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The Role of Zinc Deficiency in the Alcohol Lung Phenotype

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The Role of Zinc Deficiency in the Alcohol Lung Phenotype

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Abstract

The Role of Zinc Deficiency in the Alcohol Lung Phenotype
By Ashish Mehta

Alcohol use disorders are a major burden to society. The medical consequences for alcoholism include an increased susceptibility to lung injury and infection. Currently, there are no therapeutic options for the pulmonary complications of alcohol abuse, but animal models have suggested that chronic alcohol ingestion impairs the absorption and distribution of zinc to the lung. This pulmonary zinc deficiency impairs function of the alveolar macrophage, which is the resident immune cell of the lower airways. However, human studies are lacking. The objective of this research project is to better characterize zinc status and immune function in the lung among individuals who abuse alcohol. Otherwise healthy alcoholic subjects (n=17) and matched non-alcoholic subjects (n=17) were recruited to receive a blood draw for serum zinc measurement and to undergo bronchoscopy for collection of alveolar macrophages. These isolated phagocytes were analyzed for intracellular zinc and immune function, which was determined by measuring phagocytic capacity and cell surface expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor. Alcoholic subjects had normal serum zinc values, but alcohol abuse was associated with significantly lower alveolar macrophage intracellular zinc levels even after adjustments are made for important confounders such as cigarette smoking (adjusted mean difference (se) = -277.8 (121.7) RFU/cell; $p = 0.0305$). Importantly, there was no relationship between serum zinc and alveolar macrophage intracellular zinc levels, suggesting that serum measurements are a poor surrogate marker of pulmonary zinc status (Spearman's $r = -0.129$; $p = 0.48$). Further, alcoholism was associated with significantly decreased immune function as measured by both phagocytosis and GM-CSF receptor expression. There was a significant correlation between intracellular zinc level and immune function, providing provocative evidence that alcohol-induced immune dysfunction may be the result of zinc deficiency (Spearman's $r = 0.68$; $p < 0.0001$). These data provide important validation of animal models of chronic alcohol ingestion and argue that clinical trials be undertaken to further investigate the potential therapeutic value of zinc supplements in this vulnerable population.

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INTRODUCTION

Alcohol is the most used and abused drug in the world, and alcoholism poses a significant health burden to society. A recent national survey suggests that more than half of the population in the United States above the age of 12 consumes alcohol and national epidemiological data indicate that the lifetime prevalence of alcohol abuse is 18 percent (1, 2). Tragically, alcohol abuse has significant life-threatening consequences and takes over 100,000 lives annually, making it the third leading cause of preventable death in the United States (3). There are also chronic non-fatal health implications for individuals whose alcohol consumption is excessive, and overall the annual cost of alcohol abuse to American society has been estimated to be upwards of $200 billion dollars (4).

The consequences of chronic alcohol ingestion span multiple organ systems, including the heart, liver, brain, and skeletal muscle (5, 6). Among these widespread effects, alcohol abuse renders individuals susceptible to pulmonary infections and acute lung injury (7-10). Detailed research investigating the effects of chronic alcohol ingestion on the lung have been driven by the initial epidemiological observation nearly two decades ago that alcoholics were twice as likely as non-alcoholics to develop the acute respiratory distress syndrome (ARDS) during a critical illness such as sepsis or trauma, and that their mortality was also increased (11). This initial observation has subsequently been confirmed in other studies (12, 13). The potential mechanisms underlying the association between alcohol abuse and pulmonary infection have been explored in experimental animal models and have determined that chronic alcohol ingestion causes

previously unrecognized zinc deficiency and immune dysfunction within the alveolar space that renders the lung susceptible to infections such as pneumonia (14, 15).

While there have been numerous studies in experimental animals that have characterized the "alcohol lung" phenotype, human studies are lacking. The purpose of this research is to translate experimental animal model findings to human subjects in order to better understand the effects of alcohol abuse on the lungs and specifically to determine if zinc deficiency is an important contributor to pulmonary immune dysfunction in alcoholism. A proposed causal diagram is illustrated in Figure 1. The primary aim is to determine if there is a difference in overall zinc status between alcoholics and non-alcoholics. In addition, this research seeks to determine if there is a difference in pulmonary immune function between alcoholics and non-alcoholics and to assess the association between zinc status and immune function. Finally, zinc status can be assessed using several different methods. Serum zinc measurement is the most commonly utilized approach in the clinical setting, however serum zinc accounts for only 0.1% of total body zinc stores. Therefore, it stands to reason that serum zinc values may not be an accurate reflection of global zinc status in the individual. Intracellular zinc status may be measured in the research setting to determine the zinc status of a specific cell type, and these measurements may better reflect zinc bioavailability at the organ level. The final aim of this project is to assess the association between serum zinc level and intracellular zinc level obtained from immune cells within the lung.

In order to address the aims of this study, a group of otherwise healthy alcoholic subjects were recruited to undergo a blood draw and bronchoscopy, a procedure to isolate immune cells from the lung. Non-alcoholic subjects were also recruited as a comparison

group and were matched based on age, gender, race, and smoking status. The primary hypothesis is that alcoholics will have lower zinc levels compared to non-alcoholics. Further, it is hypothesized that alcoholics will have decreased immune function in the lung, and this decreased function will be associated with zinc deficiency. Finally, it is hypothesized that there will be no association or relationship between serum zinc levels and intracellular zinc measurements. The findings from this project will help to answer these important questions, to determine the relevance of extensive animal model research, and to improve our understanding of the effects of alcohol abuse in the human lung.

BACKGROUND

Zinc is one of the most abundant minerals in the human body, and it plays a crucial role in normal homeostasis within multiple organ systems and various cellular processes. In particular, zinc is a co-factor for catalytic activity for over 300 enzymes (16). This role is encompasses such enzymatic processes as antioxidant function and renders zinc at least partly responsible for the activity of over a thousand different transcription factors (17). In addition to these crucial life-sustaining roles, zinc sufficiency is essential for normal immune function. Various studies have shown that zinc deficiency causes atrophy of the thymus, which results in lymphopenia and compromises overall T lymphocyte response and cell-mediated immunity (18). Experimental models of zinc deprivation also result in bone marrow defects and affect the B-cell lineage (19). In addition to these observed derangements in acquired immunity, zinc deficiency has been shown to cause various deficits in innate immune function as well. Specifically, studies have shown that zinc depletion results in dysfunction of macrophages (15, 20). This is of particular consequence in the respiratory system where alveolar macrophages are constantly exposed to environmental pathogens and debris and are the first line of defense against various pulmonary infections including pneumonia.

The burden of nutritional zinc deficiency is much more evident in third world countries where poverty-stricken individuals have limited access to necessary food sources required to formulate a well-balanced diet. This disposition is especially apparent among young children. Studies show children in several African countries are much more prone to the development of pneumonia, and this deficiency has been linked specifically to zinc deficiency (21, 22). Interesting experimental models have also

verified the key role of zinc deficiency specifically in pulmonary immune function. These researchers demonstrated that immunization is ineffective in zinc-deprived animals, and further that pneumonia is much more severe in the setting of zinc deficiency (23, 24). Taken together, these studies illustrate that zinc is not only intimately connected with the global immune response, but is also crucial for immunity in the respiratory system in particular.

Given the clinical observations that zinc deficiency leads to an increased susceptibility to pneumonia and other pulmonary infections, groups of researchers began to investigate zinc metabolism at the organ level. Using the zinc fluorophore Zinquin, groups led by Truong-Tran and Carter were the first to image pools of zinc in respiratory cells (25, 26). They were able to demonstrate the presence of zinc in the apical region of ciliated airway epithelial cells as well as in alveolar epithelial cells. Further, these researchers demonstrated that pools of zinc in these cell types were strongly decreased by treatment with zinc chelators. Subsequent researchers have demonstrated that several important zinc transporters are also localized to the lung (27, 28) and are involved in regulation of zinc metabolism within the respiratory system. These early studies highlight the importance of zinc metabolism in the respiratory system.

Alcoholism is an important contributor to zinc deficiency, especially in patients who have progressed to frank liver disease (29). Studies show that individuals with alcoholic cirrhosis have both serum zinc deficiency and decreased tissue levels of zinc in the liver (30). However, much less is known about zinc levels in the alcoholic patient who has not developed hepatic dysfunction, but studies suggest that there are alterations in zinc metabolism (31, 32). The mechanisms responsible for creating zinc deficiency in

the setting of alcohol abuse are not entirely clear. While the majority of Americans have no difficulty meeting the recommended dietary allowance for zinc, studies have shown that the vast majority of alcoholics are not able to meet this goal (32). This is not surprising when taken in context of the generally poor dietary habits that exist in this population. However, even with sufficient intake, experimental studies have demonstrated that the absorption of zinc impaired by chronic alcohol ingestion, and there is also an increase in urinary zinc excretion among alcoholics (33, 34). In an experimental animal model, researchers demonstrated that alcohol abuse directly decreased the expression of an important zinc transporter responsible for zinc absorption from the small intestine (15). Finally, one group of investigators showed that albumin—which primarily binds zinc in the plasma—has a much lower affinity for zinc in subjects with cirrhosis, a finding that may additionally contribute to the observed zinc deficiency in this population (35). Taken together, these studies highlight that chronic exposure to alcohol has significant effects on zinc metabolism.

In addition to its widespread medical consequences in other organ systems, chronic alcohol exposure causes various derangements in the respiratory system. Clinically, patients who abuse alcohol have a predisposition towards the development of pulmonary infections such as community-acquired pneumonia (9, 36, 37). The key factors associated with alcoholism that contribute to this increased susceptibility include a change in oral bacterial flora, diminished gag and cough reflexes that occur during inebriation, dysfunction of the cilia in the airway that assist with secretion clearance, and impairment of the alveolar macrophage, which is the primary immune cell in the lower respiratory system (7, 38). The effect chronic alcohol ingestion on the alveolar

macrophage has been examined in several experimental models, all of which show that there is an alcohol-induced alveolar macrophage immune dysfunction function that is characterized by the inability to phagocytose bacteria (14, 39, 40). The mechanism for this finding is not entirely understood, but research has revealed that diminished signaling through granulocyte-macrophage colony-stimulating factor (GM-CSF) appears to play an important role (7). GM-CSF is a 23-kDa peptide that is secreted by several cell types and has many important functions in the lung. Specifically, GM-CSF signaling is crucial for alveolar macrophage differentiation, maturation, and function. GM-CSF signaling occurs through receptors on the cell surface of alveolar macrophages and this receptor expression is an important measure of immune function. Evidence from experimental animal models have revealed that chronic alcohol exposure decreases cell surface expression of GM-CSF receptors on alveolar macrophages and results in impairment of immune function (14).

In addition to the important role of GM-CSF in immune function, other mechanisms of alcohol-induced alveolar macrophage dysfunction have been explored. Specifically, experimental models of alcoholism have established that extracellular zinc in lung lavage samples is decreased about 30 percent in animals on an alcohol-fed diet compared to control-fed diet and dietary zinc supplementation restores alveolar macrophage function (15). Intracellular zinc in the alveolar macrophage is similarly decreased in this particular model. Further, these recent experimental studies suggest that there is an important link between zinc and GM-CSF such that treatment of alcohol-induced zinc deficiency with dietary zinc supplementation also improves GM-CSF receptor expression (15). The clinical relevance of these alveolar macrophage defects has

been investigated through the use of an infection models in setting of chronic alcohol ingestion. In one particular study, dietary zinc supplementation restored function to the alveolar macrophage and augmented bacterial clearance when alcohol-fed animals develop pneumonia (41). This finding illustrates that alcohol-induced zinc deficiency appears to promote susceptibility to lung infection in the alcoholic host. Despite an abundance of experimental evidence demonstrating that alcohol exposure causes zinc deficiency and immune dysfunction in the alveolar macrophage, the effects of alcohol abuse on zinc metabolism and immunity in the human lung remain largely unknown.

METHODS

The primary goal of this research project was to determine if alcoholic subjects have impaired zinc distribution to the lung, and if pulmonary zinc status has any effect on lung immunity. The primary immune cell in the lung is the alveolar macrophage, and these cells were isolated from alcoholic and matched non-alcoholic subjects. The primary hypothesis is that alcoholism subjects will have lower intracellular zinc in alveolar macrophages compared to non-alcoholics, and that lower intracellular zinc will be associated with lung immunity. The study design of this research is cross sectional in nature. There was recruitment of alcoholics and matched non-alcoholics who had study measurements taken at the time of enrollment and did not have longitudinal follow-up. Enrolled subjects underwent a bronchoscopy procedure for isolation of alveolar macrophages, on which further study evaluations were carried out.

Characteristics of the study population

The target population was otherwise healthy adults (18-55 years of age) who were diagnosed with an alcohol use disorder (AUD) at the time of enrollment. An AUD was defined as having a positive Short Michigan Alcohol Screening Test (SMAST) and Alcohol Use Disorders Identification Test (AUDIT) (42, 43). All subjects were recruited from the Substance Abuse Treatment Program (SATP) at the Atlanta Veterans Affairs Medical Center (VAMC) in Decatur, Georgia. Non-alcoholic subjects were matched by age, gender, race, and smoking status.

The primary inclusion criteria included the presence of an AUD and most recent alcoholic drink within 8 days of undergoing the bronchoscopy procedure. Therefore, subjects were recruited on the basis that had an active AUD and not merely a history of

prior alcohol abuse. Key exclusion criteria included 1) primary substance of abuse being something other than alcohol (e.g., cocaine), 2) active medical problems, 3) HIV-positive status, and 4) an abnormal chest radiograph signifying underlying lung disease. A primary goal of the study was to recruit subjects with a current AUD but who were otherwise healthy so as to minimize the effects of confounding factors such as chronic infections or lung disease. Non-alcoholic subjects were recruited through advertisements placed throughout the Atlanta VAMC and were likewise healthy but did not have an AUD. Each alcoholic subject was matched to a non-alcoholic subject (1:1) by age (within 5 years, but still falling in the range of 18-55 years of age), gender, race, and smoking status. The majority of individuals with an AUD are cigarette smokers, and therefore smoking status was used as a matching factor to avoid possible confounding.

A research coordinator screened subjects enrolled at the SATP clinic and discussed the study with those who met the specified inclusion criteria. Those individuals that expressed interest in participating in the study underwent more formal screening with the SMAST and AUDIT as well as a review of their medical records to screen for the specified exclusion criteria. Each subject underwent a screening chest radiograph if one had not been performed in the previous year. All non-alcoholic subjects were screened similarly, but in general medical clinics rather than in the SATP clinic. The majority of non-alcoholic participants contacted the research team directly after viewing a recruitment flyer. Once test and control subjects were enrolled in the study, they had blood drawn and sent to the VA clinical laboratory for measurement of serum zinc levels. All study subjects were compensated for their participation with a grocery store gift card for $150.

Bronchoscopy and laboratory procedures

All study subjects underwent a bronchoscopy and bronchoalveolar lavage (BAL) for collection of alveolar macrophages using standard techniques that have been previously described (44). BAL fluid was passed through sterile gauze and centrifuged at 8000 rpm for 5 min. The cell pellets contained predominantly alveolar macrophages with ~90% purity and were re-suspended as previously described (45). Serum zinc levels were determined by sending samples to a commercial laboratory (Quest Diagnostics, Chantilly, VA). Isolated alveolar macrophages were incubated with FluoZin-3AM dye (Invitrogen, Carlsbad, CA) for 30 min per the manufacturer's instructions in order to determine intracellular zinc level. The specificity of the assay is > 85% according to manufacturer documentation. Immune function was determined through the measurement of phagocytosis and GM-CSF receptor expression. Phagocytosis of bacteria was measured by incubating macrophages with 1 x 10^6 particles of pH-sensitive pHrodo Staphylococcus aureus BioParticles conjugate (Invitrogen) for 2 hours. They were then analyzed using an Olympus confocal microscope containing an argon/krypton laser. To measure S. aureus internalization, laser confocal microscopy was performed at 50% of cell depth using identical background and gain settings. Alveolar macrophages with internalized bacteria were considered positive for phagocytosis. Phagocytosis was measured as the relative fluorescence units (RFU) of S. aureus per cell. Fluorescence measurements for intraceullar zinc and GM-CSF were performed using FluoView (Olympus) via quantitative digital analysis and expressed as mean RFU per cell. The lower limit of detection for the confocal microscope is 300 RFU and the upper limit of detection is 2900.

Variables

There were several outcome variables in this research project, including serum zinc, alveolar macrophage intracellular zinc, phagocytosis, and GM-CSF receptor expression. The measurement processes for these variables are described above. The primary predictor variable is alcoholism, which is defined by a positive SMAST and AUDIT test. For this study, there were two separate groups: alcoholic or non-alcoholic. Covariates that were considered include age, race, smoking frequency, and body mass index (BMI). As there were differences in frequency of smoking between the two groups, the linear regression modeling strategies used total number of cigarettes smoked in the previous 30 days instead of merely smoking status, and therefore making it a continuous variable. Given that there were several individuals in the alcohol group who also had some recreational drug use, additional analysis was performed by splitting the subjects into three distinct groups: non-alcoholic, alcoholic with history of recreational drug use, and alcoholic without history of recreational drug use.

Power and sample size considerations

The primary outcome of this research project is intracellular zinc levels intracellular zinc levels in alveolar macrophages. Unfortunately, this is not a routinely measured variable in the clinical setting and there are no specific data on the range of measurements for human beings, much less on the expected differences in value between alcoholic and non-alcoholic individuals. However, similar studies have been performed in experimental animal models and show that there is about a 30% difference in zinc levels between alcohol-fed and control-fed rats (15). Given the paucity of human

research, these animal data were extrapolated in order to calculate sample size considerations for a similar finding in humans. Standard deviation values are also extrapolated from studies done in animal models since no human data exists prior to this study, however a more conservative estimate was used since it is likely that there is greater variability among human subjects. Table 1 summarizes the statistical power to detect a 20%, 30%, and 40% difference in zinc levels using a sample size of 15 patients. Based on these calculations, a sample size of 15 subjects in each group will have greater than 80% power to detect a 30% difference in intracellular zinc levels between alcoholics and non-alcoholics.

Analytic plan

The analysis for this research was performed using SAS 9.3 (46). Descriptive statistics were carried out on all demographic variables collected and presented by group (i.e., alcoholic, non-alcoholic). For all comparisons between alcoholics and non-alcoholics, linear regression was performed. For each of the outcome variables, two separate models were utilized. First, a simple univariate model using the presence of alcoholism as the only predictor of the outcome was executed to determine the effect of alcohol abuse without any adjustment. Next, a more comprehensive multivariate linear regression model was carried out using presence of alcoholism plus other covariates including age, race, BMI, and total number of cigarettes smoked in the previous 30 days.

RESULTS

Subject enrollment

A total of 375 subjects were screened for enrollment into the study; 175 with an alcohol use disorder and 200 without any history of alcohol abuse as shown in Figure 2. Among the screened subjects with an alcohol use disorder, 112 met at least one of the exclusion criteria and 46 declined to participate in the study. Among the non-alcoholics who were screened, 118 met at least one of the exclusion criteria, 10 declined to participate, and 55 did not have a match among the alcoholic subjects. Overall, 17 subjects with an active AUD and 17 matched non-alcoholics subjects were enrolled and underwent bronchoscopy.

Subject characteristics

Alcoholic and non-alcoholic subjects were similar for gender and race (both groups were all male and 88% were Black/African-American), average age (45.2 years v 45.8 years), and body mass index (28.0 v 28.6) as shown in Table 2. Eighty-eight percent of both groups smoked cigarettes; however, those in the alcoholic group smoked more heavily (median: 360 v 150 cigarettes in the previous 30 days). Illegal drug use was reported by 7 (44%) of the alcoholic subjects but not by any of the control subjects. Per the inclusion criteria, all alcoholic subjects had a current alcohol use disorder and were actively drinking at the time of enrollment (mean SMAST score (se): 6.9 (3.4)), whereas the control subjects were either non-drinkers or drank socially but did not meet criteria for an AUD (mean SMAST score (se): 0.9 (0.7)). The median time since the most recent alcohol drink was one day in the alcoholic subjects and 14 days in the control subjects.

Serum zinc levels

There were no appreciable differences in serum zinc levels between the alcoholic subjects and the matched non-alcoholic subjects as shown in Figure 3. Since serum zinc measurements fell within the normal range for all of the subjects, no further linear regression analysis was performed to evaluate for the influence of covariates. However, the relationship between serum and intracellular zinc measurements was performed and shown in Figure 5. There was no significant association between serum zinc and alveolar macrophage intracellular zinc levels either overall or among the two different groups (Spearman's $r_{overall}$=-0.13; $r_{alcoholic}$=0.22, p=0.43 v $r_{non\text{-}alcoholic}$ =-0.18, p=0.48).

Intracellular zinc levels

Alveolar macrophages isolated from alcoholic subjects had lower intracellular zinc levels compared to alveolar macrophages isolated from their matched non-alcoholic subjects (adjusted mean difference (se): -277.8 (121.7) RFU/cell; p=0.031). Table 3 illustrates the values for alveolar macrophage intracellular zinc with respect to both univariate and multivariate linear regression analysis including adjustment for covariates. As shown, the only significant predictor of intracellular zinc is alcoholism, which results in approximately 30% decrease in the zinc level. These results also are graphically represented as box plots in Figure 4, which show the range distribution of values for the two groups.

Immune function

Both phagocytosis and GM-CSF receptor expression are measures of immune function. Phagocytosis is a measurement that represents the ability of the alveolar

macrophage to ingest bacteria. In this study, the bacterial phagocytic function of alveolar macrophages was lower in alcoholics as compared to non-alcoholics. The findings from the univariate and multivariate analysis are displayed in Table 4, which reveals that alveolar macrophages isolated from alcoholic subjects had a phagocytic capacity that was more than 30% less than that of their non-alcoholic counterparts (adjusted mean difference (se): -523.9 (119.6) RFU/cell; $p=0.0002$). The only statistically significant predictor of phagocytosis in this model was the presence of alcoholism. The distribution of phagocytosis values for the two groups is also represented graphically as a box plot in Figure 6.

GM-CSF receptor expression is another measure of immune function. GM-CSF is a peptide that is crucial for macrophage maturation and function. Table 5 illustrates the univariate and multivariate regression model for GM-CSF receptor expression. In parallel to the findings for phagocytosis, GM-CSF receptor expression is about 30% lower in alveolar macrophages isolated from alcoholic individuals compared to non-alcoholics (adjusted mean difference (se): -632.5 (79.5) RFU/cell; $p<0.0001$). The distribution of GM-CSF receptor expression values is also graphically illustrated as a box plot in Figure 7.

A linear regression was also performed between the measures of immune function. As they are both different measurements of a similar outcome, the expectation would that there is a high degree of correlation between the two values. The results from this analysis are shown in Figure 8, which illustrates that there is a very strong positive relationship between alveolar macrophage phagocytosis and GM-CSF receptor expression (Spearman's $r=0.84$, $p<0.0001$).

Relationship between intracellular zinc and immune function

One of the important goals of this research is to assess the relationship between zinc status and immune function. The causal diagram in Figure 1 proposes that alcohol abuse causes zinc deficiency, which in turn causes immune dysfunction. In this study, alcoholism is associated with both decreased alveolar macrophage intracellular zinc levels and immune function (i.e., phagocytosis and GM-CSF receptor expression). The association between intracellular zinc and immune function is assessed using two different linear regressions, one between intracellular zinc and phagocytosis and one between intracellular zinc and GM-CSF receptor expression. The results are shown in Figure 9 and demonstrate a positive relationship between intracellular zinc and immune function (Spearman's $r_{phagocytosis}=0.68$, $p<0.0001$; $r_{GM\text{-}CSF\ receptor}=0.67$, $p<0.0001$).

Impact of drug abuse

There were a total of 7 alcoholic subjects who used recreational drugs, but none of the non-alcoholics reported any history of drug use. In order to assess for a possible effect of drug use, linear regression analyses were performed on the outcome variables after dividing subjects into three separate groups: non-alcoholics, alcoholics with no history of drug use, and alcoholics with a positive history of drug use. These results are summarized in Table 6, and show that there is no significant effect from drug use. Alcoholics continued to demonstrate lower intracellular zinc levels and immune function, but there were no significant differences in the outcomes between those alcoholics who had a history of drug use and those that did not have a history of drug use.

DISCUSSION

This study determined that alveolar macrophages from otherwise healthy alcoholic subjects have significant intracellular zinc deficiency even in the presence of normal serum zinc levels. Further, it confirmed that features of the alcoholic lung phenotype identified in the animal model, namely alveolar macrophage immune dysfunction and decreased GM-CSF receptor expression, also occur in human subjects. Taken together, these results provide new evidence that chronic alcohol abuse, even in the absence of clinically apparent zinc deficiency or end-organ damage, causes significant zinc depletion and immune dysfunction within the alveolar space. Therefore, this study extends our basic understanding of the association between alcoholism and pulmonary outcomes such as pneumonia (47-49) and provides novel evidence that increasing zinc bioavailability within the alveolar space with dietary zinc supplements may be able to mitigate these pathophysiological consequences.

Zinc deficiency has been established in alcoholic liver disease for over 50 years (29, 50). These individuals have significantly lower serum zinc levels compared to non-alcoholic subjects, and both human studies and animal models of chronic alcohol ingestion demonstrate the presence of zinc deficiency in liver cells (51-53). However, much less is known about zinc balance in alcoholics without liver disease, and the pulmonary zinc status in these individuals has never been examined. This is an important area for research since zinc therapy remains absent from clinical practice guidelines involving the management of alcohol-related disorders including alcohol withdrawal (54). In this study, alcoholic subjects all had normal blood zinc measurements despite having significantly lower alveolar macrophage intracellular zinc levels compared to non-

alcoholics. Further, there was no appreciable relationship between serum zinc levels and intracellular zinc levels in any of the subjects in this study. These findings suggest that serum measurements are unreliable surrogate markers for zinc metabolism at the organ level. Specifically, alveolar macrophage intracellular zinc was about 30% lower in alcoholic subjects compared to matched non-alcoholics in this study. While the clinical relevance of this finding requires additional investigation, this degree of intracellular zinc depletion has been shown to cause significant cellular and clinical derangements in other studies (55, 56). Moreover, these clinical findings are remarkably consistent with published findings in an animal model of chronic alcohol ingestion in which there is a similar 30% decrease in lung zinc levels, and where correction of this deficiency with dietary supplements reversed immune dysfunction in the alveolar macrophage (15).

The most devastating pulmonary manifestations of alcoholism are the increased susceptibility to pneumonia and lung injury. Animal models have been instrumental in characterizing what has been termed the 'alcoholic lung phenotype', which includes increased oxidative stress in the lower airways, immune dysfunction of the alveolar macrophage, and disruption of alveolar epithelial barrier function (7). Human studies are limited, but have confirmed the presence of oxidative stress in the alveolar space (57) and revealed alteration of gene expression in alveolar macrophages isolated from alcoholics (58). While these studies add to our understanding of the alcohol lung phenotype, there remains a clear knowledge gap between experimental animal models and research in human alcoholism. In this study, isolated macrophages from human alcoholics have significantly decreased phagocytic capacity compared to matched controls. This finding is parallel to observations in the animal model and confirms the presence of alcohol-

induced immune dysfunction in the human lung (7). Previous research has demonstrated that decreased GM-CSF signaling secondary to alcohol exposure is at least partly responsible alveolar macrophage immune impairment in the rat lung (14, 59). This study evaluated the role of the GM-CSF pathway in the human lung, and found that subjects with an AUD exhibit significantly decreased alveolar macrophage GM-CSF receptor β-subunit expression compared to matched non-alcoholic subjects. This is similar to findings in the animal model in which there was decreased expression of the signaling component of the GM-CSF receptor. Therefore, these data suggest that dampened signaling via the GM-CSF pathway mediates at least some of the macrophage immune dysfunction in humans with alcohol use disorders. Further, in this study there was a strong positive relationship between GM-CSF receptor expression and alveolar macrophage phagocytosis, suggesting that these are two comparable measures of immune function and may used interchangeably.

There are no currently available treatments to mitigate the adverse effects of chronic alcohol use on the lung. Animal models have shown that dietary zinc supplementation may have a therapeutic role, but to date no human trials have been done (15). The use of this modality is of particular interest as zinc supplements are readily available and inexpensive. In this study, alveolar macrophage zinc levels are significantly decreased in alcoholic subjects compared to non-alcoholic subjects, and there is a positive relationship between intracellular zinc levels and immune function as measured by both phagocytosis and GM-CSF receptor expression. While this association is not direct evidence that correction of this zinc deficiency will result in an improvement in immune function, evidence from the animal model has demonstrated that this does

indeed occur (15). In addition, the effect of alcoholism in causing zinc deficiency appears to stand firm even when adjustments are made for possible confounders such as cigarette smoking and recreational drug use. These data provide sufficient evidence that alcoholism creates a state of zinc deficiency in the lung and argues for further investigation of the use of dietary zinc supplements in this vulnerable population.

While this study provides novel evidence for the role of pulmonary zinc deficiency in alcoholism, it does have some important limitations. First, this is a relatively small, single-center study, making it difficult to control for many potential confounding factors simultaneously. Due to the nature of the veteran population at this center, all enrolled subjects were male and most were African American, which limits generalizability to the overall population of alcoholics. Despite these limitations, a matched population of non-alcoholics was utilized in order to control for confounding. In particular, smoking is a known confounder in most studies that involve lung disease and control subjects were matched on smoking status as the majority of individuals with an alcohol use disorder also smoke. In fact, alcoholic subjects were heavier smokers in general, and the modeling strategies used in the analysis adjusted for the number of cigarettes smoked in the previous 30 days. Second, the clinical importance of measuring intracellular zinc levels is not known and such testing is not commercially available for individuals seen by health care providers. Currently, the evaluation of pulmonary zinc status is on a research basis only and requires a relatively invasive technique (i.e., bronchoscopy) to perform. Therefore, initial studies to test the efficacy of these dietary supplements in alcoholic human subjects will have to be relatively small in scope. Finally, the findings from this study are still observational in nature. While there appears

to be strong evidence for the presence of zinc deficiency in the setting of alcohol use in animal models and now human studies, the true utility of correcting these abnormalities remains to be determined.

In summary, this research furthers our understanding of the effects of alcoholism on the human lung. Specifically, this study demonstrates that intracellular zinc levels are significantly decreased in alveolar macrophages from alcoholic subjects compared to non-alcoholics even when serum zinc levels are normal. In parallel, alveolar macrophages from alcoholic subjects have impaired immune function as characterized by decreased GM-CSF receptor expression and decreased phagocytic capacity. Further, there is a positive relationship between intracellular zinc levels and immune function, providing provocative evidence that suggests it is not the alcohol *per se*, but rather the alcohol-induced zinc deficiency that impairs their host immune function. While many factors contribute to the alcoholic lung phenotype, experimental and clinical evidence now implicates decreased zinc bioavailability within the alveolar space as fundamental mechanisms by which alcohol impairs host immunity. In fact, the findings from this study are the basis for a future clinical trial to evaluate the impact of dietary zinc supplementation on lung health in individuals with chronic alcohol use disorders.

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TABLES/GRAPHS

Table 1 – Power and sample size considerations

Sample Size	Intracellular Zinc Non-Alcoholics	Intracellular Zinc Alcoholics	Standard Deviation Non-Alcoholics	Standard Deviation Alcoholics	Alpha Level	Power *
15	100	60	30	30	0.05	97.8%
15	100	70	30	30	0.05	86.3%
15	100	80	30	30	0.05	57.2%

* Based on two sample t-test

Table 2 – Demographic characteristics of the study population

Characterisitics	Non-Alcoholic n=17 Mean (sd), Median [Q1,Q3], *or* n %	Alcoholic n=17 Mean (sd), Median [Q1,Q3], *or* n %
Demographics		
Male	17 100%	17 100%
African Am./Black	15 88%	15 88%
Age, years	45.2 (7.5)	45.8 (8.4)
BMI kg/m^2	28.0 (4.0)	28.6 (7.8)
Health Habits		
Drinking-AUDIT score[1]	2.2 (1.9)	20.3 (8.6)
Drinking-SMAST score[2]	0.9 (.7)	6.9 (3.4)
Days since last drink	14 [7, 60]	1 [1, 3]
Smokes cigarettes	15 88%	15 88%
If yes- #cig. in last 30 days	150 [40, 150]	360 [120, 600]
Illegal drug use	0 0%	7 44%

[1] AUDIT = Alcohol Use Disorders Identification Test
[2] SMAST = Short Michigan Alcohol Screening Test

Table 3 – Determinants of alveolar macrophage intracellular zinc levels

Univariate Linear Regression Model

Variable	Estimate (RFU)	Standard Error	t Value	p Value
Intercept (Non-alcoholics)	1017.74			
Alcohol	-295.59	105.96	-2.79	0.0089

RFU = Relative Fluorescence Units per cell (measure of intracellular zinc status)
Alcohol = 0: Non-alcoholic, 1: Alcoholic

Multivariate Linear Regression Model

Variable	Estimate (RFU)	Standard Error	t Value	p Value
Age	4.05	7.89	0.51	0.6119
Race	-30.90	206.85	-0.15	0.8824
Alcohol	-277.79	121.66	-2.28	0.0305
Cigarettes	-0.18	0.32	-0.57	0.5731
BMI	4.17	9.70	0.43	0.6707

RFU = Relative Fluorescence Units per cell (measure of intracellular zinc status)
Age = in years
Race = 1: Caucasian, 2: African American
Alcohol = 0: Non-alcoholic, 1: Alcoholic
Cigarettes = Total cigarettes smoked in the last month
BMI = measured as kg/m^2

Table 4 – Determinants of alveolar macrophage phagocytosis

Univariate Linear Regression Model

Variable	Estimate (RFU)	Standard Error	t Value	p Value
Intercept (Non-alcoholics)	1569.30			
Alcohol	-550.72	113.09	-4.87	<.0001

RFU = Relative Fluorescence Units per cell (measure of phagocytic index)
Alcohol = 0: Non-alcoholic, 1: Alcoholic

Multivariate Linear Regression Model

Variable	Estimate (RFU)	Standard Error	t Value	p Value
Age	-3.36	7.57	-0.44	0.6612
Race	-140.14	234.78	-0.60	0.5562
Alcohol	-523.91	119.64	-4.38	0.0002
Cigarettes	-0.48	0.33	-1.45	0.1605
BMI	18.99	9.55	1.99	0.0584

RFU = Relative Fluorescence Units per cell (measure of phagocytic index)
Age = in years
Race = 1: Caucasian, 2: African American
Alcohol = 0: Non-alcoholic, 1: Alcoholic
Cigarettes = Total cigarettes smoked in last month
BMI = measured as kg/m^2

Table 5 – Determinants of alveolar macrophage GM-CSF receptor expression

Univariate Linear Regression Model

Variable	Estimate (RFU)	Standard Error	t Value	p Value
Intercept (Non-alcoholics)	2135.02			
Alcohol	-653.53	74.43	-8.78	<.0001

RFU = Relative Fluorescence Units per cell (measure of GM-CSF receptor expression)
Alcohol = 0: Non-alcoholic, 1: Alcoholic

Multivariate Linear Regression Model

Variable	Estimate (RFU)	Standard Error	t Value	p Value
Age	-1.00	5.15	-0.19	0.8479
Race	-87.80	135.17	-0.65	0.5215
Alcohol	-632.45	79.50	-7.96	<.0001
Cigarettes	-0.19	0.21	-0.93	0.3626
BMI	11.90	6.34	1.88	0.0713

RFU = Relative Fluorescence Units per cell (measure of GM-CSF receptor expression)
Age = in years
Race = 1: Caucasian, 2: African American
Alcohol = 0: Non-alcoholic, 1: Alcoholic
Cigarettes = Total cigarettes smoked in last month
BMI = measured as kg/m^2

Table 6 – Impact of drug use on intracellular zinc and immune function

Effect of drug use on alveolar macrophage intracellular zinc

Variable	Estimate (RFU)	Standard Error	t Value	p Value
Intercept (Non-alcoholics)	1017.74			
Alcoholic, no drug use	-278.63	127.34	-2.19	0.0366
Alcoholic, drug use	-317.39	138.73	-2.29	0.0294

RFU = Relative Fluorescence Units per cell (measure of intracellular zinc status)

Effect of drug use on alveolar macrophage phagocytic index

Variable	Estimate (RFU)	Standard Error	t Value	p Value
Intercept (Non-alcoholics)	1569.30			
Alcoholic, no drug use	-548.64	134.01	-4.09	0.0003
Alcoholic, drug use	-554.04	159.02	-3.48	0.0017

RFU = Relative Fluorescence Units per cell (measure of phagocytic index)

Effect of drug use on macrophage GM-CSF receptor expression

Variable	Estimate (RFU)	Standard Error	t Value	p Value
Intercept (Non-alcoholics)	2135.02			
Alcoholic, no drug use	-615.52	88.60	-6.95	<.0001
Alcoholic, drug use	-702.40	96.52	-7.28	<.0001

RFU = Relative Fluorescence Units per cell (measure of GM-CSF receptor expression)

Figure 1 – Proposed causal diagram for the effect of alcohol abuse on the lung

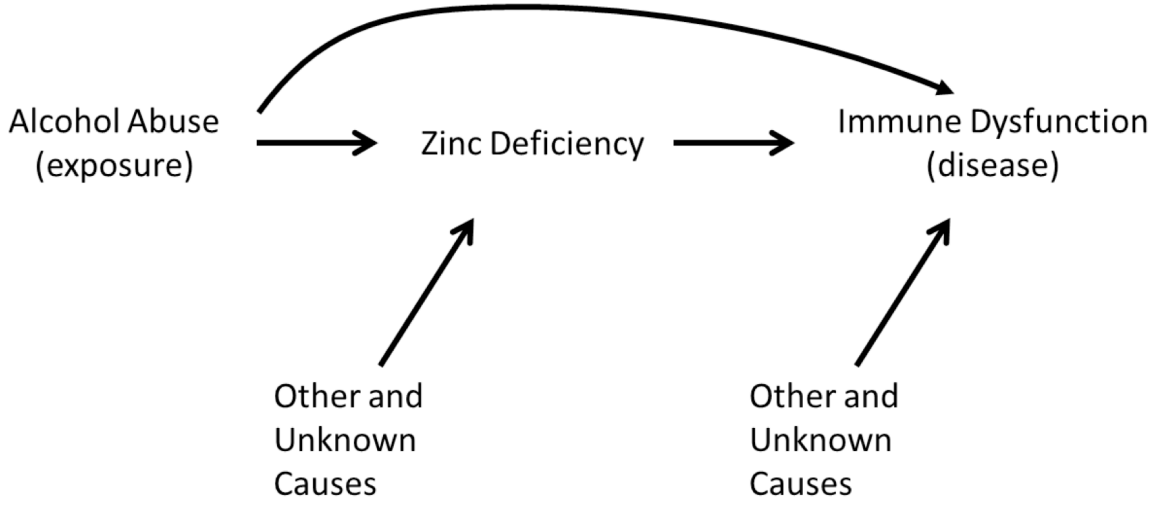

Figure 2 – Recruitment of study subjects

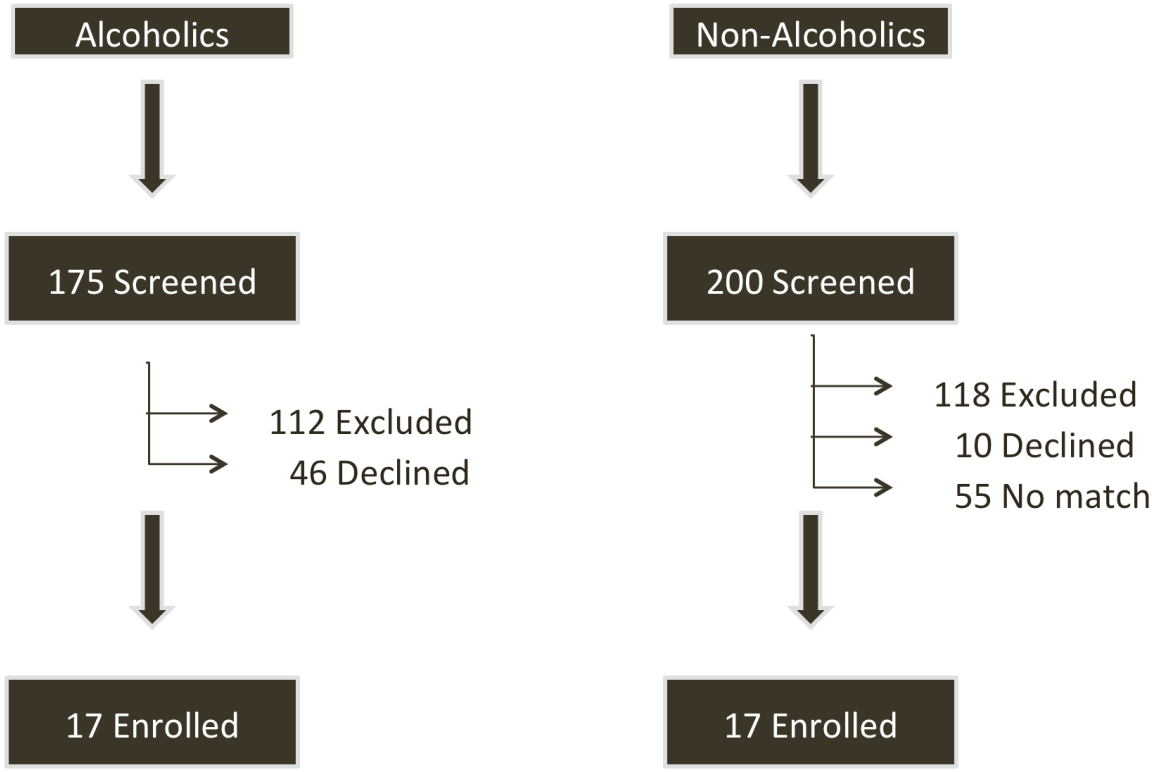

Figure 3 – Distribution of serum zinc measurements

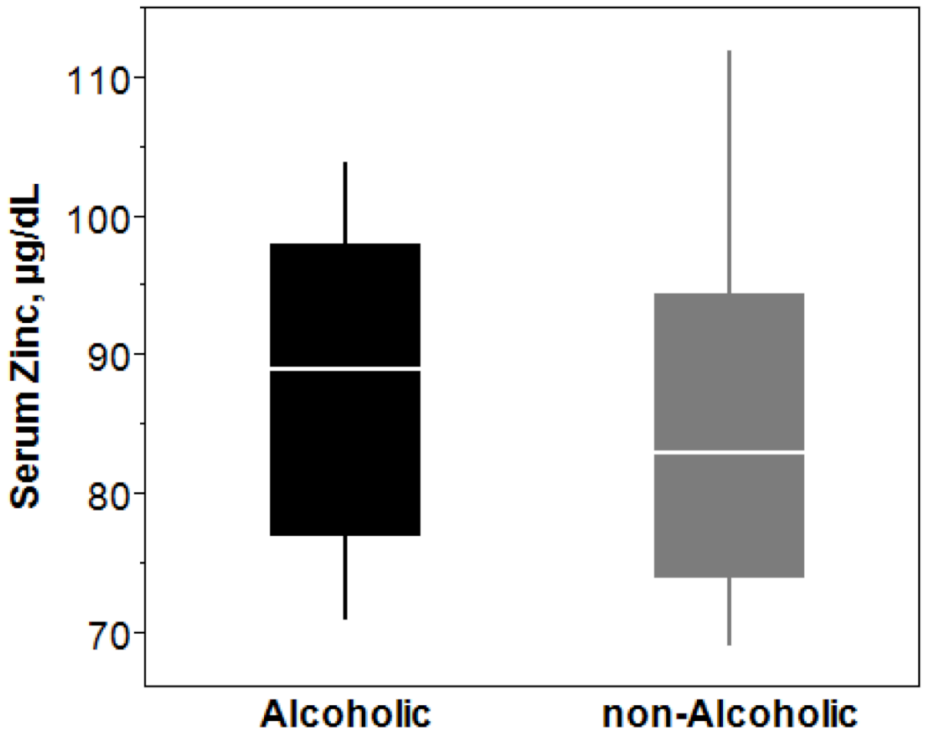

Figure 4 – Distribution of alveolar macrophage intracellular zinc levels

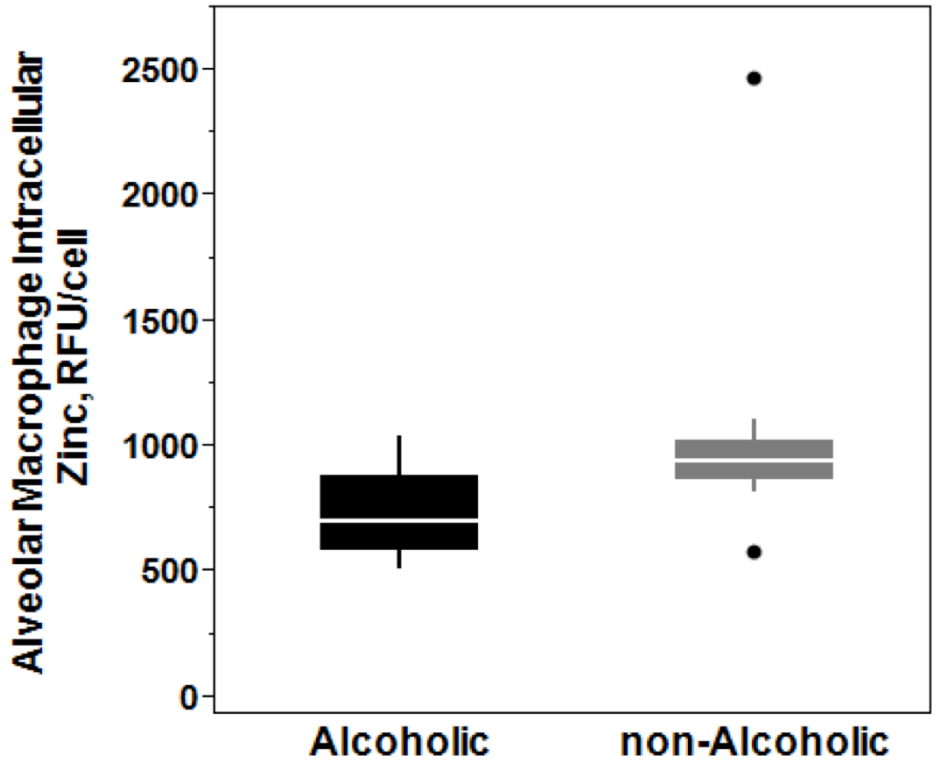

Figure 5 – Relationship between serum zinc and intracellular zinc levels

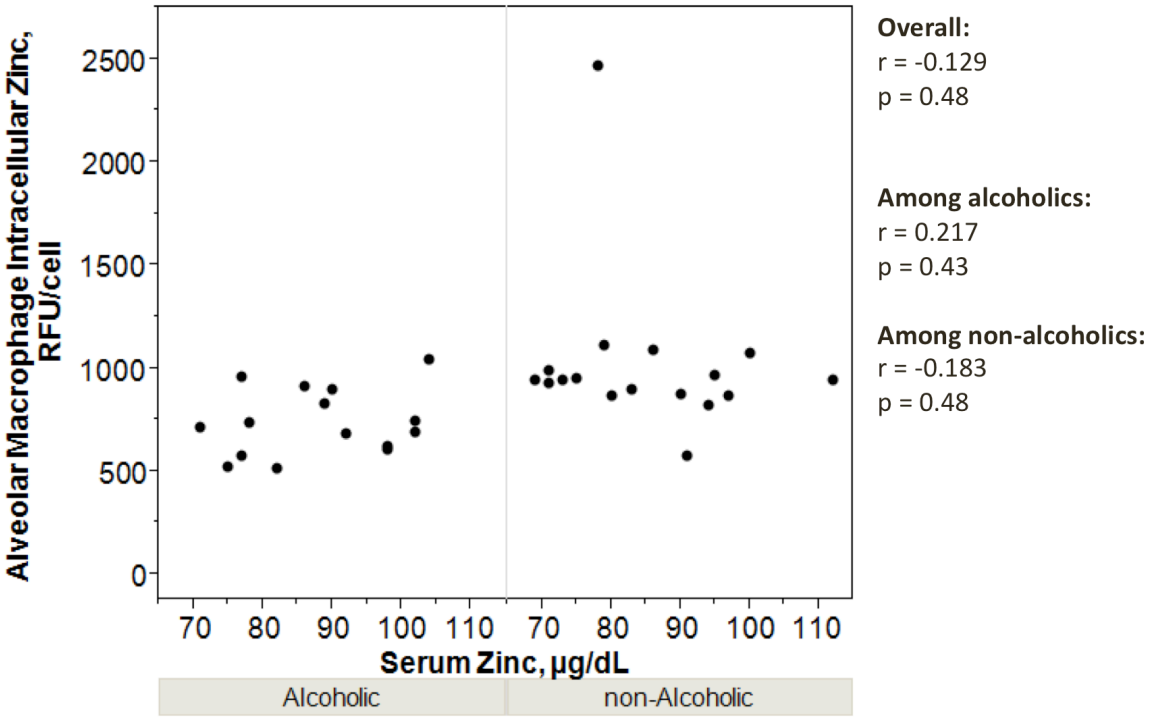

Figure 6 – Distribution of alveolar macrophage phagocytosis

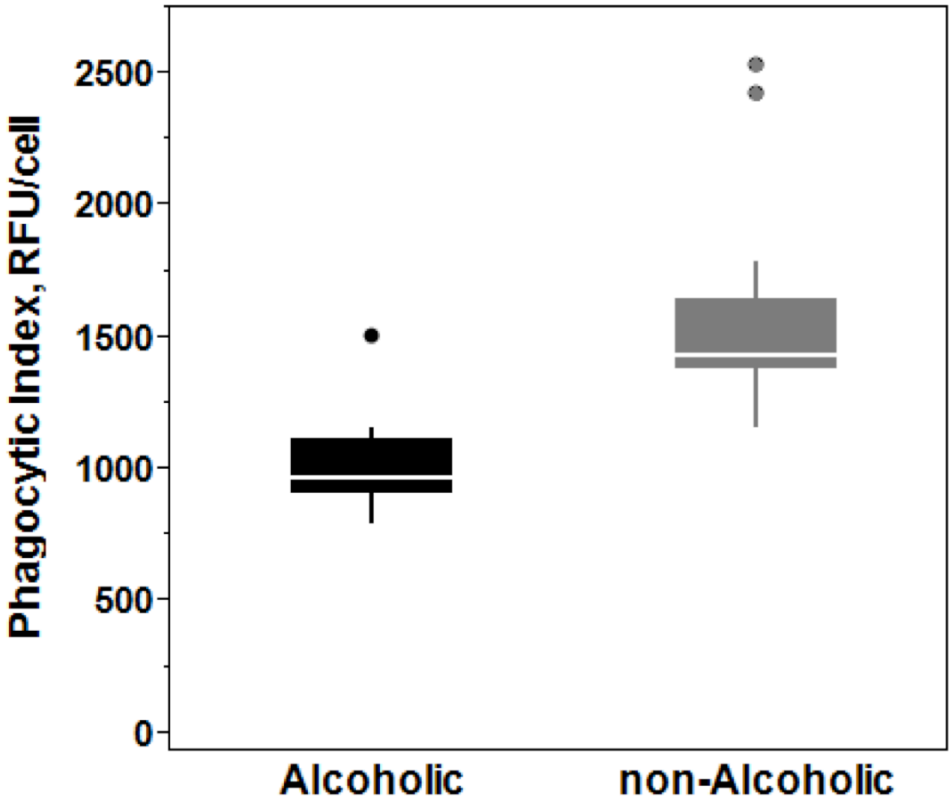

Figure 7 – Distribution of alveolar macrophage GM-CSF receptor expression

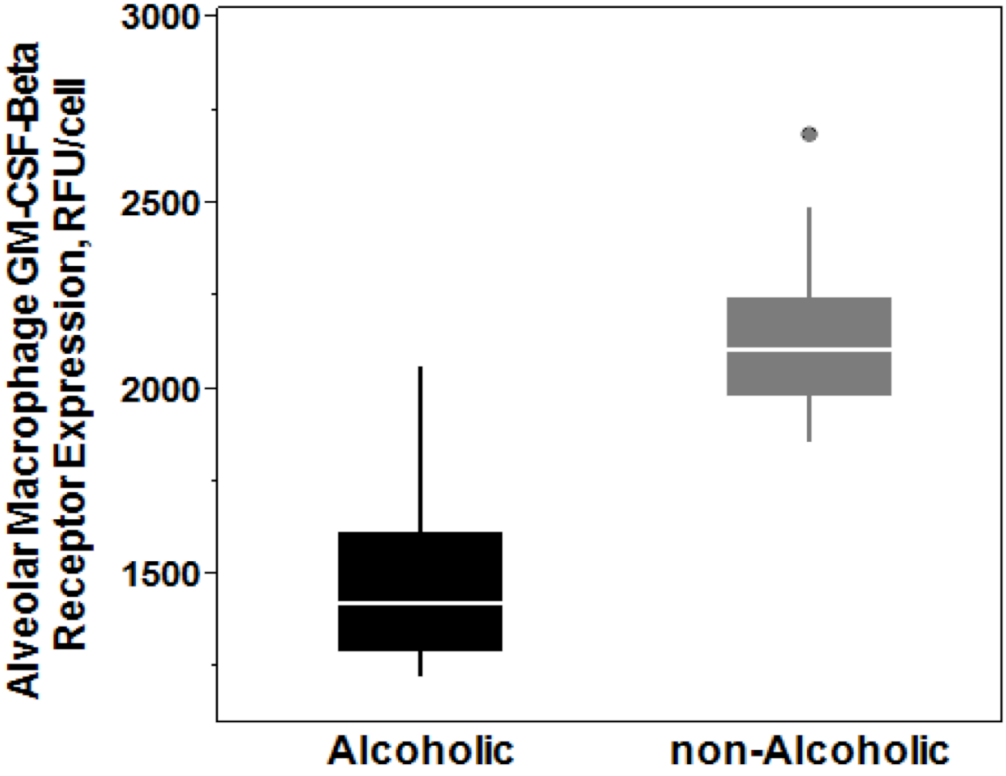

Figure 8 – Relationship between phagocytosis and GM-CSF receptor expression

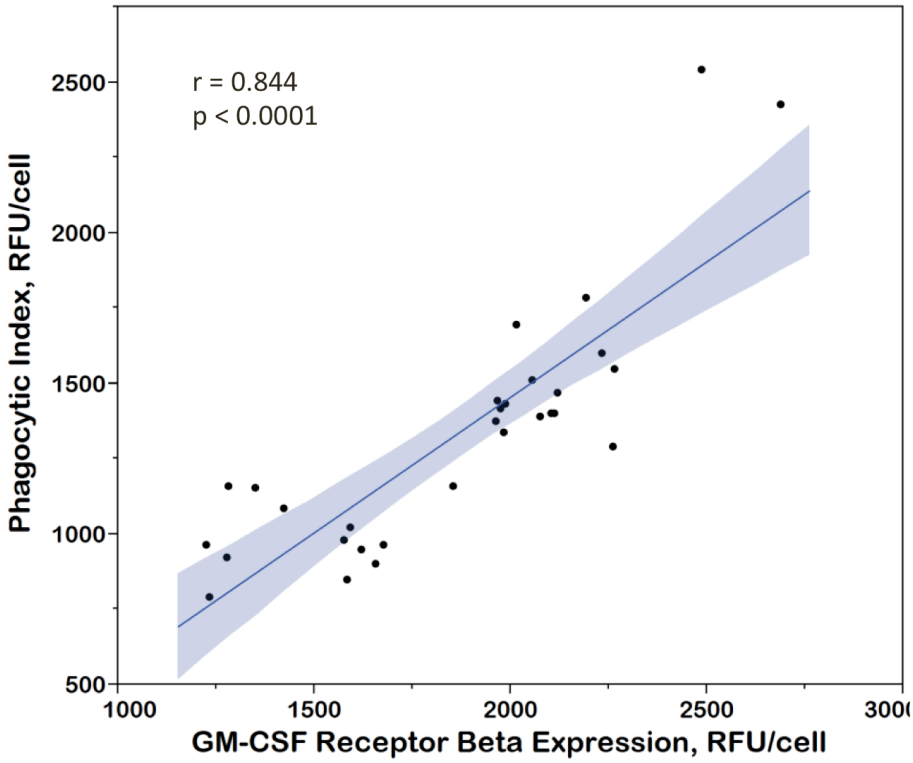

Figure 9 – Relationship between intracellular zinc levels and immune function