority of infants who are exposed to alcohol *in utero* do not display the phenotypic characteristics associated with Fetal Alcohol Syndrome [435] and, as a result, exposure often goes undetected by primary care providers. Early identification of infants exposed to alcohol *in utero* allows for early intervention which has shown to reduce secondary disabilities including neurocognitive and attention deficits often observed during the childhood years [545, 546]. Maternal self-report is widely used to identify affected infants; however, it is well-known that mothers consuming alcohol during pregnancy underreport the amount and frequency of consumption. To date, there is not a laboratory test screening that can accurately identify and quantify prenatal alcohol exposure and clinicians have to still rely on maternal self-report even though they do not accurately depict consumption [426] or patterns of consumption [427, 428].

Alcohol is metabolized through oxidative and non-oxidative pathways with the latter resulting in formation of fatty acid ethyl esters (**FAEE**) and phosphatidylethanol. FAEE are synthesized in almost all tissues and are transported in the blood bound to albumin or incorporated in the core of lipoproteins. The use of FAEE as markers of prenatal alcohol exposure has gained a lot of momentum in the past decades and researchers continue to examine their correlation to alcohol consumption in different tissues and in matrices such as meconium and hair. Measurement of FAEE in meconium has been validated in term newborns as a sensitive biomarker of heavy alcohol exposure in second and third trimesters of pregnancy [427]. However, in a study assessing agreement between maternal self-reported drinking and quantification of ethanol metabolites, no agreement was found between reported alcohol intake and FAEE concentrations in meconium [429]. Furthermore, most studies to date have studied FAEE concentrations in full-term infant populations and limited research has focused on the premature infant given the many

complications this population suffers from due to premature delivery. Cut-offs established for full-term models may not be sensitive for the premature infant who has less fat deposition which occurs in late pregnancy [547] and reduced enzyme activity [548]. Since newborns exposed to alcohol *in utero* have a high risk of being born pre-term and experiencing post-natal complications, it is important to develop markers of fetal alcohol exposure in the premature born that are non-invasive yet have high sensitivity and specificity.

The placenta acts primarily to protect and transport nutrients and oxygen to the fetus [518]. Placental transfer of nutrients largely impacts fetal growth and has been linked with lifelong health outcomes for the fetus [535, 549]. It is well recognized that alcohol passes through the placenta and affects fetal growth, development, and the fetal immune system [523]. In animal models, chronic ethanol exposure compromises placental circulation and nutrient exchange by preventing expansion of maternal uterine blood vessels and by impairing placentation and placental development [525]. Our group has recently investigated placental FAEEs as potential negative predictive biomarker of prenatal alcohol exposure and has proposed cutoffs for detection of alcohol-exposed infants in a population of premature newborns born <1500g using a combination of fatty acid ethyl esters (ethyl oleate, linoleate, linolenate, and stearate) (Gauthier 2014; manuscript under review).

Cytokines are mainly, but not exclusively, immunoregulatory proteins produced by immune cells in response to an external stimulus that modulate immune cell differentiation and function [526]. A successful pregnancy is characterized by a balance of pro- and anti- inflammatory cytokines which play an integral role in implantation, placental growth and development, and pregnancy maintenance [526]. Disruptions in the pro- and anti- inflammatory balance lead to maternal and fetal complications such as pre-term labor, preeclampsia and intrauterine growth restriction. Exposure to high levels of alcohol can disrupt cytokine functions and delay onset of humoral and cell-mediated immunity [533]. In the current study, we investigated whether the balance between

pro- and anti- inflammatory placental cytokines from a vulnerable population of preterm infants born under 1500g differed in the presence of prenatal alcohol exposure. We hypothesized that placental pre-term tissues exposed to alcohol during pregnancy would display an imbalance of Th-1 and Th-2 cytokines characterized by increased levels of pro-inflammatory cytokines when compared to unexposed pre-term placental tissues.

Materials and Methods

Study participants. Subjects were enrolled from Emory University Hospital Midtown and Grady Memorial Hospital in Atlanta, GA, after approval from Emory IRB (Emory IRB 00000976, Gauthier, PI) from 11/2009-12/2012. Eligibility for the study included mothers of all neonates born weighing less than 1500g who were admitted to the Newborn Intensive Care Unit at either Emory University Midtown or Grady Memorial Hospital. Exclusion criteria included refusal to participate, multiple congenital anomalies on physical exam, and clinically suspected or confirmed chromosomal aberrations in the infant. Mothers whose babies were deemed not viable by the attending neonatologist were not approached for study enrollment. Mothers with HIV history were excluded due to the potential risk on laboratory personnel handling and processing the samples. Informed consent was obtained prior to maternal questionnaire administration and placental collection.

Placental collection. Placentas were harvested after delivery using the Human Tissue Procurement Service (Winship Cancer Institute, Emory University). A tissue sample was uniformly collected as a full thickness section from the edge of the placenta, approximately 5 cm from the point of umbilical cord insertion. The sample was frozen at -80°C until batched analysis via gas-chromatography mass spectrometry (**GC/MS**) (Emory + Children's Healthcare of Atlanta Biomarker Core) in the Brown laboratory.

Maternal questionnaire. The subjects were interviewed using a structured, extensive questionnaire administered by trained research staff. The questionnaire was modeled after those used in previous studies focusing on maternal alcohol use and outcomes of pregnancy [550, 551], as we have previously standardized and reported [20]. During the interview, the subjects were asked about lifestyle and behaviors including alcohol consumption (beer, wine or liquor) three months before conception and in each trimester. A calendar was used to assist in maternal timing of alcohol consumption before and during pregnancy. Data on subject education, marital status, income, tobacco smoking and illicit drug usage were also obtained. Subjects were identified with a study number and strict confidentiality was maintained. The subject's medical record was reviewed for maternal demographics and delivery room information. Data were abstracted by study staff into a secure de-identified electronic database (Emory Alcohol Lung Biology Center, Guidot PI) and then to a SPSS database for analyses.

Fatty acid ethyl ester measurement. After collection, the placental samples were labeled with a de-identified study number and stored at -80°C until batch analysis by GC/MS using methods mirroring those first described by Bearer *et al.* [430]. Thawed placental samples (0.01-1.0g) were homogenized and spiked with a surrogate standard (**SS**) of pentadecanoic acid ethyl ester (MP Biomedicals, LLC, Santa Ana, CA). FAEEs were extracted using methanol (3000 rpm, 15 min) (Fisher Scientific, Pittsburgh, PA). The samples were filtered across extraction columns (UCT, Bristol, PA), dried under nitrogen gas, and then reconstituted in 200 μl of methanol prior to analysis. Samples were analyzed across a single column GC/MS using a Hewlett-Packard 5890 Series II GC and a Hewlett-Packard 5972A Mass Selective Detector with analysis via Chemstation Productivity Software G1701BA (Version B.01/.01). An internal standard (**IS**, heptadecanoic acid ethyl ester, 100 μg/ml, Nu-Chek-Prep, Inc., Elysian, MN) was added to all samples and standards before analysis by GC/MS. Individual FAEEs were identified *via* their unique retention times with confirmation by a calibration standard of a mixture of all FAEEs of

interest (final concentration of 1000 µg/ml in methanol, Cayman Chemicals, Ann Arbor, Michigan). The calibration curve of each FAEE standard of interest displayed a linear fit with a mean (± SD) coefficients of determination (r^2) of 0.972 ± 0.027. Final concentration of each sample (ng) was normalized to dry weight of the placenta (g). The inter-assay coefficient of variation of the SS was 16.7% while the intra-assay coefficients of variation of the IS was 7.5 ± 2.1%. The mean recovery rate (± SD) was 98.5 ± 2.1%. The lower limit of detection was 0.003 ng/g and the limit of quantification was 0.01nM.

Cytokine measurement. Cytokine profiles in placental homogenates were measured using Human Cytokine 10-Plex panel (Invitrogen, Carlsbad, CA) and BioPlex Manager 4.0 (BioRad Laboratories, Hercules, CA) following provided protocol with kit. Briefly, antibody-conjugated beads tagged with a specific fluorophore were plated in a 96-well plate along with filtered placental supernatants and known protein standards and incubated for 2 hrs. After washing the beads, protein-specific biotinylated detector antibodies were added to the plate and incubated for an additional hour, to allow for detector antibodies to bind to the appropriate immobilized proteins. After the wells were washed to remove excess biotinylated detector antibodies, the streptavidin conjugated to fluorescent protein, R-Phycoerythrin (**streptavidin-RPE**), was added and wells were incubated for 30 min to allow binding of streptavidin-RPE to biotinylated cytokine complex. Beads were washed again and the concentration of the target proteins was measured using BioPlex Manager 4.0. Analytes quantified in placental homogenates included human IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, GM-CSF, IFNγ, and TNFα.

Statistical analysis. Data analysis was performed using IBM SPSS version 21 (IBM, Armonk, NY). Cytokines in placental homogenates were not normally distributed and non-parametric tests were performed to examine differences between groups. Values represent median and interquartile range. A $p < 0.05$ was considered statistically significant.

Results

Study demographics

For this study, 86 placental samples from 80 mothers were evaluated for cytokine concentration where 31.2% of mothers (n = 25) admitted to drinking during pregnancy (**Figure 5-1**) while 51.3% (n = 41) fell above the OLL + Stearate positive cutoff (**Table 5-1**). There was a 68% positive percent agreement in identification of exposed newborns between maternal reported alcohol use during pregnancy and detected OLL + Stearate (kappa = 0. 239) in the placental tissues and a 56.3% negative percent agreement (**Table 5-1)**. For the analyses, samples that fell above the positive cutoff value for OLL + Stearate were classified as "alcohol-exposed" during pregnancy while those below the cutoff as "non-exposed".

The majority of subjects in the study were African-American (86%), with Caucasians and other races contributing to 9% and 5%, respectively. No differences were observed between "alcohol-exposed" and "non-exposed" groups in race, education, income distribution, maternal age at delivery, prenatal care, delivery mode, weight gain and prevalence of diabetes, and drug or cigarette use (**Table 5-2**). Additionally, no statistical significant differences were observed in gestational age or birth weight between infants with or without fetal alcohol exposure.

Cytokine concentrations were higher in alcohol-exposed pre-term placentas when compared to non-exposed pre-term placentas. Placental tissue pro-inflammatory cytokine levels were higher in alcohol-exposed pre-term placentas when compared to unexposed pre-term placentas (**Table 5-3**). Additionally, anti-inflammatory cytokines IL-2 and IL-10 were significantly increased in alcohol-exposed placentas while no significant differences were observed in IL-4 levels (**Table 5-3**).

Cytokine concentrations in placental tissues of patients that self-reported "NO" to drinking during pregnancy were different when OLL + Stearate cutoffs were used to classify exposure. Based on the questionnaire, 55 mothers (68.8%) self-reported "NO" to drinking during pregnancy. When OLL + Stearate cutoffs were used to classify placentas of the newborns *within* this "NO" group, 24 newborns (44%) were identified as alcohol-exposed, while 31 newborns (56%) as non-exposed. Comparison of cytokine profile between the classified groups revealed statistically significant differences in pro-inflammatory cytokines IL-1β, IL-6, GM-CSF, and IFNγ with higher concentrations in the alcohol-exposed vs. non-exposed pre-term placental tissues (**Table 5-4**). On the other hand, 25 mothers self-reported "YES" to drinking during pregnancy and further classification within this group based on the placental OLL + Stearate values showed no statistically significant differences in cytokine profile (data not shown).

Discussion

The etiology of premature delivery is complex and is often associated with an increased release of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8 [530]. Prenatal alcohol exposure also impairs fetal growth and development *in utero*, increases the risk for pre-term birth and postnatal morbidity and mortality associated with prematurity, among other complications [14, 414, 415]. High levels of alcohol exposure can disrupt cytokine functions and delay onset of humoral and cell-mediated immunity [533], and are also associated with increased expression of pro-inflammatory cytokines during pregnancy [533, 534]. Given the social stigma associated with alcohol use during pregnancy, women often underreport or do not report alcohol use during pregnancy [426, 552], making it challenging to identify infants that do not display the characteristics of Fetal Alcohol Syndrome but are still highly vulnerable to complications from alcohol exposure *in utero*.

In recent decades, fatty acid ethyl esters (**FAEE**), non-oxidative metabolites of alcohol, have attracted attention as a useful and cost-effective screening method for prenatal alcohol exposure [553, 554] and have been measured in blood, tissues, and other matrices such as meconium and hair. Maternal self-report cannot be considered a reference standard due to its unreliability, therefore, calculating specificity and sensitivity estimates will produce biased results [555]. In the current study, estimates of agreement measures (kappa value, overall percent agreement, positive and negative percent agreement) between maternal self-report and a panel of placental FAEEs were calculated. Maternal self-report for alcohol consumption the selected panel of FAEEs agreed on classification 60% of the time. The low overall agreement and (Cohen's) kappa agreement between maternal self-report and FAEE is not surprising given the poor reliability of maternal self-report for alcohol use.

Most studies with FAEE measurements are conducted in full-term models and it is unknown whether the same cutoffs apply to pre-term infants. Our laboratory has examined placental FAEE as biomarkers of prenatal alcohol exposure in a pre-term, low birth weight population (Gauthier, manuscript under review). In the current study, we first examined the placental cytokine profiles stratified by maternal self-report of alcohol use during pregnancy but observed no statistical differences (data not shown). Within the group that self-reported "NO" to alcohol use during pregnancy, we next stratified the samples based on the OLL + Stearate concentrations and observed significant differences in pro-inflammatory cytokines. IL-1β, IL-6, GM-CSF and IFNγ were increased in those placental tissues above the OLL + Stearate cutoff. We next categorized fetal alcohol exposure solely based on the OLL + Stearate cutoffs and independent of maternal self-reporting of alcohol use. In that analysis, placental pro-inflammatory cytokine levels IL-1β, IL-5, IL-6, TNFα, IFNγ, and GM-CSF were statistically increased in alcohol-exposed placentas when compared to unexposed placentas. Additionally, levels of anti-inflammatory cytokines, IL-2 and IL-10, increased in alcohol-exposed placental tissues when compared to unexposed but IL-4

was not statistically different. Even though premature delivery is associated with increased pro-inflammatory cytokines in placental tissues, the results from this study suggested that fetal alcohol exposure exacerbated the increased cytokine production associated with premature delivery. Limitations in this study include small sample size and, consequently, less power, inability to ascertain "true" exposed cases since due to low agreement between maternal self-report and placental FAEE, and a high number of undetected cytokine concentrations for both exposed and non-exposed groups. Additionally, the estimate of agreement measures between maternal self-report and FAEE concentrations cutoffs should be interpreted with caution, as agreement does not mean correct and agreement will change as the prevalence in the self-report groups changes [555].

It is important to address that FAEE exist naturally in food flavorings, beverages, lotions, hair care products [556], and in some omega-3 fish oil supplements [557]. However, these ethyl esters are rapidly removed from the body and have not shown to produce any toxic effects [558]. Furthermore, basal FAEE concentrations are low or undetected in those that abstain from alcohol use [428]. Animal studies of mid-gestation acute alcohol exposure have shown accumulation of FAEE in maternal and fetal tissues, while saline-treated animals show complete absence of FAEE [430]. Placental FAEEs as biomarkers of fetal alcohol exposure have not been extensively explored and future research to refine and validate placental FAEE as indicative of alcohol exposure *in utero* is warranted. Additionally, the correlation and estimate of agreement between meconium and placental FAEE should be investigated since meconium FAEE is a more established marker of prenatal alcohol exposure and the combination of the two markers may aid in earlier and more accurate identification of infants exposed to alcohol during gestation.

In summary, these studies suggested that fetal alcohol exposure was associated with an increase in pro-inflammatory cytokines in the placenta. Placental inflammation is often associated with a fetal inflammatory response as defined by increased systemic inflammatory cytokine

concentrations. Clinical and epidemiological studies have demonstrated that a fetal inflammatory response leads to poor cardiorespiratory, neurological, and renal outcomes [559]. Whether fetal alcohol exposure leads to a fetal inflammatory response or exacerbates that associated with pre-term delivery remains to be determined.

Figure 5-1. Summary of subject recruitment

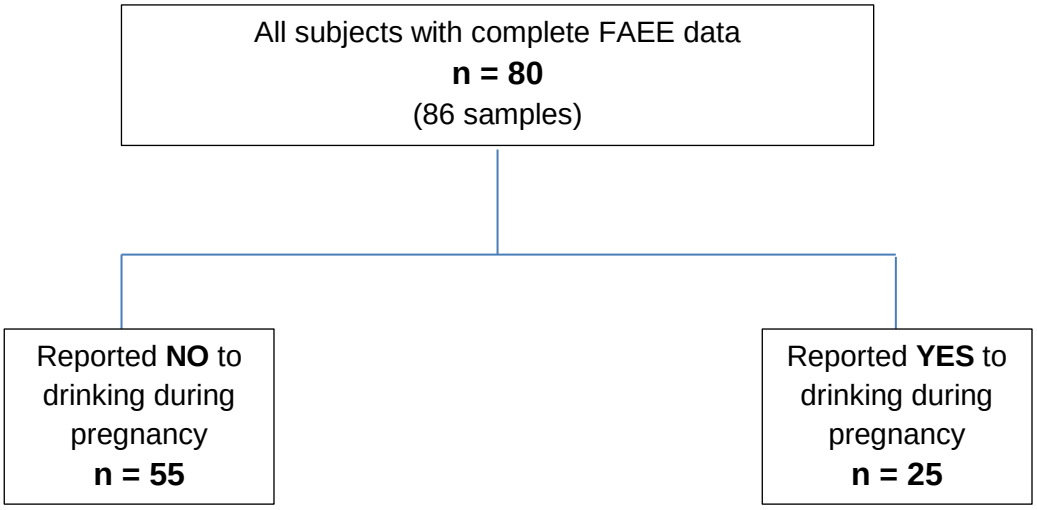

Table 5-1. Agreement between maternal self-report alcohol use during pregnancy and detected fatty acid ethyl esters above positive cutoff in placenta of 80 subjects

Maternal self-report	OLL + Stearate	Number
Positive	Detected	17
Positive	Undetected	8
Negative	Detected	24
Negative	undetected	31
Kappa = 0.239, overall percent agreement: 60 % **positive percent agreement: 68 %** **negative percent agreement: 56.3 %**		

Table 5-2. Study demographics

	Non-exposed (n=38)	**Exposed** (n=42)	**p**
Maternal race			0.108[b]
African-American	29 (76.3%)	38 (90.5%)	
Caucasian	7 (18.4%)	2 (4.8%)	
Other	2 (5.3%)	2 (4.8%)	
Maternal age at delivery (Years ± SD)	28.1 ± 7.4	28.6 ± 6.8	0.706[a]
Marital status			0.173[b]
Married	10 (26.3%)	6 (14.3%)	
Separated	20 (52.6%)	35 (83.3%)	
Undetermined	8 (21.1%)	1 (2.4%)	
Maternal education			0.499[b]
High school or less	18 (47.4%)	16 (38.1%)	
Technical school/Jr. college	9 (23.7%)	19 (45.2%)	
Finished college	3 (7.9%)	5 (11.9%)	
Graduate or other post college degree	2 (5.3%)	2 (4.8%)	
No answer	6 (15.8%)	NA	
Maternal Income			0.175[b]
< 25,000	13 (34.2%)	23 (54.8%)	
25,000 - 54,999	7 (18.4%)	14 (33.3%)	
55,000 - 70,000	1 (2.6%)	1 (2.4%)	
> 70,000	5 (13.2%)	2 (4.8%)	
No answer	12 (31.6%)	2 (4.8%)	
Delivery mode			0.302[b]
Cesarean	25 (65.8%)	27 (64.3%)	
Vaginal	9 (23.7%)	15 (35.7%)	
No answer	4 (10.5%)	NA	
Maternal fever	2 (5.3%)	1 (2.4%)	0.463[b]
Drug Use during pregnancy			
Antibiotic use during pregnancy	12 (31.6%)	16 (38.1%)	0.921[b]
Illicit drugs	4 (10.5%)	3 (7.1%)	0.465[c]
Smoking during pregnancy	4 (10.5%)	9 (21.4%)	0.144[b]
Weight gain in pregnancy (lbs)	18.4 ± 2.07	18.07 ± 1.84	0.899[a]
Prevalence of diabetes	2	5	0.454[c]
Infant demographics			
Male	18 (47.4%)	27 (64.2%)	0.165[b]
Gestational age (weeks ± SD)	28.2 ± 2.7	27.9 ± 2.4	0.597[a]
Birth weight (g)	1038.4 ± 283.3	1041.2 ± 251.0	0.962[a]

a t-test
b Chi- square
c Fisher's Exact Test

Table 5-3. Cytokine concentrations in all placental tissues

Pro-inflammatory	**Alcohol-exposed**	**n**	**Non-exposed**	**n**	***p****
IL-1β	0.055 (2.426)	44	0.000 (0.000)	31	0.003
IL-5	0.022 (0.409)	27	0.000 (0.000)	15	0.005
IL-6	0.085 (1.380)	30	0.000 (0.000)	22	0.001
IL-8	1.483 (9.211)	12	0.000 (0.063)	11	0.079
GM-CSF	0.480 (3.754)	43	0.000 (0.287)	29	0.003
IFNγ	0.503 (7.337)	33	0.000 (0.048)	26	<0.001
TNFα	0.352 (1.021)	30	0.000 (0.291)	24	0.011
Ant-inflammatory					
IL-2	0.031 (30.277)	22	0.000 (0.000)	18	0.019
IL-4	0.000 (10.401)	14	0.000 (0.000)	11	0.202
IL-10	2.773 (13.839)	30	0.000 (2.201)	21	0.003
Values represent median (interquartile range)					
*Data was not normally distributed and non-parametric tests were used for statistical analysis					

Table 5-4. Cytokine concentrations in placental tissues from patients that reported "NO" to drinking during pregnancy classified by OLL + Stearate cutoff

Pro-inflammatory	**Alcohol-exposed**	**n**	**Non-exposed**	**n**	***p****
IL-1β	0.000 (2.304)	25	0.000 (0.000)	27	**0.014**
IL-5	0.011 (0.467)	14	0.000 (0.000)	15	**0.123**
IL-6	0.044 (0.910)	17	0.000 (0.000)	18	**0.049**
IL-8	0.059 (17.214)	7	0.000 (0.032)	9	**0.299**
GM-CSF	0.000 (13.695)	25	0.000 (0.258)	26	**0.038**
IFNγ	0.196 (9.351)	17	0.000 (0.055)	24	**0.038**
TNFα	0.350 (1.111)	18	0.000 (0.284)	21	**0.069**
Ant-inflammatory					
IL-2	0.000 (53.656)	14	0.000 (0.000)	17	**0.109**
IL-4	0.000 (7.011)	11	0.000 (0.000)	8	**0.206**
IL-10	2.902 (18.090)	15	0.000 (2.444)	18	**0.086**
Values represent median (interquartile range)					
*Data was not normally distributed and non-parametric tests were used for statistical analysis					

CHAPTER 6: CONCLUSIONS, LIMITATIONS, FUTURE DIRECTIONS, PUBLIC HEALTH IMPLICATIONS

CONCLUSIONS

Experimental studies

Ethanol ingestion prior to and during pregnancy perturbed maternal AM zinc balance resulting in impaired bacterial clearance, but these effects were improved by *in vitro* zinc treatments

Our goal was to mimic societal patterns where pregnant women who routinely abuse alcohol probably were drinking prior to pregnancy recognition and may therefore have decreased zinc pools compared to women who do not drink prior to pregnancy. To model this, we established a fetal ethanol model where chronic ethanol ingestion by the dams started prior to conception and continued for the duration of pregnancy. We demonstrated that chronic ethanol ingestion prior to pregnancy (≥ 3 weeks) decreased maternal zinc pools in the AM and its immune functions when compared to the control group. *A priori*, we expected that zinc insufficiency would be exacerbated when chronic ethanol ingestion was superimposed on pregnancy. However, comparison of non-pregnant with pregnant dams with ethanol exposure showed no statistical significant differences in intracellular zinc levels. The failure to observe an effect could be due to increased calorie intake by the pregnant groups.

Continued ethanol ingestion (25% of calories) during pregnancy was associated with perturbations in zinc homeostasis and zinc transporter protein expression (ZIP1, ZnT1, ZnT4) in

the maternal AM despite adequate zinc and other nutrients in the diet. Moreover, chronic ethanol ingestion prior to and during pregnancy resulted in decreased AM phagocytic function (54% of control) when compared to pair-fed control group. A central role for zinc availability in the ethanol-induced suppression of phagocytosis was validated by the ability of *in vitro* zinc treatments to restore phagocytosis to control levels. As expected, *in vitro* zinc treatment to AMs isolated from ethanol-fed dams normalized intracellular zinc pools presumably through the restoration of protein expression of the zinc transporters ZIP1, ZnT1 and ZnT4. The control group maintained normal zinc balance in the maternal AM and the dams consumed equivalent amounts of chow, independent of ethanol ingestion. Therefore, chronic ethanol ingestion resulted in depletion of the zinc pool and transporters in the maternal AM despite adequate nutritional intake. Furthermore, the data suggested that the increased risk of respiratory infections due to impaired AM bacterial clearance can be reversed by the simple addition of zinc supplements, however additional studies are necessary.

Zinc availability, zinc transporter expression, and bacterial clearance in the newborn AMs were depressed with fetal alcohol exposure but *in vitro* zinc treatments ameliorated these effects

In the newborn lung, the expression of zinc transporters has received little attention. Although studies in the adult model demonstrated that chronic ethanol ingestion suppresses expression of zinc transporters [31], the effects of fetal ethanol exposure have not been explored. To address this question, we examined the effects on zinc homeostasis in pups born to dams that ingested ethanol $\geq$ 3 weeks prior to conception and during pregnancy. We speculated that the combination of ethanol-induced zinc insufficiency prior to conception and the zinc requirements that occur

during pregnancy would exacerbate zinc insufficiency in the newborn with fetal ethanol exposure.

Pups exposed to chronic ethanol exposure *in utero* displayed ~70% decrease in AM intracellular zinc when compared to the AMs from unexposed pups. Additionally, chronic fetal ethanol exposure was associated with significantly decreased protein expression of Zip1, ZnT1, and ZnT4 when compared to unexposed pups. These results suggested that decreased zinc availability in the AM associated with fetal ethanol exposure correlated with decreased protein expression of the zinc transporters Zip1, ZnT1, and ZnT4. Furthermore, ethanol exposure *in utero* was associated with decreases in phagocytosis, characterized by decreases in the number of cells that internalized bacteria as well as the overall mean fluorescence per cell when compared to control groups.

In vitro treatment with zinc not only normalized the zinc pool and transporters in the AM, it also restored phagocytosis to the AMs derived from the pups with fetal ethanol exposure, as reflected by the increases in mean fluorescence and the percent of cells that internalized bacteria. This data demonstrated that fetal ethanol exposure resulted in decreased expression of zinc transporters and the subsequent decrease in zinc pools in the fetal AMs despite adequate maternal nutritional intake. Additionally, these studies suggested that the impaired immune functions and capacity to clear infectious microbes associated with fetal ethanol exposure may be reversed by zinc supplements however *in vivo* studies are needed.

Clinical study

Cytokine concentrations were higher in alcohol-exposed pre-term placental tissues when compared to unexposed pre-term placental tissues

Several mechanisms may explain the decrease of zinc availability in the newborn pulmonary AM and may derive from complications on the maternal side, placental interface, or neonatal end. It is

known that alcohol exposure impairs placental transfer of nutrients and may affect maternal-placental-fetal transfer by altering zinc transporter expression in the placental surface or by inducing an inflammatory state in the placenta that impairs zinc movement to the fetus. Since placental inflammation is associated with fetal inflammation and the zinc status of the fetus, we first wanted to establish whether fetal alcohol exposure exacerbated placental inflammation, independent of premature birth. To address this question, we focused on characterization of cytokine profiles in human placental tissues from a pre-term population where the in utero alcohol exposure was known. Using a panel of fatty acid ethyl esters as biomarkers of alcohol exposure, we demonstrated that placental pre-term tissues exposed to alcohol during pregnancy had increased levels of pro-inflammatory cytokines when compared to unexposed placental pre-term tissues. Additionally, levels of anti-inflammatory cytokines, IL-2 and IL-10, increased in alcohol-exposed placental tissues when compared to non-exposed, while IL-4 did not change. Results from this study suggested that fetal alcohol exposure exacerbated placental inflammation beyond that associated with premature delivery.

LIMITATIONS

Experimental studies

One of the limitations of isolating AMs from newborn mouse pups is the low number of AMs obtained which limits the types and number of assays one can do. In these studies, after plating the cells needed to measure protein expression of zinc transporters, intracellular zinc levels, and AM function, few cells remained and it was not feasible to extract RNA to measure mRNA expression of zinc transporters. Additionally, the low number of AMs isolated at birth limited us in the number of zinc transporters we could measure. Moreover, the amounts of AMs isolated from newborn pups do not allow for exploration of dose-response experiments with zinc

treatments. It is unclear if the observed effects of fetal ethanol exposure on zinc homeostasis and AM bacterial clearance improve with maturation. Furthermore, while AMs are the primary immune response in the lung, other cells display anti-microbial activity and it is unclear if they are similarly affected by ethanol exposure *in utero*.

Experimental models portray controlled scenarios and may not translate as clearly in human studies where compliance may be poor and alcohol consumption is underreported. Additionally, even though zinc deficiency is a cause of disease and can affect disease progression, single nutrient deficiencies are rare and it is necessary to study nutrient deficiencies that often occur together in human population studies. Pregnant women that are consuming a diet deficient in one element, most likely are consuming suboptimal amounts of other nutrients because of food disparity or insufficient availability, among others.

Clinical study

Limitations in our clinical study include small sample size and consequently less power, inability to ascertain “true” exposed cases due to low agreement between maternal self-report and placental FAEE, and a high number of undetected cytokine concentrations for both exposed and non-exposed groups. The estimate of agreement measures between maternal self-report and FAEE concentrations cutoffs should be interpreted with caution, as agreement does not mean correct and agreement will change as the prevalence in the self-report groups changes [555]. Furthermore, more studies are required to establish placental FAEE as biomarkers for fetal alcohol exposure and to explore whether a dose-response curve exists between placental FAEE accumulation and amounts of alcohol consumed during pregnancy.

FUTURE DIRECTIONS

In the United States 1 in 2 women of child-bearing age use alcohol and 15% of women who drink alcohol in this age group binge drink [4]. Since nearly 50% of pregnancies are unplanned, many women may unintentionally expose their developing fetus to continuous or acute high levels of alcohol exposure until pregnancy recognition and possibly beyond that. The effects of different alcohol patterns on zinc homeostasis and lung immune defense in the newborn have not been well-explored and animal models of binge ethanol ingestion and trimester exposures may provide some insight. Additionally, *in vivo* maternal zinc supplementation in combination with ethanol exposure during pregnancy could be explored to see if fetal derangements are inhibited when zinc is administered alongside ethanol.

Realistically, pregnant women consuming alcohol during pregnancy are less likely to take prenatal supplements or eat healthy, thus it is important to explore if postnatal zinc supplementation to the infant can reverse the negative effects of alcohol on the newborn lung. The premature infant exposed to alcohol *in utero* is at risk for zinc deficiency from a combination of alcohol exposure and premature birth since alcohol may impair zinc transfer and since nearly 60% of fetal zinc is acquired during third trimester of pregnancy [560] and can often display AE-like symptoms, such as skin lesions [561]. In exploring a safe and effective dose in treatment of the premature infant, factors such as interference with absorption of other metals, toxicity, and mode of administration need to be taken into account. Zinc may interfere with absorption of copper and iron, and mode and time of supplementation matter, where administration in aqueous solution and at a fasting state inhibits iron absorption, however at normal fortification levels absorption should not be inhibited [181].

The American Society for Clinical Nutrition recommends zinc supplementation at 400 μg/kg/day for premature infants [562]. In a case report study, zinc supplementation at 146%-195% from the

recommended amounts of 400ug/kg/day, given with parenteral nutrition to infants born at 24-31weeks with a birth weight of 650-940g and at risk for zinc deficiency, improved skin lesions with no signs of toxicity displayed [561]. Another case study reported that oral zinc supplementation (3mg zinc gluconate/kg/day) healed skin lesions in an infant born 29 weeks gestation with no apparent toxicity [563]. Future studies examining the effects of zinc supplementation on immune function of the premature infant exposed to alcohol *in utero* can start with doses 800 μg/kg/day of elemental zinc added to parenteral nutrition.

The ability of oral zinc supplementation to decrease the risk of respiratory infections in the alcohol-exposed newborn could be determined by bacterial challenge *in vitro* and *in vivo*. *In vitro* experiments would involve isolation of AMs from pups and incubation with fluorescent-tagged inactivated bacteria, as demonstrated in current analyses of this dissertation. *In vivo* experiments would involve administration of group B streptococcus (**GBS**) *pneumonia* intratracheally [564] or intranasally [18] to the pups, followed by a recovery period, after which AMs would be isolated as described in **Chapter 4.** Measurement of ingested GBS would be done by incubating isolated AMs with antibodies, and analyses would be conducted with fluorescent microscopy. In these studies we focused on AMs and it is unclear if other immune cells are similarly affected and further studies are warranted. Also, other zinc transporters that express in the lung such as ZIP8 and ZIP14, could be examined.

Further investigations can explore the relationship between zinc and anti-inflammatory regulator, nuclear factor erythroid 2-related factor 2 (**Nrf2**). Nrf2 plays a crucial role in resistance to oxidative stress, with Nrf2 target genes regulating drug metabolism and disposition, antioxidant defense, and oxidant signaling (reviewed in [565]). In the anti-oxidant defense Nrf2 is involved 1) in induction of catabolism of superoxides and peroxides through superoxide dismutases, peroxiredoxins, and glutaredoxins, 2) in reduction of glutathione disulfide (**GSSG**) and synthesis

of glutathione (**GSH**), 3) and induction of stress response, among others [565]. Nrf2 knockout mice display heightened inflammation and susceptibility to chemical toxicity [565] while overexpression of Nrf2 protects from ethanol-induced oxidative stress and lipid accumulation in the liver of ethanol treated mice [566]. In the lung, experimental models show that alcohol causes alveolar epithelial oxidative stress by down-regulation of Nrf2 pathways [567] and zinc supplementation improves binding of Nrf2 in AMs and decreases oxidative stress [163]. Our laboratory has extensively shown that alcohol-induced depletion of major anti-oxidant GSH in the lung results in alveolar macrophage and epithelia cell dysfunction in adult and fetal models [17, 18, 150, 168]. Nrf2 modulates GSH pathways by transcriptional regulation of glutathione reductase [568], which reduces GSSG to GSH. Future studies can explore whether ethanol exposure *in utero* alters Nrf2 expression in the newborn AM and whether zinc supplementation can restore Nrf2 expression, GSH levels, and bacterial clearance in the alcohol-exposed newborn pulmonary AMs.

Furthermore, Nrf2 may be necessary for placental development and protection against oxidative damage [569]. Pregnancy complications such as preeclampsia are often characterized by increased oxidative stress in the placenta and Nrf2 mRNA expression is significantly decreased in preeclamptic placentas when compared to placentas from normal pregnant women [570]. It would be important to investigate how alcohol exposure *in utero* alters Nrf2 expression in the placenta and whether zinc supplementation can neutralize alcohol-induced derangements through restoration of Nrf2 and its target genes.

As it pertains to biomarkers of prenatal alcohol exposure, more studies are needed to examine placental FAEE not only as biomarkers of chronic alcohol exposure but also as biomarkers of partial alcohol exposure *in utero*. Additionally, the correlation and estimate of agreement between meconium and placental FAEE should be investigated since meconium FAEE is a more

established marker of prenatal alcohol exposure and the combination of the two markers may aid in earlier and more accurate identification of infants exposed to alcohol during gestation. As it pertains to zinc, future studies can examine the relationship between zinc status and placental transfer in response to inflammatory stimuli and how alcohol affects zinc transporter expression in the placenta. Of importance may be measurement of ZIP8 and ZIP14 which are known to be up-regulated in response to inflammatory stimuli such as TNFα or LPS challenge.

IMPLICATIONS FOR PUBLIC HEALTH

To date there is no conventionally accepted biomarker of long-term fetal alcohol exposure. Early identification of infants exposed to alcohol *in utero* allows for early intervention which has shown to reduce secondary disabilities including neurocognitive and attention deficits often observed during the childhood years [545, 546]. Maternal self-report is widely used to identify infants that have been exposed to alcohol during gestation however, it is well-known that mothers consuming alcohol during pregnancy underreport the amount and frequency of consumption possibly due to difficulty to recall and due to the social stigma associated with alcohol use during pregnancy [426]. Further research refining placental FAEEs as biomarkers of alcohol exposure will aid clinicians in promptly identifying infants exposed to ethanol *in utero* and immediately intervening with zinc supplementation to prevent pulmonary infections. Modification of immune responses with zinc supplementation can also prove beneficial in the alcohol-exposed placenta, where there is an increase of pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines with alcohol exposure. Even though zinc supplementation will not solve all problems created by fetal alcohol exposure, it will ameliorate some alcohol-induced lung injury of affected infants.

Our findings could have implications outside the alcohol-exposed infant. Zinc deficiency is linked with pulmonary conditions such as cystic fibrosis (**CF**) and asthma [571, 572]. In CF, zinc supplementation appears to be promising as an adjuvant therapy to antibiotic treatment, which has been increasingly ineffective due to the emergence of antibiotic-resistant bacteria [573]. A small study in pediatric CF patients showed reduction of oral antibiotic use, marginal reduction of pro-inflammatory cytokine production, and increased anti-inflammatory cytokine levels with zinc supplementation (30mg/day) [574].

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