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## Detection of silica particles in lung tissue of non-occupationally exposed individuals by computer controlled scanning electron microscopy

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### Recommended Citation

Coleman, Kristen. "Detection of silica particles in lung tissue of non-occupationally exposed individuals by computer controlled scanning electron microscopy." MS (Master of Science) thesis, University of Iowa, 2013.

<https://doi.org/10.17077/etd.9nt9ofsi>

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DETECTION OF SILICA PARTICLES IN LUNG TISSUE OF NON-  
OCCUPATIONALLY EXPOSED INDIVIDUALS BY COMPUTER CONTROLLED  
SCANNING ELECTRON MICROSCOPY

by

Kristen Lindsey Coleman

A thesis submitted in partial fulfillment  
of the requirements for the  
Master of Science degree in Occupational and Environmental Health  
in the Graduate College of  
The University of Iowa

December 2013

Thesis Supervisors: Professor R. William Field  
Professor James A. Merchant

Graduate College  
The University of Iowa  
Iowa City, Iowa

CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

Kristen Lindsey Coleman

has been approved by the Examining Committee for the thesis requirement for the Master of Science degree in Occupational and Environmental Health at the December 2013 graduation.

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## **ACKNOWLEDGMENTS**

In addition to my advisor and mentor, Professor R. William Field, I thank the other members of my thesis committee, Professor James A. Merchant and Professor Charles F. Lynch for their assistance and also for their insight. I also would like to thank Professor Charles Platz for his contributions to the development of this study, as well as his expertise which made this study possible. I thank Freda Selk of the Iowa Cancer Registry for her assistance with the handling and selection of the blocks used for this study. Furthermore, this research would not have been completed without the funding support provided by The University of Iowa Center for Health Effects of Environmental Contamination as well as the fellowship support granted to me by the Occupational Epidemiology Training Program of the Heartland Center for Occupational Health and Safety.

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## INTRODUCTION

The hazard of silica exposure to human health is not just a recent scientific concern, but rather one that has persisted for hundreds of years (Harber et al. 1996). However, despite the widely recognized lung toxicity of silica, a complete working mechanism of injury has yet to be elucidated. Occupational exposures to silica, in high concentrations, have long been the primary focus of research in the field. Despite this fact, research performed in the past few years (Fassina et al 2009) has suggested that non-occupational exposure to silica, at lower concentrations, may also cause lung toxicity. Since the 1997 International Agency for Research on Cancer (IARC) classification of crystalline silica as a class 1 human carcinogen, several potential sources of environmental silica exposure have been identified. This has resulted in concerns about silica exposure from agricultural activities like tilling (IARC, 2012) and land modification practices such as hydraulic fracturing, which have the potential to affect a much broader subset of the population (Field and Withers, 2012).

The scope of the current scientific literature involving silica toxicity spans from large cohorts of occupationally exposed populations to small *in vitro* laboratory experiments looking at cellular damage (NTP, 2011). However, the advent of innovative high resolution microscopy expands the opportunities for silica-related health research. For example, one of the primary obstacles for performing large population-based epidemiologic studies has been poor silica exposure assessment methods that frequently rely on self-reported information or limited personal sampling to approximate exposure. The utilization of this microscopy technology may help fill an important void in developing and validating other less sophisticated methods to assess silica exposure.

The long term objectives of this research are to: 1) identify whether silica plays a role in the development of lung cancer and other pulmonary diseases at environmental exposure levels or at silica concentrations associated with some occupations previously thought to be at lower risk, such as farming (NIOSH, 2002); 2) identify the general

sources of silica found in lung tissues; 3) relate the occurrence of silica in lung tissues to cellular histology; 4) assess the agreement between modeled retrospective exposures and lung silica content; and 5) enhance future *in vitro* research by allowing direct observations of the co-occurrence of silica particles and cellular structures in the lung by performing an overlay of a H&E stained histological slide section imaged with a light microscope onto a scanning electron microscope image from the adjacent ultrathin microtomed lung section. The overall objectives of the research presented in this thesis are to determine whether silica particles are present in lung tissues of non-occupationally-exposed individuals with lung cancer and to provide preliminary observations regarding factors that may affect the burden and type of silica detected in the lung. The objectives include a preliminary investigation into whether silica may be deposited in the lung as a result of smoking cigarettes and whether silica deposition may preferentially play a role in the development of morphologic types of lung cancer. Furthermore, observations are made in regard to silica clustering in lung tissue and its association with other particles in the lung tissue with hope that this information may help to inform future research efforts addressing the underlying mechanisms of silica toxicity.

The Iowa Radon Lung Cancer Study (IRLCS) serves as an excellent resource for lung tissue samples as well as provides access to a robust dataset that can be used to retrospectively assess factors that may be associated with silica exposure. Due to the extremely large population at risk from low level exposure to silica, even small increases in adverse health outcomes related to silica deposition in the lung can produce large impacts on public health. Therefore, it is of paramount importance to determine if there should be concern about non-occupational silica exposure throughout the population.

These objectives are further clarified in the exploration of the following hypotheses: 1) silica particles will be present in lung samples, which contain tumor tissue, from patients without a known history of occupational exposure to silica; 2) smokers will have more silica particles deposited within their lung tissue than non-

smokers due to the potential for exposure to silica through tobacco smoke; 3) squamous cell carcinoma tissue will contain more silica particles than adenocarcinoma tissue due to its strong association with smoking, and also to the synergistic inflammatory effect seen when smokers are exposed to silica (Brown, 2009); and 4) the silica particles will be deposited in clusters within the lung tissue, indicating the need for further inquiry to determine if their clustering is associated with scar tissue, specific cellular structures, or the tumor edge.

The long-term benefits and applications of this study are still unclear due to the early phase of the research, however it does serve to highlight the previously undocumented potential for a measureable silica lung burden in individuals not thought to be exposed to silica. This finding raises the concern of the potential for adverse health-related events even in individuals not thought to be at risk from silica exposure. It also may indicate the need for environmental monitoring of silica exposures to protect the population from this non-occupational hazard. This research is particularly applicable in light of current land manipulation practices which include farming, quarrying, and hydraulic fracturing, all of which have the potential to affect the entire population as a whole. It is hoped that this research may help to inspire further inquiry into the inflammatory response of the lung to silica exposure and its role in the development of lung cancer, and to also offer insight into the mechanism of tobacco smoke-related toxicity including the potential contributions from silica. This insight may eventually help lead to a decrease in the overall toxicity of cigarette smoke through efforts to reduce silica contamination of tobacco. In pursuing these challenging goals, the above-mentioned hypotheses will be explored further in the specific aims that follow.

## **SPECIFIC AIMS**

### **Specific Aim I**

Determine if silica is present in lung tissue from patients without occupational exposure.

#### **Sub Aim 1**

Determine how many particles are present per  $\text{mm}^2$  of scanned lung tissue.

#### **Sub Aim 2**

Determine what percentage of the particles deposited in the lung tissue are free silica, soil-bound silica and other particles.

#### **Sub Aim 3**

Determine what the size distribution of the particles deposited in the lung tissue is.

### **Specific Aim II**

Determine if cancer type affects the particle deposition within the lung.

#### **Sub Aim 1**

Determine if the number of particles deposited in the lung vary in adenocarcinoma, squamous cell carcinoma, and carcinoid lung tissue.

#### **Sub Aim 2**

Determine if the mean size of the deposited particles vary between adenocarcinoma, squamous cell carcinoma and carcinoid lung tissue.

### **Specific Aim III**

Determine if smoking history affects the particle deposition within the lung tissue.

#### **Sub Aim 1**

Determine if the number of particles deposited in the lung tissue vary between smokers and never smokers.

**Sub Aim 2**

Determine if the number of particles deposited in the lung tissue vary in the same manner according to pack-years.

**Sub Aim 3**

Determine if the mean size of particles deposited in the lung tissue vary between smokers and never smokers.

**Sub Aim 4**

Determine if the mean size of particles deposited in the lung tissue vary over pack-years.

**Specific Aim IV**

Determine how silica particles are dispersed throughout the lung tissue.

**Sub Aim 1**

Determine if the free silica is randomly dispersed throughout the tissue.

**Sub Aim 2**

Determine if the soil-bound silica is randomly dispersed throughout the tissue.

**Sub Aim 3**

Determine if the deposition of silica particles within the lung is associated with the deposition of other particles within the lung.

## **BACKGROUND AND SIGNIFICANCE**

The inherent hazards of silica to human health have been studied and documented over hundreds of years (Harber et al, 1996). Silicosis, which is one of the world's oldest known occupational diseases, was written about as long ago as the ancient Greeks, with Hippocrates noting the association between silica dust and disease (Goldsmith et al, 1986). Silicosis, even to this day, remains a disease of concern for workers in some occupations, including hard rock mining, tunnel drilling, granite quarrying and carving, foundry work, sandblasting, silica flour production, diatomaceous earth mining and milling, glass making, and farming (Rees and Murray, 2007). The disease itself is described as a chronic diffuse interstitial fibronodular lung disease resulting from prolonged inhalation of crystalline silica and leading to protracted disability (Banks and Parker, 1998).

In 1997, IARC classified crystalline silica as a class 1 human carcinogen based on what was deemed sufficient evidence displayed by studies in humans that indicated a causal relationship between silica exposures and increased lung cancer rates in the workplace (NTP, 2011). This classification broadened the potential population affected by silica exposure from the relatively few afflicted with silicosis to the much larger number of people suffering from lung cancer. IARC has classified only the crystalline form of silica as a human carcinogen, but recent research on the toxicity of amorphous silica indicates that the other silicate forms may also present an increased health risk (Ghiazza et al, 2010). The generally reported relative risk attributed to the contribution of crystalline silica to the development of lung cancer is 1.3 to 1.5 from protracted exposure (NTP, 2011).

As a result of the National Occupational Exposure Survey and the National Occupational Hazard Survey, the National Institute of Occupational Safety and Health (NIOSH) estimated that over one million workers per year are potentially exposed to crystalline silica, prompting the release of exposure guidelines (NIOSH, 2002). The

current NIOSH guidelines include a recommended exposure limit (REL) of  $0.05 \text{ mg/m}^3$  and an immediately dangerous to life and health (IDLH) of  $25 \text{ mg/m}^3$  (NIOSH, 2002). The Occupational Safety and Health Administration (OSHA) and the American Conference of Governmental Industrial Hygienists (ACGIH) followed suit releasing their own guidelines of permissible exposure limit (PEL) of  $10 \text{ mg/m}^3$  and threshold limit value-time weighted average limit (TLV-TWA) of  $0.025 \text{ mg/m}^3$  respectively (NTP, 2011).

However, these guidelines and regulations only apply to indoor air quality leaving many workers susceptible to potential unregulated exposure. Furthermore, new research has indicated a potential for substantial exposures of silica within the agricultural sector, thus adding to the amount of workers potentially exposed to toxic silica through unregulated practices (Schenker et al, 2005). As a result of some of these findings, there have been limited attempts to protect the workforce from occupational exposures to crystalline silica with moderate success.

## LITERATURE REVIEW

### Introduction

Silica is a group IV metal oxide, known also as silicon dioxide, which exists in nature in both crystalline and amorphous forms (Goldsmith et al, 1986). It is the second most abundant element in the earth's crust, found to a large extent in many different rock types, the most common of which is quartz. Silica is also a major component of most sands and soils around the world (Brown, 2009).

Occupational silica exposure has been well documented, especially in mining, granite quarrying, crushed stone industries, foundries, ceramics industries, construction and sandblasting (Weill and Turner-Warwick, 1981). While proof of the existence of non-occupational exposures to crystalline silica in sizes and concentrations large enough to cause lung toxicity is still controversial, NIOSH has attributed potential exposures to crystalline silica to both anthropogenic and natural sources in non-occupational settings. NIOSH further indicates that people who live near quarries and sand and gravel operations may be at increased risk for both exposure and its resultant health effects. However, there are no current guidelines to determine how closely the residents must live to be considered at risk or for the quantification of their level of risk (Heyder, 2004). Further non-occupational exposure to crystalline silica through the smoking of cigarettes has been sporadically documented in research dating back to the 1970s with the discovery of silica-contaminated tobacco (Fleischer, 1974). In addition, with the advent of electrical cigarettes, which commonly use parts made from silica, a substantial potential for exposure has been demonstrated (Williams et al, 2013). The perpetuation of hydraulic fracturing also contributes to the overall potential for non-occupational exposure to the general population, making land-manipulation practices perhaps the most hazardous threats to human health on a grand scale (Phillips, 1972).



### **Smoking and Silica**

Cigarette smoking and silica have been tied together in the literature spanning decades after research demonstrated a synergistic perpetuation of health effects. In 1993, Nery and associates were able to concisely outline the mechanism of this synergism as an additive effect on the permeability of pulmonary epithelium. They further postulated that the congruence of inflammatory changes in the lung, by both the silica and the components of tobacco smoke, lead to more severe anatomical abnormalities than would ever be seen resulting from the effects of one of those agents alone. This paper helped to solidify that control of smoking is paramount to minimizing the adverse effects of silica-related injury in the workforce (Nery et al,1993).

However, this assessment of the relationship between silica and tobacco smoke may be too conservative. In the early 1970's, Martell was researching explanations for how atmospheric radioactivity could be found in the lungs of tobacco smokers. He postulated that the radioactive products in the atmosphere, primarily polonium-210, were attaching to soil particles, which in turn would become attached to the sticky trichomes of the tobacco plant. He observed that when the smoker inhaled the tobacco smoke that the soil particles would be deposited in the lung taking with them their radioactive passengers (Brown et al, 2002). Fleisher built upon these observations using scanning electron microscopy to demonstrate that both silica alone and silica as a component of feldspar from the soil could commonly be found on the trichomes of tobacco (Fleischer, 1974).

There does not appear to be any research to date that has explored the question of whether the silica contained on the trichomes of tobacco is a significant hazard to the individual in addition to the many other hazards introduced by tobacco smoking (Brown, 2009).

With the recent surge in popularity of electronic cigarettes, there is yet another source of potential silica exposure, which should be addressed in any scientific

investigations regarding silica and smoking. In March of 2013, Williams and associates analyzed the particulate material from the aerosol produced by electronic cigarettes in an attempt to provide preliminary analysis on the safety of electronic cigarettes compared to traditional tobacco cigarettes (Williams et al, 2013). The group analyzed the particulate utilizing scanning electron microscopy with energy dispersive x-ray spectroscopy (EDS) to determine the elemental makeup. They found 2.34 micrograms of silica particles were inhaled per ten puffs on the electronic cigarette with an average particle size small enough for deposition within the small airway. While the use of electronic cigarettes is still in its infancy, the potential health hazards as a result of silica exposure require further examination.

### **Mechanisms of Silica Toxicity**

Any discussion on silica and its associated health effects should attempt to reflect on the mechanism of silica toxicity in the lung. Banks and Parker attempted to summarize the pathogenesis of the health effects related to silica exposure in their collection of writings on occupational lung disease (Banks and Parker, 1998). The basics behind toxicity involve the inhalation of silica particles which then become embedded in the basement membrane of the alveoli of the lungs. Alveolar macrophages ingest these particles, taking them across the basement membrane and into the lung parenchyma where they begin to make their way to the lymphatic system for clearance. It is indicated that macrophages release cytokines that recruit inflammatory cells which produce reactive oxygen species that lead to lung damage and resultant fibrosis.

It was believed until quite recently that silica-induced macrophage apoptosis was exclusively a laboratory-associated phenomenon seen as the result of treating cell cultures with too high of a concentration of silica to be biologically plausible. As a result of this, it was believed that deposition of silica particles in the parenchyma of the lungs would happen rarely when not exposed to overwhelming amounts of silica, with lung

damage occurring primarily as a result of silica-induced chemical processes without any contribution from mechanical ones (Banks and Parker, 1998).

In 2010, Mara Ghiazza and associates began to change the paradigm, helping to highlight several potential new mechanisms behind the lung toxicity of silica (Ghiazza et al, 2010). The researchers reported, as a part of their investigation on the lung toxicity of amorphous silica, that in the presence of silica, macrophages would take up the silica, become activated and then the overwhelming majority would enter into early apoptosis. As a result, the non-degraded silica would be deposited directly into the parenchyma. In light of this finding, a new mechanism was proposed indicating that direct mechanical insult by the silica particle on the lysosome of the macrophage may play a role in the induction of apoptosis. However, even with the overwhelming evidence of apoptosis in both lab-based experiments and in the lung *in vivo*, it is still apparent that chemical inflammatory processes have a role in the induction of toxicity as well. Ghiazza et al. have been able to demonstrate that silica enhances the production of Nitric Oxide (NO) by macrophages. NO has been shown to contribute to the development of inflammatory lung disease. Furthermore, ingestion of silica by macrophages promotes the macrophages to release the cytokines IL-1b and TNF-a which recruit inflammatory cells (Ghiazza et al, 2010).

Research by Dostert et al. supports this information and indicates that the stimulation of macrophages with silica results in the secretion of IL-1b dependent on the Nalp 3 inflammasome (Dostert et al, 2008). The importance of the inflammatory mechanism of silica toxicity is further supported by Srivastava et al. who demonstrated that mice unable to produce IL-1b and TNF-a were more resistant to developing pulmonary pathology in the presence of silica than those who were able to mount the appropriate inflammatory response (Srivastava et al, 2002).

### **Population-Based Studies of Silica Toxicity**

Ever since IARC announced in 1997 that silica was a class 1 human carcinogen there has been a large number of studies conducted at the population level aimed at providing insight into the level of risk that accompanies exposure to silica. As can be expected, many of these studies are in an occupational setting.

In 2007, Cassidy and colleagues published a case-control study that assessed occupational exposure to crystalline silica and the resulting lung cancer risk. Their study included 2,056 male and 576 female lung cancer incidence cases and 2,144 male and 727 female controls, frequency-matched for sex and age, from across Europe. They reported an increased risk of lung cancer as the cumulative exposure increased and also with increasing duration of exposure. In light of this, the authors noted their findings further supported a causal association between silica and lung cancer (Cassidy et al, 2007). Despite the demonstration of a causal relationship, this study highlights some of the drawbacks of exposure assessment in a traditional population-based study.

Most population-based studies employed to study the lung effects of silica exposure are retrospective in nature due to the long latency period between exposure and disease manifestation. Such was the case in Cassidy's study, which used both interview and case-by-case exposure assessment by highly trained individuals to establish exposure information. However, in spite of the training provided to the exposure assessors, the inter-rater agreement for how much silica each individual was exposed to was very poor. Due to this, the researchers had to resort to algorithms to achieve a viable exposure assessment, demonstrating the difficulties in obtaining valid information about exposures in diseases with long latent periods at the population level (Cassidy et al, 2007).

A slightly more effective approach toward assessing silica toxicity at the population level was utilized by Chen and associates in their 2007 study of Chinese miners and pottery workers. This study, however, failed to see an association between silica and lung cancer after adjustment for confounding variables. A dose-response

relationship was reported when the regression equation was not stratified by occupation, but was not observed otherwise. The methods used to assess exposure were interesting for two reasons: the cohort size was very large (65,285 workers) and industrial hygiene data were available as far back as 1950 for indoor exposure to total dust, particle size and percent of free silica enabling the researchers to match these data to individual work history providing a better estimate of exposure than recall alone (Chen et al, 2007).

However, even though the latter example of exposure assessment seems to approximate actual exposure to a reasonable degree, errors in exposure assessment remain a daunting challenge when using population-based epidemiologic studies to assess the relationship between silica exposure and lung cancer. In order to reduce exposure misclassification when examining the association between silica exposure and lung cancer, it is often helpful to utilize techniques that can provide a more accurate estimate of how much silica was deposited in the tissue.

### **Approaches to Assessing Silica Within Lung Tissue**

Historic methods of examining particulates in lung tissue, especially silica, are now considered quite primitive and in many cases uninformative. The gold standard for establishing the presence of silica within the lungs was, for many years, polarizing light microscopy, where silica could be detected on the basis of its birefringence. This method was prone to operator error and was quite time-consuming. However, one of the drawbacks of this method is that light microscopy is limited in its ability to detect small particles within the tissue and it cannot clearly identify the composition of those particles. The benefit to this method, however, was that once particles were located and marked, the researcher could then easily observe the tissue architecture around the particle using normal light microscopy (Tsuchiya et al, 2007).

Tissue digesting and ashing of samples for use with both scanning and transmission electron microscopes was the next step in the study of particulate in lung tissue. However, while quick and easy to accomplish and allowing high levels of

accuracy in particulate count and elemental make-up when utilized with electron microscopy, this methodology is not ideal for the study of lung pathology for the obvious reason that the tissue is destroyed for analysis and therefore correspondence of particle deposition with tissue structures is impossible (Attanoos and Gibbs, 2009).

The newest approach to particle detection in tissue involves the use of scanning electron microscopy and x-ray diffraction microanalysis of the particles *in situ*. This approach is described in Fassina et al.'s 2009 paper in which the researchers use environmental scanning electron microscopy (ESEM) to analyze silica particles in lung tissue. Energy dispersive x-ray fluorescence microanalysis (EDX) was used to identify each particle found by the ESEM. The analysis covered an area that measured 16.835 mm<sup>2</sup> and each silica particle within this area was counted by hand as the researcher moved through the slide frame by frame (Fassina et al, 2009). The main drawbacks to this approach are the time commitment and potential for operator error. These concerns are both alleviated by the next generation of microscopy technology called computer-controlled scanning electron microscopy (CCSEM), which is utilized for this present study.

## **RESEARCH DESIGN**

### **Background**

The Iowa Radon Lung Cancer Study (IRLCS) was a population-based case control epidemiologic study conducted at the University of Iowa in the 1990's. The cohort established by the IRLCS formed the source population for the current study. For inclusion into the IRLCS, lung cancer cases had to be newly diagnosed with an invasive lung carcinoma confirmed by histology, be a female resident of Iowa at the time of cancer diagnosis, between the ages of 40 and 84, and maintain their current residence for the past 20 years or more. 1,974 eligible cases were identified by the Iowa Cancer Registry for inclusion in the IRLCS. 431 of these eligible cases participated and completed all required materials. The required materials included questionnaires covering demographics, smoking history, diet, and information about the home. Pathologic sections or tissue blocks were obtained from 423 of the 431 cases and were reviewed separately by two pathologists to confirm histologic type. At initial review, the two pathologists were blinded to both the diagnosis on the original pathology report and to each other's findings. Tissue blocks were collected for IRLCS subjects as part of the Iowa Cancer Registry's effort to develop a bio-repository of lung cancer samples for Iowa (Field et al, 2000).

### **Study Design**

This study is primarily intended to be an investigation of methods that can then be utilized for future research. It makes use of pathology samples and questionnaire information from the IRLCS case-control study. For the purpose of the analysis of differences between lung cancer morphology, a nested study will be performed, using samples and data from 24 IRLCS cases (Table 1). The IRLCS has received continuing approval for projects using IRLCS data from the University of Iowa's Institutional Review Board and an extension of this approval was granted for the current research. No further materials were collected for this research outside of what was obtained within the

Table 1. Demographic information for 24 Iowa Radon Lung Cancer Study members who met criteria for inclusion

<b>Characteristic</b>	<b>n</b>	<b>%</b>
<b>Gender</b>		
Female	24	100
<b>Race</b>		
White, not Spanish Origin	24	100
<b>Education</b>		
Grade School or less	1	4
High School	13	54
Some College or Technical School	6	25
College with degree	2	8
Graduate School or more	2	8
<b>Pack-Years</b>		
Never Smoker	4	17
1-10	1	4
11-20	1	4
21-30	3	13
31-40	5	21
41-50	5	21
51-60	2	8
61+	3	13
<b>Secondhand Smoke Exposure</b>		
No	7	29
Yes	17	71
<b>If Smoker, Non-Filtered Cigarette Use</b>		
No	9	45
Yes	11	55
<b>Other Health Conditions</b>		
Chronic Bronchitis	2	8
Emphysema	5	21
Asthma	2	8
Pneumonia	5	21
Stroke	1	4
High Blood Pressure	7	29
Other Cancer	4	17
<b>Cancer Morphology</b>		
Adenocarcinoma	15	63
Squamous Cell Carcinoma	8	33
Carcinoid	1	4



scope of the IRLCS. Permission was received from the Institutional Review Board, and a contract established, that allowed sending samples to the RJ Lee Group for analysis.

### **Subject Selection and Source Population**

The eligible cohort was defined as IRLCS cases whose cancer was determined to be adenocarcinoma or squamous cell carcinoma and whose blocks were available at the Iowa Cancer Registry.

The source population was Iowa women between the ages of 40 and 84 who were diagnosed with an invasive lung carcinoma between May 1, 1993 and October 30, 1996 (Field et al, 2000).

### **Subject Inclusion Criteria**

Study subjects met the following inclusion criteria: 1) involved in the IRLCS, 2) diagnosed with either adenocarcinoma or squamous cell carcinoma, 3) blocks of pathologic material available at the Iowa Cancer Registry, and 4) the tissue for the block was obtained as a result of a biopsy or resection procedure. Only female cases were eligible for the original IRLCS inclusion. Additional inclusion criteria for the IRLCS proper were discussed above.

### **Subject Exclusion Criteria**

For the purposes of this research, cases which did not have complete questionnaires in the IRLCS were excluded. Furthermore, any case with a lung cancer that was not determined, by consensus, by the two pathologists working in the IRLCS to be adenocarcinoma or squamous cell carcinoma was excluded. One carcinoid tumor from the IRLCS was included in the study, to serve as a control since a neuroendocrine tumor is unlikely to arise due to mechanisms associated with silica exposure, but was not included in the original IRLCS analysis. Controls from the IRLCS did not have a history of lung cancer and consequently were excluded from this study due to lack of pathologic material. Cases without available blocks from the Iowa Cancer Registry were also excluded. In addition, any blocks with tissue obtained through fine needle aspirate were

excluded based on the lack of an appropriate amount of tissue to examine with scanning electron microscopy.

Eighty-six members of the original IRLCS met the inclusion criteria in the beginning of this study. Twenty-six samples were randomly chosen from the eighty-six to establish the cases used in this study. This sample size was chosen based off of the budgetary constraints of this pilot study. Upon analysis of the blocks by a pathologist, two samples were excluded due to not having any tumor tissue present on the blocks that were made available by the Iowa Cancer Registry.

## METHODS

### **Methods Development**

The majority of the early work on this research was the development of a workable method for examining silica particles within lung tissue. In the beginning, the focus was centered around the question of whether there was silica in the lung tissue of non-occupationally exposed individuals. In order to investigate this question, existing randomly selected H&E stained slides from the IRLCS were examined under a polarizing light microscope. When birefringent particles were recognized and confirmed by a pathologist, it was noted that these particles were associated with carbon deposits in the lung tissue. Given this observation, it was hypothesized that an association may exist between smoking and silica particles in the lung.

At this point it was necessary to identify a method that would allow not only the quantification but also the determination of size of the silica particles within the lung. CCSEM was selected to address this because it is the only process available today that removes human subjectivity from the ascertainment of particle location within the lung tissue.

RJ Lee is a company that specializes in microscopy, and was recommended due to their use of CCSEM to detect particulates in ashed samples. Since they had never used CCSEM to examine particulates in lung tissue, several months of this study were spent in trial and error attempting to determine the best protocol for sample preparation. The resulting method produced the findings outlined in this study.

### **Selection of Blocks**

The Iowa Cancer Registry maintains a repository of cancer tissue fixed in paraffin blocks from patients diagnosed with cancer in Iowa. Many blocks from the Iowa Radon Lung Cancer Study are maintained there as well. In many cases, several blocks were taken of tumor and surrounding lung tissue. Once the process of sample selection was completed, it was necessary to determine which of the blocks for each patient should be

selected for use in the study. Since it was only important to establish that there was both tumor tissue and surrounding non-tumor lung tissue on the chosen block, the first block of choice was always the same block analyzed by the pathologists in the IRLCS. These blocks were submitted to the University of Iowa sample preparation laboratory and cuts were made off of the block in order to create a H&E stained slide. This H&E slide was reviewed by one of the original pathologists from the IRLCS in order to establish that both tumor and non-tumor tissue were present on the slide. The pathologist, who was blinded to any identifiers of the slide, also marked the delineation of the tumor on the slide. If it was determined that there were not both tumor and non-tumor tissue present, the next block, numerically, from the registry was analyzed in the same fashion. Furthermore, it enabled a uniform way to move through the tissue blocks across participants. There were two cases where there were no blocks available that contained both tumor tissue and surrounding lung tissue on the same slide, possibly due to depletion of those blocks. In both of these cases, the participants were excluded from the study due to lack of an appropriate sample.

### **Preparation of Samples**

Once the blocks were established as acceptable for inclusion in the study, they were sent again to the University sample preparation laboratory. This laboratory was provided with 7.5 mm diameter highly-polished carbon, silica-free, planchettes and carbon, silica-free, adhesion tape from Ted Pella, Inc. in order to mount the samples. Two micron thick slices were then taken off of the paraffin-fixed blocks and mounted on the planchette with the adhesion tape via a water bath of distilled, deionized water to prevent curling of the edges of the tissue. The planchettes were then sealed in a plastic membrane box for transport to RJ Lee Company. No coverslip was used due to concerns about interference with the CCSEM. The H&E slides that were marked by the pathologist as to delineation of the tumor tissue were sent to RJ Lee Company as well. Upon arrival, RJ Lee took digital scans of both the H&E slides and the planchettes. The digital scan of the

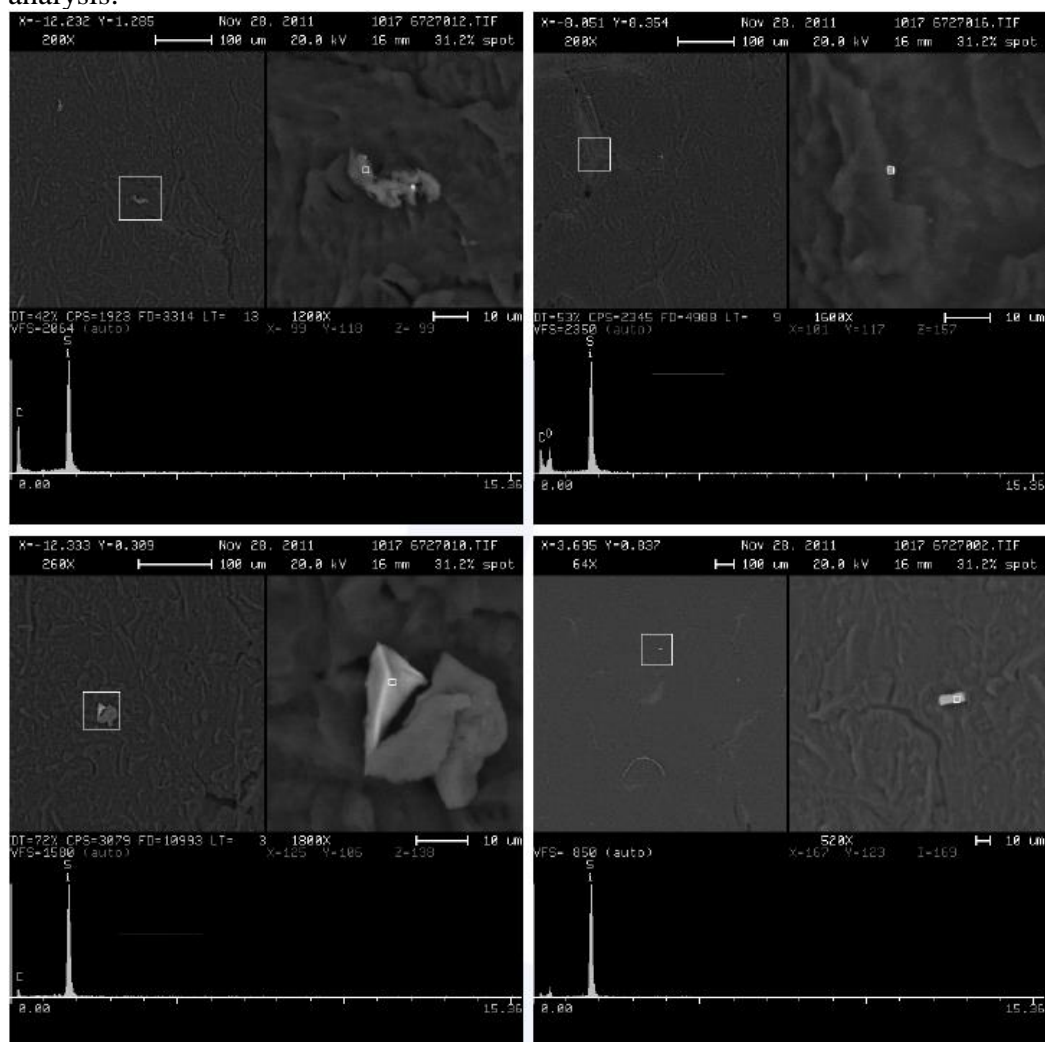
H&E slide was then marked with a box of 5 square millimeters at a place on the line delineating tumor and non-tumor tissue where the box could contain approximately 2/3 normal tissue and 1/3 tumor tissue. This box was then transcribed to the same location on the scan of the planchette and was used as a guide for scanning of the sample by CCSEM. The placement of the boxes was entirely random and was done prior to any analysis so as to be completely blinded to particle deposition. The only concern in placement of the boxes was to maintain a consistent ratio between amount of tumor tissue and amount of non-tumor tissue included in each scan.

### **Detection of Silica Particles by CCSEM**

To prepare the samples for processing, RJ Lee spatter-coated the samples on the planchette with carbon and then each sample was analyzed by the CCSEM software. The CCSEM performed at RJ Lee is an analysis program that includes a computer-guided scanning electron microscope coupled with energy dispersive spectroscopy (EDS) x-rays. This program enables the simultaneous measurement of particle size, shape and elemental composition. The analysis is performed by moving the electron beam over the sample and recording any signal above the present threshold value as a particle. All particles are then processed by the EDS which measures the x-rays emitted by the particle and compares them to energy spectra from all scannable elements to determine the composition of the particle (Figure 1).

Once a particle was analyzed, its coordinates within the scanning plane were recorded and plotted using spatial software developed at RJ Lee. The particle was then labeled free silica, soil-bound silica or other particle depending on its makeup. At the conclusion of the sample runs, two samples were chosen at random to be run again for quality control purposes. One of these samples was scanned exactly the same way over the same area to demonstrate calibration of the microscope. This was done twice for a total of three identical scans over the same area. The other randomly chosen sample was

Figure 1. Example of detection of free silica particles by the computer controlled scanning electron microscope and x-ray energy dispersive spectrometry elemental analysis.



scanned at a new location which was identified and marked by the above-described procedure in order to examine continuity within the same sample at different sites.

### **Statistical Analysis**

SAS 9.3 statistical software was used for all statistical analysis with the exception of the spatial analysis which was performed using ESRI Arcmap. The carcinoid sample was identified through descriptive statistics to be an outlier and was excluded from all analysis for all aims.

#### **Specific Aim I – Sub Aim 1-3**

These aims were simple quantifications and descriptions of the particles present in the tissue that was scanned by CCSEM, therefore no statistical analysis was warranted.

#### **Specific Aim II – Sub Aim 1**

This aim first involved comparing the number of free silica particles between adenocarcinoma and squamous cell carcinoma groups. A Shapiro-Wilk Normality Test was conducted, which failed ( $p < 0.050$ ), indicating that the data were not normally distributed. As a result, a Mann-Whitney Rank Sum Test was used instead of a standard parametric t-test.

Secondly, the number of soil-bound silica particles was assessed in the same manner. The data again failed the Shapiro-Wilk Normality Test ( $p < 0.050$ ), and a Mann-Whitney Rank Sum Test was used instead of a t-test in this analysis as well.

#### **Specific Aim II – Sub Aim 2**

To evaluate the mean size of free silica between the adenocarcinoma and squamous cell carcinoma groups, a Shapiro-Wilk Normality Test was conducted, which passed ( $p = 0.067$ ) indicating the data are normally distributed. A test for variance was then conducted, which also passed ( $p = 0.271$ ) indicating that the data displayed equal variance. A two-tailed t-test was used to address the aim with an alpha of 0.050.

The same procedure was followed for the assessment of soil-bound silica. The data was determined to be both normal ( $p=0.234$ ) and to exhibit equal variance ( $p=0.153$ ). As a result, a two-tailed t-test could be used with an alpha of 0.050.

#### **Specific Aim III – Sub Aim 1**

This aim compares the number of both free silica and soil-bound silica particles between ever smoker (smoker or past smoker) and never smoker groups. For both the free silica and soil-bound silica data, the Shapiro-Wilk Normality Test failed ( $p<0.050$ ) indicating that neither set of data were normally distributed. Therefore a Mann-Whitney Rank Sum Test was used.

#### **Specific Aim III – Sub Aim 2**

To further investigate the association of smoking on the number of particles of free silica and bound silica, pack-year smoking data were analyzed. For both free silica and bound silica datasets, a Shapiro-Wilk Normality Test was conducted, which failed ( $p<0.001$ ) in both cases, indicating that neither dataset was normally distributed. Linear regression was then used to evaluate the association of each data set with pack-years.

#### **Specific Aim III – Sub Aim 3**

This aim was addressed in a similar manner to specific aim III – sub aim 1, with mean particle size replacing number of particles. For free silica, a Shapiro-Wilk Normality Test was conducted, which passed ( $p=0.264$ ), along with a variance test, which also passed ( $p=0.059$ ), indicating that the data were both normally distributed and displayed equal variance. Therefore, a two-tailed t-test was conducted with an alpha of 0.050. The bound-silica data passed the Shapiro-Wilk Normality Test ( $p=0.244$ ) but failed to demonstrate equal variance ( $p<0.050$ ), therefore a Mann-Whitney Rank Sum Test was performed for non-parametric data.

#### **Specific Aim III – Sub Aim 4**

This aim was addressed in a similar manner to specific aim III – sub aim 2, with number of particles being replaced by mean particle size. The datasets for both free silica



and soil-bound silica failed the Shapiro-Wilk Normality Test ( $p < 0.001$ ) indicating that neither data set was normally distributed. Linear regression was then used to evaluate the association of each data set with pack-years.

#### **Specific Aim IV – Sub Aim 1-2**

Sub Aims 1 and 2 were concerned with the question of the dispersion of silica and soil-bound silica in the tissue. Spatial analysis statistics were utilized to address this question. Average Nearest Neighbor was used to indicate whether free silica or soil-bound silica were more clustered or dispersed than one would expect in a random sample. This statistic calculates a nearest neighbor index based on the average distance from each particle to its nearest neighboring particle of the same type.

#### **Specific Aim IV – Sub Aim 3**

Global Moran's  $i$  was used in order to determine whether silica particles in the lung tissue were associated spatially with another particle type. This statistic determines spatial autocorrelation based off of both location and value simultaneously. Global Moran's  $i$  is able to analyze whether a particle is located more closely to a particle of its own type or one of a different type.

## RESULTS

### **Sample Size**

Of the twenty-four final cases, fifteen were adenocarcinoma cases, eight were squamous cell carcinoma cases, and a single carcinoid case was included to serve as a control based on the postulated mechanism of silica toxicity in the lung. The data pertaining to this carcinoid sample will be presented, however due to the fact that it is a statistical outlier, it was not included in any analysis. Out of the twenty-four samples, four were from never smokers while the rest of the cohort smoked for varying amounts of time. The mean pack-years for the twenty-four participants was approximately thirty-five.

Demographic data about this group were provided in Table 1 above. The study cohort was composed entirely of females and was entirely white, non-Hispanic. Most were high school graduates. About 71% had been exposed to second-hand smoke within their home and 83% smoked themselves at some point in their lives. 55% acknowledged smoking unfiltered cigarettes, and several reported health issues aside from lung cancer. Table 2 identifies the diagnosis and lists smoking history in pack-years for each sample.

### **Specific Aim I Results**

The primary objective of the first Specific Aim was to obtain descriptive information about silica particles found within lung tissue scanned with computer-controlled scanning electron microscopy. Table 3 presents the particle count per  $\text{mm}^2$  of scanned tissue as well as the percentage of particle type per sample. The amount of free silica detected in the samples ranges from 0 particles to 219.8 particles, with an average of 28.2 free silica particles per  $\text{mm}^2$  across all samples. Soil-bound silica was detected in the tissues of the samples in a range from 0 particles to 229.8 particles, which the amount of other non-silica particles ranges from 0.3 to 61.7 particles per  $\text{mm}^2$  of tissue, with averages of 37.9 and 14.0 particles per  $\text{mm}^2$ , respectively. Out of the twenty-three samples analyzed, soil-bound silica particles were most abundant in twelve samples, free silica and other non-silica particles were most abundant in six samples each.

Table 2. Lung cancer morphology and smoking history of study members

<b>Study Member</b>	<b>Morphology</b>	<b>Smoking History in Pack-years</b>
1	Adenocarcinoma	29.75
2	Squamous Cell Carcinoma	38
3	Squamous Cell Carcinoma	51
4	Adenocarcinoma	58
5	Squamous Cell Carcinoma	0
6	Squamous Cell Carcinoma	47
7	Adenocarcinoma	0
8	Adenocarcinoma	38
9	Adenocarcinoma	48
10	Adenocarcinoma	48.5
11	Squamous Cell Carcinoma	28.5
12	Adenocarcinoma	0
13	Squamous Cell Carcinoma	33.5
14	Adenocarcinoma	78
15	Squamous Cell Carcinoma	62
16	Adenocarcinoma	1
17	Adenocarcinoma	0
18	Adenocarcinoma	27
19	Adenocarcinoma	37
20	Adenocarcinoma	50
21	Adenocarcinoma	46
22	Squamous Cell Carcinoma	72
23	Adenocarcinoma	31
24	Carcinoid	12

Table 3. Particle count per mm<sup>2</sup> of scanned tissue and percentage of free silica, soil-bound silica, and other particles by sample

<b>Count of Particles per mm<sup>2</sup> and Percent Distribution by Type of Silica</b>						
<b>Sample</b>	<b>Free Silica</b>		<b>Soil-Bound Silica</b>		<b>Other Particles</b>	
	<b>n</b>	<b>%</b>	<b>N</b>	<b>%</b>	<b>n</b>	<b>%</b>
1	16.3	61.0	5.0	18.7	5.4	20.2
2	8.3	34.4	5.5	22.8	10.3	42.7
3	1.1	22.9	0.0	0.0	3.7	77.1
4	41.8	68.9	8.4	13.8	10.5	17.3
5	219.8	52.2	194.8	46.3	6.4	1.5
6	42.9	30.5	69.8	49.7	27.8	19.8
7	13.4	60.4	5.4	24.3	3.4	15.3
8	0.7	12.3	3.4	59.6	1.6	28.1
9	3.9	22.2	3.1	17.6	10.6	60.2
10	9.5	18.8	13.9	27.5	27.1	53.7
11	2.2	15.6	6.4	45.4	5.5	39.0
12	0.0	0.0	0.4	16.3	2.0	83.7
13	14.7	36.5	18.1	44.9	7.5	18.6
14	176.4	38.3	229.8	49.9	54.5	11.8
15	4.5	21.7	10.1	48.8	6.1	29.5
16	4.9	24.6	9.3	46.7	5.7	28.6
17	7.1	86.6	0.8	9.7	0.3	3.7
18	15.5	21.2	43.4	59.5	14.1	19.3
19	12.2	17.3	41.1	58.2	17.3	24.5
20	31.6	13.7	136.9	59.5	61.7	26.8
21	6.7	15.3	7.9	18.1	29.1	66.6
22	6.5	14.1	32.0	69.6	7.5	16.3
23	9.5	23.8	26.8	67.0	3.7	9.3
24	805.4	58.7	406.4	29.6	159.3	11.6

Table 4 displays the breakdown of the particle sizes of free silica found in the samples. Mean sizes of free silica particles for each sample ranged from 0.43 microns to 2.41 microns. The mean size of all particles of free silica across the samples is 1.22 microns.

Table 5 presents the breakdown of the particle sizes for soil-bound silica that was found deposited in the tissue. Mean sizes of soil-bound silica particles for each sample range from 0.20 microns to 1.98 microns. The mean size of all particles of soil-bound silica across the samples is 1.10 microns. Figure 2 compares the mean particle size between free silica and soil-bound silica.

Overall, soil-bound silica is most predominate in the tissue samples, followed by free silica and then other particles. The largest number of particles in any sample was free silica particles, while there was one sample that had no free silica particles present and one sample that had no soil-bound particles present. On average, soil-bound silica particles were present in greater quantity, but were of a smaller size than free silica particles.

### **Specific Aim II Results**

Specific Aim 2 investigated the association of lung cancer diagnosis with the quantity and size of silica particles in the lung.

In total, fifteen of the samples had a diagnosis of adenocarcinoma and eight of the samples were squamous cell carcinoma. Table 6 presents descriptive statistics for Aim II.

To investigate the association between the number of free silica particles and the sample's morphology, a Mann-Whitney Rank Sum Test was used. The Mann-Whitney U statistic was 56.0. Since the p-value was large (0.821) the difference in median values between the two groups is not great enough to exclude the possibility that the difference is due to randomness alone. Therefore, there is no statistically significant difference between the number of free silica particles per  $\text{mm}^2$  in the adenocarcinoma samples and the number of free silica particles per  $\text{mm}^2$  in the squamous cell carcinoma samples.

Table 4. Mean and distribution of free silica particle size by sample

Sample	Number of Free Silica Particles (Microns)													Mean size
	0.2-0.3	0.3-0.4	0.4-0.5	0.5-0.6	0.6-0.7	0.7-0.8	0.8-0.9	0.9-1.0	1.0-1.5	1.5-2.5	2.5-5.0	5.0-10.0	>10.0	
	1	26	13	6	4	2	3	4	1	4	8	7	8	
2	6	4	0	2	0	1	0	0	1	7	1	2	0	1.17
3	0	0	0	0	0	0	1	0	2	0	0	0	0	0.93
4	36	11	9	5	8	6	3	2	10	16	15	12	2	1.33
5	3	6	14	22	25	32	42	24	154	206	185	40	1	1.65
6	2	4	5	15	16	12	11	11	41	26	11	5	0	1.13
7	6	3	2	2	3	2	1	1	13	15	2	2	0	1.14
8	0	0	0	0	0	0	1	0	3	0	0	0	0	0.95
9	0	0	0	1	1	0	0	1	2	6	4	0	1	2.06
10	1	3	3	2	9	2	3	1	8	7	9	0	0	1.13
11	0	0	0	0	1	2	1	0	1	0	0	0	0	0.76
12	0	0	0	0	0	0	0	0	0	0	0	0	0	-
13	2	1	1	6	0	9	5	7	20	10	13	1	0	1.24
14	5	23	67	89	92	80	71	57	202	148	48	2	0	0.96
15	1	0	0	1	2	1	1	2	11	4	0	0	0	0.97
16	2	3	2	1	1	0	1	0	4	6	2	2	1	1.68
17	1	1	1	1	3	4	5	2	17	23	28	17	4	2.41
18	25	14	17	7	5	2	2	0	4	1	1	0	0	0.43
19	0	3	3	4	2	6	9	7	14	8	5	1	0	1.07
20	5	13	13	17	18	17	13	14	39	10	2	0	0	0.77
21	0	1	2	1	2	1	1	2	7	12	5	0	0	1.28
22	0	2	2	2	1	7	1	4	7	3	3	1	0	1.10
23	1	1	3	3	2	6	1	2	14	9	5	1	0	1.17
24	8	16	58	143	177	192	200	203	956	1295	760	27	0	1.39

Table 5. Mean and distribution of soil-bound silica particle size by sample

Sample	Number of Soil-Bound Silica Particles													Mean size
	(Microns)													
	0.2-0.3	0.3-0.4	0.4-0.5	0.5-0.6	0.6-0.7	0.7-0.8	0.8-0.9	0.9-1.0	1.0-1.5	1.5-2.5	2.5-5.0	5.0-10.0	>10.0	
1	3	2	1	3	0	5	0	0	6	5	2	0	0	0.93
2	1	1	0	1	0	0	3	1	5	3	1	0	0	1.02
3	0	0	0	0	0	0	0	0	0	0	0	0	0	-
4	2	3	6	0	2	0	2	1	3	6	2	0	0	0.91
5	17	24	30	30	42	30	30	34	131	146	126	28	0	1.41
6	3	7	14	11	14	19	15	14	71	67	21	3	0	1.15
7	1	1	0	0	1	2	2	1	8	3	2	0	0	1.07
8	6	1	4	0	1	1	1	0	2	5	0	0	0	0.70
9	2	1	1	2	0	1	0	1	2	0	3	0	0	1.02
10	0	4	3	5	6	9	3	2	17	13	8	0	0	1.08
11	2	2	0	0	0	0	1	0	3	3	2	1	0	1.38
12	2	0	0	0	0	0	0	0	0	0	0	0	0	0.20
13	1	3	3	4	5	7	6	4	27	25	7	0	0	1.12
14	40	57	84	112	91	90	83	86	316	338	191	14	0	1.16
15	0	1	1	9	8	2	3	2	8	11	6	0	0	1.08
16	5	0	2	3	2	1	2	3	7	10	8	4	0	1.52
17	2	1	0	0	1	0	3	0	1	1	1	1	1	1.98
18	51	33	29	27	14	12	9	4	29	8	2	0	0	0.56
19	7	4	10	11	11	14	23	12	41	42	29	5	0	1.24
20	53	45	54	63	46	43	57	44	147	101	44	0	0	0.90
21	9	1	4	0	1	2	1	0	5	6	7	4	0	1.45
22	5	6	10	18	16	12	9	17	26	26	16	0	1	1.06
23	1	1	7	10	6	6	13	11	36	25	19	1	0	1.18
24	16	26	64	106	114	132	131	125	527	550	237	8	0	1.20

Figure 2. Comparison of mean particle size between free silica and soil-bound silica for each of 24 samples

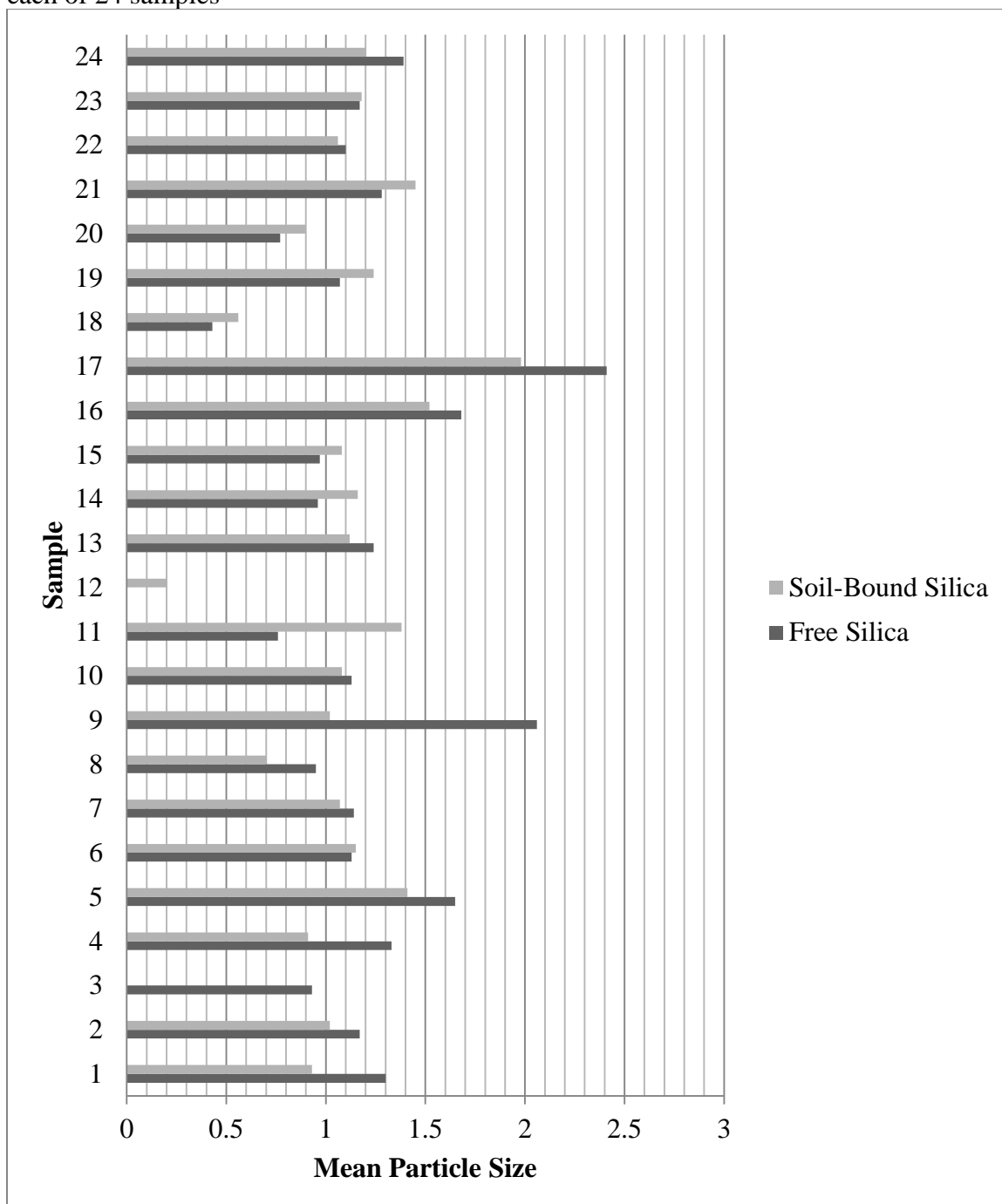




Table 6. Descriptive Statistics for Specific Aim II.

	<b>Diagnosis</b>	<b>Size</b>	<b>Mean</b>	<b>Std Dev</b>	<b>Std Error</b>	<b>Max</b>	<b>Min</b>	<b>Median</b>	<b>25%</b>	<b>75%</b>
<b>Free Silica Particle Number</b>										
	A	15	23.3	43.8	11.3	176.4	0.0	9.5	4.9	16.3
	S	8	37.5	74.9	26.5	219.8	1.1	7.4	2.8	35.9
<b>Bound-Silica Particle Number</b>										
	A	15	35.7	63.9	16.5	229.8	0.4	8.4	3.4	41.1
	S	8	42.1	65.7	23.2	194.8	0.0	14.1	5.7	60.3
<b>Free Silica Mean Size</b>										
	A	14	1.3	0.5	0.1	2.4	0.4	1.2	1.0	1.4
	S	8	1.1	0.3	0.1	1.7	0.8	1.1	0.9	1.2
<b>Bound-Silica Mean Size</b>										
	A	15	1.1	0.4	0.1	2.0	0.2	1.1	0.9	1.2

For the association between the number of soil-bound silica particles and the sample's morphology, the Mann-Whitney U statistic was 51.0. Since the p-value was large (0.583) there is no statistically significant difference between the number of soil-bound silica particles per  $\text{mm}^2$  in the adenocarcinoma samples and the number of soil-bound silica particles per  $\text{mm}^2$  in the squamous cell carcinoma samples.

To investigate the association between the mean size of free silica particles and the sample's morphology, a two-tailed t-test was used. The t-value was 0.744 with 20 degrees of freedom. The difference between the means was 0.144, and a 95% two-tailed confidence interval for the difference of means was (-0.260, 0.548). The p-value was again large ( $p=0.466$ ) leading to the conclusion that there is no statistically significant difference between the mean size of free silica particles in the adenocarcinoma samples and the mean size of free silica particles in the squamous cell carcinoma samples. The power of the two-tailed t-test with an alpha of 0.050 was 0.109. This power is below the desired power of 0.800 and indicates that the detection of a difference between the two means when one actually exists is less likely.

For the association between the mean size of soil-bound silica particles and the sample's morphology, the t-value was -0.692 with 20 degrees of freedom. The difference between the means was -0.114, and a 95% two-tailed confidence interval for the difference of means was (-0.459, -0.230). The p-value was large (0.497) indicating that there is no statistically significant difference between the mean size of soil-bound silica particles in the adenocarcinoma samples and the mean size of soil-bound silica particles in the squamous cell carcinoma samples. The power of the two-tailed t-test with an alpha of 0.050 was 0.101, which is also below the desired power of 0.800.

Overall, there is not a statistically significant association between lung cancer diagnosis and the quantity or size of silica particles deposited in the lung.

### **Specific Aim III Results**

Specific Aim 3 investigated smoking history to determine if smoking is associated with the quantity and size of silica particles within the lung tissue. In total, 19 of the samples were from current or past smokers and 4 of the samples were from never smokers. Descriptive statistics for Aim III are presented in Table 8.

For the association between the number of free silica particles and smoking history, the Mann-Whitney U statistic was 37.0. The p-value was large ( $p=0.968$ ) indicating that there is no statistically significant difference between the number of free silica particles and smoking status.

The association between the number of soil-bound silica particles and smoking history had a Mann-Whitney U statistic of 24.0. Since the p-value is large (0.274), there is no statistically significant difference between the number of soil-bound silica particles and smoking status.

To investigate the association between the mean size of free silica particles and smoking history, a two-tailed t-test was used. The t-value was -2.525 with 20 degrees of freedom. The difference between means was -0.605, and a 95% two-tailed confidence interval for the difference of means was (-1.106,-0.105). Since  $p=0.0201$  ( $<0.050$ ) the sample mean of the never smoker group exceeds the sample mean of the smoker group by an amount that is greater than would be expected by chance. Therefore, there is a statistically significant difference between the mean size of free silica particles and smoking status. This result indicates that the mean particle size of free silica is smaller in those who have smoked than in those who have never smoked.

For the association between the mean size of soil-bound silica and smoking history, a Mann-Whitney Rank Sum Test was used. The Mann-Whitney U statistic was 30.0. Since the p-value was large (0.639) there is no statistically significant difference between the mean size of soil-bound silica and smoking status.

Table 7. Descriptive Statistics for Specific Aim III.

	<b>Ever Smoked</b>	<b>Size</b>	<b>Mean</b>	<b>Std Dev</b>	<b>Std Error</b>	<b>Max</b>	<b>Min</b>	<b>Median</b>	<b>25%</b>	<b>75%</b>
<b>Free Silica Particle Number</b>										
	Yes	19	21.5	39.6	9.1	176.4	0.7	9.5	4.5	16.3
	No	4	60.1	106.6	53.3	219.8	0.0	10.3	1.8	168.2
<b>Bound-Silica Particle Number</b>										
	Yes	19	35.3	57.3	13.1	229.8	0.0	10.1	5.5	41.1
	No	4	50.4	96.3	48.2	194.8	0.4	3.1	50.5	147.5
<b>Free Silica Mean Size</b>										
	Yes	19	1.1	0.3	0.1	2.1	0.4	1.1	1.0	1.3
	No	3	1.7	0.6	0.4	2.4	1.1	1.7	1.2	2.4
<b>Bound-Silica Mean Size</b>										
	Yes	18	1.1	0.2	0.1	1.5	0.6	1.1	0.9	1.2
	No	4	1.2	0.7	0.4	2.0	0.2	1.2	0.4	1.8

To address the association between the number of free silica particles and smoking history in pack-years, linear regression was used. The R-value was 0.0289. The p-value was 0.896 and was not statistically significant indicating that there is no significant association between the number of free silica particles and smoking history in pack-years.

For the association between the number of soil-bound silica particles and smoking history in pack-years, linear regression was used with an R-value was 0.193. The p-value was 0.378 indicating that there is no significant association between the number of soil-bound silica particles and smoking history in pack-years.

For the association between the mean size of free silica particles and smoking history in pack-years, the R-value was 0.159 for the regression model. The p-value was not statistically significant at 0.469. Therefore, it can be concluded that there is no significant association between the mean size of free silica particles and smoking history in pack-years.

The R-value for the association between the mean size of soil-bound silica particles and smoking history in pack-years was 0.197. The p-value was 0.367 and was not statistically significant indicating that there is no significant association between the mean size of soil-bound silica particles and smoking history in pack-years.

Overall, the only statistically significant association was between the mean size of free silica particles and smoking status, indicating that smaller particles were present in samples with a history of smoking compared to samples from never smokers.

#### **Specific Aim IV Results**

Specific Aim 4 utilized spatial statistics to determine if the silica particles in the samples were clustered, dispersed or random. The first statistic used was Average Nearest Neighbor, which looks at how closely each particle of the same type is to each other. The analysis was conducted on each sample individually. For sample 1, free silica, soil-bound silica and other particles all were distributed in a random fashion. When compared by the

test statistic to what would be expected from a random sample, they were neither clustered nor dispersed. None of the p-values were significant (Table 10). Sample 2 had free silica particles that were significantly dispersed when compared to a random sample, with a z-score of 3.30 and a p-value of 0.00. This indicates that there is less than a 1% chance that this distribution would occur by random chance. The remaining particles in sample 2 were randomly distributed, with large p-values. The free silica in sample 4 approached significance for clustering; however the other types of particles were all distributed randomly throughout the tissue, with large p-values. The free silica in sample 8 is strongly dispersed with a z-score of 14.05 and a p-value of 0.00, indicating that there is less than 1% chance that this pattern would have happened randomly. The other particles in sample 8 are significantly clustered with a p-score of 0.0233, and soil-bound silica approaches significance for clustering with a p-value of 0.0776. Sample 17 exhibits significant dispersion of both soil-bound silica and other particles, with p-values of 0.025 and 0.000 respectively, while the free silica is randomly distributed with a large p-value.

Moran's global  $i$  was also calculated in order to determine how the three types of particles are related to each other. All samples demonstrated a strongly significant clustering pattern other than sample 8 which was just approaching significance for clustering. The p-values for 1,2,4,8, and 17 were 0.006, 0.000, 0.001, 0.071, and 0.000 respectively. This indicates that all particles were more closely associated with particles of their own type than with particles of another type.

Table 8. Spatial analysis of five random samples.

Sample	Average Nearest Neighbor (z-score/p-value)			Moran's global i
	Free silica	Soil-bound silica	Other particles	
1	-1.39/0.1656	-0.74/0.4615	0.27/0.7886	2.74/0.0061
2	3.30/0.0009	0.04/0.9671	0.70/0.4821	4.14/0.00004
4	-1.80/0.0713	-0.91/0.3631	-0.27/0.7903	3.36/0.0008
8	14.05/0.0000	-1.76/0.0776	-2.27/0.0233	1.81/0.0707
17	-0.79/0.4307	2.24/0.0253	5.13/0.0000	3.90/0.00009

## DISCUSSION

The overall objective of this study was to develop a successful methodology that could be utilized for the future study of silica particle distribution in lung tissue. With detection of silica particles in the lung tissue of non-occupationally exposed individuals, this methodology has demonstrated its usefulness in estimating exposure to inhaled particulates.

Computer-controlled scanning electron microscopy removes all subjectivity in the analysis of particulate, increasing detection capacity and uniformity of results. Nowhere else in the literature has silica been reported in this magnitude before, especially not in non-occupationally exposed individuals. These CCSEM techniques to analyze silica particles in lung tissue are unique in the literature with the results of this study demonstrating an unsurpassed ability to detect silica particles in quantities far greater than those reported by other popular methods (Fassina et al, 2009).

Regarding the first hypothesis of this study, silica particles were identified in lung samples, which contain tumor tissue, of non-occupationally exposed individuals. Although it seems simple, this fact is the most important discovery of this project. Finding silica particles in the lungs of individuals who have not had a sustained, large-scale exposure to silica is previously undocumented. This result will hopefully help promote research of environmental causes of silica exposure and hazard assessment. In their 2009 study, Fassina et al. reported an average occupational exposure of 12.5 free silica particles per  $\text{mm}^2$  of lung tissue and an average non-occupational exposure of 0.27 free silica particles per  $\text{mm}^2$  of lung tissue containing tumor (Fassina et al, 2009). The discrepancy between the results of Fassina's study and this study demonstrate the increased utility of the CCSEM tool. Furthermore, the differences in the results highlights that the lung burden of silica in both occupationally-exposed individuals and non-occupationally exposed individuals may be greater than previously assessed.



The second hypothesis indicated that smokers would have more silica particles in their lung tissue than non-smokers. The belief was that there would be demonstration of a dose-response with increasing pack-years being associated with an elevated silica burden in the lung. This belief was based off of pilot data from the beginning of this study that demonstrated an association between the amount of silica particles present in the lung tissue and smoking history. However, this association diminished upon analysis of all of the study data.

The third hypothesis of this study postulated that lung tissue from samples with squamous cell carcinoma would contain more silica particles than lung tissue from samples with adenocarcinoma due to its association with cigarette smoking. However, this hypothesis appears to be inconclusive, with analysis indicating that there is no significant association between cancer morphology and either the amount or size of silica particles in the lung. Part of this lack of demonstrated association could perhaps be contributed to the differences between the locations of adenocarcinoma and squamous cell carcinoma in the lung. Adenocarcinomas tend to arise more in the periphery of the lung, while squamous cell carcinomas are more commonly found peribronchially. The bronchial location is less ideal for particle deposition than the alveoli due to air turbulence and the size of the airway, potentially contributing to fewer silica particles in the tissue. This may be counteracted by the increased particle load from cigarette smoke, diminishing any difference between particle deposition at the two locations (Brown et al, 2002).

The final hypothesis for this study involved conducting spatial analysis to determine the clustering of silica particles in the lung tissue. The results from the Average Nearest Neighbor analysis were inconclusive, with both clustering and dispersion of silica particles seen. This discrepancy could be due to the imperfection of the statistic when used to analyze particles rather than places on a map. The presence of outliers can cause the results to completely change and the analysis is dependent on the

precise definition of the scanning area limiting conclusions to within the samples and making it impossible to compare between the samples. The Global Moran's I statistic indicated that particles are more likely to be associated with like particles than with particles unlike themselves, hence demonstrating a cluster. The next step in applying the information obtained from spatial analysis would be to combine it with the architecture of the tissue to determine if there is clustering relative to lung features. This will be achieved through the realization of a method, currently in production in collaboration with RJ Lee, to optically overlay particles found in the lung tissue with CCSEM onto an H&E slide.

An unexpected finding of this study was the amount of particles contained in the sample of carcinoid tissue. The carcinoid sample was added to act as a possible control for the other morphologies of lung cancer since it is believed that the neuroendocrine tumor would not arise in the same way as the other tumors, indicating less of a potential role for silica. The carcinoid sample contained the largest amount of all three particle types when compared to the 23 other samples. This result was highly unexpected since the carcinoid patient had no occupational silica exposure, working as a registered nurse, with a relatively small smoking history (12 pack-years), no history of second-hand smoke within the home, and no other reported ailments. Since the history of the patient does not seem to line up with what was seen on the CCSEM analysis, it is important to investigate other potential exposures moving forward. Furthermore, it is necessary to analyze more carcinoid tissue to determine if this result is common to the morphology or if it was an outlier related to unaccounted individual exposure, which is more probable at this time.

An important association noted by this study was the association between smoking status and free silica particle size. Free silica in the lung of smokers is, on average, smaller than the free silica in the lung of never smokers. A potential explanation for this observation may be that the larger silica particles are filtered out leaving only

small particles to deposit in the lungs. Alternatively, the burning of the silica particles in the contaminated tobacco while smoking may decrease the size as well.

### **Limitations**

Most of the limitations to this study result from the high cost of operation of CCSEM and the resulting budgetary constraints. In light of this, it is likely that the biggest limitation of this study was the sample size. With a sample size so small, the power to detect associations was severely limited.

Another potential limitation of this study is that the entire sample could not be scanned, also due to cost. The arbitrary nature of choosing where on the slide to scan limits the reproducibility of the methods and opens the analysis up to subjective error.

The restriction of this study to lung cancer patients only, due to the availability of tissue from the Iowa Cancer Registry, prevented any analysis of control samples to determine if silica particle amount and size are associated with lung cancer in general. This question is important to address moving forward.

The inability to link the tissue architecture with particle location is a major limitation of this methodology. In an effort to address this limitation an optical overlay was conceived and is still under development.

### **Strengths**

One of the biggest strengths of this study is that it describes a successful method to detect previously undetectable silica particles within lung tissue in a highly uniform manner without destroying its architecture. This study has demonstrated that there are silica particles present in the lungs of people without occupational exposure to silica. Furthermore, the methodology is quick, easy to implement, and may be applied to detect particulate in tissue outside of the scope of silica research.

### **Future Research**

There are many opportunities for future research resulting from the findings of this study. First, it is important to further establish this method as a surrogate for other

forms of exposure analysis in population-based studies. Using the CCSEM to analyze a cohort of non-occupationally exposed individuals with a sufficient sample size is most likely the next step for this research. In this next step it is important to utilize controls to determine if there is a difference in silica particle behavior between cancer patients and healthy controls. This cohort would likely not be limited to adenocarcinoma and squamous cell carcinoma samples, as the results of this study indicate the need to investigate carcinoid and its association with silica particles in the lung as well.

Further research into the reason that there is a potential association between smoking and smaller free silica size is warranted as well. This research would also attempt to establish whether or not cigarette smoking, and also electronic cigarette use, is a source of silica exposure.

The continued development of the optical overlay method for examining CCSEM-analyzed particles and their association to lung architecture is a current pursuit.

## CONCLUSION

This study was constructed to outline a viable process for standardizing exposure assessment while at the same time increasing the detection capabilities for locating silica particles within lung tissue. It has demonstrated that CCSEM is not only a viable, but in some ways a preferable, alternative to standard scanning electron microscopy techniques for the study of silica particles within the lung. The findings of this study are also unique with this method demonstrating that silica particles are present, in a substantial amount in some cases, in the lung tissue of people who had no previous occupation exposure to silica. This indicates that there may be a significant pathway for environmental exposure to silica.

While this study has failed to demonstrate an association between size and quantity of silica particles with morphology of cancer, the lung burden of silica in the sample of carcinoid remains an intriguing finding. The ability of this study to address particle size enabled the detection of an association between smoking history and free silica particle size. Since the low statistical power of this study made detection of associations even more difficult, it was encouraging to find a significant result.

The hazard of silica exposure in both occupational and environmental settings is a danger to public health. Despite the lack of knowledge about the fundamental mechanism contributing to silica-induced toxicity, population-based studies have described a causal pathway between silica exposure and lung damage on several occasions (Cassidy et al, 2007). IARC's classification of silica as a class 1 human carcinogen has indicated that the potential of silica's contribution to lung toxicity is great and has ensured that reduction of exposure to silica remain an important goal in industry (IARC, 2012). NIOSH's affirmation that potential for exposure to silica extends beyond the workplace warrants further research into the risk of non-occupational activities (NIOSH, 2002).

In conclusion, this study has established a detailed method that can be used to address concerns about silica exposure in the future. Furthermore, some of these potential concerns have been highlighted within the scope of this research.

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