CHARACTERIZATION OF EXPOSURE TO AND ASSOCIATED ACUTE EFFECTS OF OUTDOOR SECONDHAND SMOKE FOLLOWING INDOOR SMOKING BANS IN ATHENS, GEORGIA

by

GIDEON ST.HELEN

(Under the Direction of Luke P. Naeher)

ABSTRACT

Objectives: (1) To investigate whether particulate matter ($PM_{2.5}$) and carbon monoxide (CO) outside establishments are directly associated with secondhand smoke (SHS); (2) to characterize systemic exposure of non-smokers to outdoor SHS using biomarkers, salivary cotinine and urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL); and (3) to assess the utility of urinary Clara cell protein (CC16) as a biomarker of SHS-induced lung epithelial permeability.

Methods: Real-time $PM_{2.5}$ and CO were monitored in outdoor patios at five locations, two restaurants, two bars, and a control site. Number of smokers and patrons at and vehicles passing each location were counted. The effects of these variables on $PM_{2.5}$ and CO were estimated through linear mixed effects models. Further, twenty-eight non-smokers were assigned to outdoor patios of a restaurant and a bar and an open-air location with no smokers (control) on three weekend days in a crossover study. Saliva and urine samples were collected before, post-3 h visits, and next-morning, and analyzed for salivary cotinine and total NNAL and CC16 in urine. CC16 was measured in post-100 mL urine from males. Number of lit cigarettes was

counted per sampling occasion. Changes in biomarkers were analyzed across locations and with cigarette count, respectively. CC16 analyses were stratified by gender.

Results: Smoker count had a significant positive effect on log(CO) ($p=0.032$) and log($PM_{2.5}$) ($p<0.001$). The vehicle effect was non-significant. Also, significant increases in salivary cotinine were measured post- and next-morning following visits outside the bar and restaurant compared to the control ($p<0.001$). Next-day–pre-exposure NNAL differences were significantly higher following visits outside the bar and restaurant compared to the control ($p=0.005$). A tendency of increasing post:pre-exposure ratios of urinary CC16 with increasing SHS was observed among females. Cigarette count had a significant effect on post-:pre-exposure urinary CC16 among females ($p=0.048$). Urinary CC16 in males were several times higher indicative of post-renal CC16 contamination.

Conclusion: $PM_{2.5}$ outside establishments where smoking is allowed is proportional to number of cigarettes smoked. Also, non-smokers exposed to outdoor SHS are exposed systemically to components of SHS and these levels may be associated to increased lung epithelial permeability. However, urinary CC16 applicability in males needs futher study.

INDEX WORDS: Outdoor secondhand smoke, SHS, salivary cotinine, urinary NNAL, urinary Clara cell protein, CC16, epithelial permeability, smoking bans, tobacco control

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DEDICATION

To my parents, Ucilla and Bernard St.Helen, who made educating their children their highest priority and calling. I am indebted to them. To my brother, Sammy, and sisters, Silvren, Emlyne, Sirjean, and Bernelle, whose love and support have not wavered. To Victoria Perez who always cared. And last but not least, to my wife, Nichelle, who endured patiently and stuck by me until the end.

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CHAPTER 1

INTRODUCTION

The US Surgeon General released a landmark report in 1986 that was the first of its kind to identify a chronic disease risk from exposure to tobacco smoke for individuals other than smokers (USDHHS 1986). Secondhand smoke (SHS) or environmental tobacco smoke (ETS), as it was referred to in the 1986 report, is defined as a combination of smoke emitted from a burning tobacco product between puffs (sidestream smoke) and the smoke exhaled by the smoker (mainstream smoke). Subsequent Surgeon General reports have validated and extended on the current knowledge on SHS (USDHHS 2006, 2010). Tobacco smoke is a complex mixture of over 7,000 chemicals including hundreds that are hazardous and at least 69 known carcinogens (IARC 2004; USDHHS 2010). Scientific evidence continues to show that SHS exposure is causally associated to lung cancer in never- or non-smokers (Vineis et al. 2007; Wakelee et al. 2007), breast cancer in non-smoking, premenopausal younger women (Miller et al. 2007), as well as a risk factor for other cancers such as bladder and pancreatic cancers (Alberg et al. 2007; Bao et al. 2009; Van Hemelrijck et al. 2009). SHS has been shown to increase the risk of cardiovascular disease by ~30% (Barnoya, and Glantz 2005) and accounts for at least 30,000 deaths annually in the United States (Adhikari et al. 2008). Other studies further support the causal link between SHS exposure and respiratory diseases such as asthma in children (Jaakkola, and Gissler 2004), chronic bronchitis (Vozoris, and Lougheed 2008), and cough and sinusitis (Hammad et al. 2010), and as a potential cause of obstructive lung disease in non-smokers (Eisner et al. 2010; Flouris et al. 2009).

The increasing and overwhelming body of evidence showing elevated disease risk among non-smokers exposed to SHS has led to the passage of smoking bans in workplaces and public places, including restaurants and bars. These bans have been shown to have had an important effect (Pirkle et al. 2006; USDHHS 2006). It seems however, that indoor smoking bans are also leading to increased smoking outside establishments or at their entrances as smokers move outside to smoke, creating potentially new or higher SHS exposure to non-smokers. The magnitude of outdoor SHS exposure and associated health risks are unknown as very few studies have characterized outdoor SHS exposure. Thus human health risk assessment and informed tobacco control policies are impeded. Based on a thorough literature search, two published studies other than those presented in this dissertation have measured SHS outside establishments using environmental markers of SHS (Kaufman et al. 2010; Klepeis, Ott, and Switzer 2007) and one using cotinine as a biomarker of exposure (Hall et al. 2009). Further, no studies have assessed immediate health endpoints of exposure to outdoor SHS.

This dissertation therefore reports the results of two studies conducted in Athens, Georgia which sought to characterize outdoor SHS using both environmental and biological markers of exposure to constituents of tobacco smoke as well as a biomarker of effect to investigate potential pre-symptomatic effects of short-term outdoor SHS exposure on non-smokers. Biological markers or biomarkers are defined as cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells or fluid (Hulka 1991). The first study was conducted in 2006 and examined the relationship between changes in particulate matter less than 2.5 μm in aerodynamic diameter ($PM_{2.5}$) and carbon monoxide (CO) concentration outside restaurants and bars with the number of cigarettes smoked outside these establishments as well as with the total number of patrons or pedestrians at the locations and the

number of motorized vehicles passing by each location. It was important to determine the associations between the environmental markers with both number of cigarettes smoked and vehicles because $PM_{2.5}$ and CO are proxies of the particulate and gaseous phases of SHS, respectively and are also produced by a number of other sources such as fuel combustion in the internal combustion engines of vehicles. The description of this study and the results are presented in Chapter 3.

Chapters 4 and 5 present the results of a second study conducted in summer 2010 in which biomarkers of exposure to constituents of tobacco smoke as well as a biomarker of increased lung epithelial permeability, an early indication of air pollution-induced injury, were measured in non-smokers exposed to SHS outside of a restaurant and a bar as well as a control location with no observed smokers. In Chapter 4 salivary cotinine (cotinine is the primary proximate metabolite of nicotine) and urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) were used to characterize exposure to outdoor SHS and tobacco-specific nitrosamines (TSNA), respectively. NNAL is a metabolic breakdown product of the parent compound 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Both NNK and NNAL are known pulmonary carcinogens (Hoffmann, and Hecht 1990). Differences in biomarker responses after 3-h site visits were investigated between the bar, restaurant, and control location. This study is the first to report NNAL levels as well as the ratio between urinary NNAL and salivary cotinine levels in non-smokers exposed to outdoor SHS. In Chapter 5, the utility of urinary Clara cell protein (CC16) as a biomarker of outdoor SHS-induced respiratory epithelial changes are investigated. Serum concentration of the 16-kDa Clara cell specific protein (CC16, CC10 or CCSP) has been proposed as a new sensitive marker to detect an increased permeability of the epithelial barrier (Broeckaert et al. 2000). Urinary CC16 has also been used to characterize lung

epithelial permeability changes following air pollution challenge (Timonen et al. 2004). Due to its relative ease of collection and ability for self-administration, urinary CC16 was considered a more appropriate choice. This study will be the first to report the use of CC16, measured in either urine or serum, to assess outdoor SHS-induced lung epithelial changes.

In addition to the studies presented in Chapters 3 to 5, this dissertation also includes a literature review of the current knowledge on SHS. The literature review, presented in Chapter 2, contains an overview of the chemical composition of SHS, the health effects of SHS which include cancer and cardiovascular and respiratory diseases, markers of SHS, and a short discussion of current smoke-free air laws. In the discussion of markers of SHS, biomarkers of exposure, cotinine and NNAL, and biomarker of effect, CC16, have been highlighted.

In Chapter 6 the summary and conclusions of the studies presented in Chapters 3 to 5 are discussed. Studies of SHS or tobacco smoke have been known to generate public interest and may prompt policy questions. Therefore, the public health implications of the major findings of the studies reported in this dissertation will be discussed in Chapter 7. Finally, the Appendices contain copies of the consent form and baseline and daily questionnaires given to the subjects in the second study reported in Chapters 4 and 5.

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CHAPTER 2

LITERATURE REVIEW

OVERVIEW

There has been an extensive documentation of scientific evidence associating active tobacco use to health risks over several decades. With this accumulating evidence, the Advisory Committee to the US Surgeon General issued the first report on smoking and health in 1964, concluding that cigarette smoking contributes substantially to mortality from certain specific diseases such as lung cancer and emphysema (USDHEW 1964). While subsequent reports were issued detailing and expanding the current knowledge on toxicity and carcinogenicity of tobacco smoke and associated disease risks in active smokers, the 1986 report of the Surgeon General was the first that identified a chronic disease risk from exposure to tobacco smoke for individuals other than smokers (USDHHS 1986). Environmental tobacco smoke (ETS) was defined in this report as a combination of smoke emitted from a burning tobacco product between puffs (sidestream smoke) and the smoke exhaled by the smoker (mainstream smoke). Exposure to ETS was referred to as *involuntary smoking* in the 1986 report to note that such exposures often occur as an "unavoidable consequence of being in close proximity to smokers" (USDHHS 1986). ETS is also known as secondhand smoke (SHS) and passive smoking. The term SHS will be used in this review.

The 1986 report presented evidence that the chemical composition of sidestream smoke is qualitatively similar to mainstream smoke inhaled by active smokers and that both sidestream and mainstream smoke were carcinogens (USDHHS 1986). This report also documented

evidence of a relationship between parental smoking and respiratory diseases in infants and children. While it was noted that a few published studies had shown some association between cardiovascular disease and other non-lung cancer diseases and SHS exposure, the 1986 Report encouraged more studies to elucidate these associations. In 2006 the Surgeon General released a second report on the health consequences of SHS (USDHHS 2006) that updated the 1986 report. Four of the six major conclusions of this report were: 1) SHS causes premature death and disease in children and in adults who do not smoke; 2) exposure to SHS has immediate adverse effects on the cardiovascular system and causes coronary heart disease and lung cancer; 3) the scientific evidence indicates that there is no risk-free level of exposure to SHS; and 4) many millions of Americans are still exposed to SHS in their homes and workplaces (USDHHS 2006). The conclusions of the Surgeon General's reports are also supported by similar reports from the National Research Council (NRC 1986), the US Environmental Protection Agency (USEPA 1992), World Health Organization (WHO 1999), and International Agency for Research on Cancer (IARC 2004).

CHEMICAL COMPOSITION OF SECONDHAND SMOKE

Numerous studies and review articles have presented information on the chemical constituents of mainstream, sidestream, and secondhand smoke (Guerin 1979; Haustein, and Groneberg 2010; Hecht 1999; Jenkins, Guerin, and Tomkins 2000; Kumar Pandey, and Kim 2010; Löfroth 1989; Rodgman, and Perfetti 2006; Stedman 1968). The Surgeon General's 2010 report, *How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease* presents the most up-to-date review of the chemical components present in tobacco smoke (USDHHS 2010). Information presented in this subtopic of the *Literature Review*

has been derived from an exhaustive literature search in Google Scholar, PubMed, and MEDLINE using keywords such as environmental tobacco smoke, secondhand smoke, mainstream, sidestream, chemical components, gas and particulate phase, among others, as well as references from the 2010 Surgeon General's report.

Tobacco smoke is a complex mixture of over 7,000 chemicals including hundreds that are hazardous and at least 69 known carcinogens have been identified in mainstream and sidestream smoke (IARC 2004; USDHHS 2010). Tobacco smoke is also very dynamic, with changes in chemical concentrations as it ages (Schick, and Glantz 2006). Mainstream smoke comprises of a fraction of the inhaled smoke exhaled by the smoker as well as smoke emitted from the butt end of the cigarette during puffs, and sidestream smoke originates from the burning cigarette when it smolders between puffs (Guerin, Higgins, and Jenkins 1987; USDHHS 2010). The concentration of chemicals in mainstream and sidestream smoke vary. Several reasons account for this difference including: (1) puff volume and time between puffs; (2) differences in cigarettes such as tobacco blend and preparation, filters used, additives; and, (3) the temperatures at which cigarettes burn during and between puffs, ~900 ^{o}C during puffs and ~400 ^{o}C between puffs (Guerin et al. 1987; Perfetti, Coleman, and Smith 1998; USDHHS 2010). While the actual concentration of chemicals may vary between mainstream and sidestream smoke, their qualitative compositions are very similar (IARC 2004; USDHHS 1986).

The main components of tobacco smoke are tar, carbon monoxide (CO), nicotine, and nitric oxide, in reducing order of concentration. These compounds are found in much higher concentrations in sidestream smoke compared to mainstream smoke, between two times higher tar and ten times higher nitric oxide concentrations (Norman et al. 1983; Rickert, Robinson, and Collishaw 1984). Sidestream smoke has higher concentrations of volatile organic compounds

(VOCs) such as ethane, propene, 1,3-butadiene, and isoprene (Löfroth 1989), polycyclic aromatic hyrdrocarbon (PAHs) like fluoranthene, benzo[a]pyrene, o-toluidine, 2-napthylamine, and quinodine (Dong et al. 1978; Grimmer, Boehnke, and Harke 1977; Patrianakos, and Hoffmann 1979), N-nitrosamines such as nitrosodimethylamine, nitrosonornicotine, and 4-(methylnitrosamino)-I-(3-pyridyl)-1-butanone (NNK), a highly carcinogenic tobacco-specific nitrosamine (TSNA) (Hoffmann et al. 1984; Schick, and Glantz 2007), and radioactive isotopes such as Polonium-210 (Ferri, and Baratta 1966). Other compounds such as cyanide and certain semi-volatile organic compounds like catechol and hydroquinone are higher in mainstream smoke (Brunnemann, Yu, and Hoffmann 1977; Norman et al. 1983; USDHHS 2010).

The chemical constituents of tobacco smoke exist in either the gas or particulate phase or both (Guerin 1979). While nicotine is easily and consistently measured in the particulate phase of SHS, it is found primarily in the gas phase (Benner et al. 1989; Eatough et al. 1989). Other constituents that are found in the gas phase include CO, nitrous oxide (N_2O), nitrogen dioxide (NO_2), ammonia, 3-ethenylpyridine, myosmine, nitrous acid, pyridine (Eatough et al. 1989), volatile organic compounds such as benzene, 1,3-butadiene, and formaldehyde (Löfroth 1989; Singer et al. 2002), among others. Particulate phase constituents include solanesol, sterols and sterenes (Benner et al. 1989) as well as carboxylic acids, phenols, terpenoids, paraffin waxes, and TSNAs (USDHHS 2010). A chemical analysis of mainstream and sidestream particulate matter had shown that most of the detected compounds were $C_{6\text{-}31}H_{2\text{-}35}N_{0\text{-}7}O_{0\text{-}9}$, with those in sidestream smoke being more unsaturated and less oxygenated than those observed in mainstream smoke (Schramm et al. 2011). This same study also reported higher particulate matter in sidestream smoke than in mainstream smoke (Schramm et al. 2011). Several heavy metals have been measured in the particulate phase of SHS, including cadmium (Cd), arsenic

(As), antimony (Sb), zinc (Zn) (Landsberger, and Wu 1995), chromium (Cr), lead (Pb), and nickel (Ni) (USDHHS 2006). Pure metallic mercury (Hg) may be released in the gas phase of SHS (Chiba, and Masironi 1992). Cd, a highly toxic heavy metal accumulates in unusually high concentrations in tobacco leaves from the soil and is one of the most important heavy metals when the adverse health effects of SHS are considered (Chiba, and Masironi 1992).

The chemical composition of SHS, as mentioned, is very dynamic, and varies with environmental conditions because of transformation processes (Singer et al. 2002). For example, the selective sorption of specific components of SHS to indoor surfaces may reduce the air concentration of these SHS constituents. Consequently, the desorption of these constituents from indoor surfaces over time into indoor environments gives rise to *thirdhand smoke* exposure, a phenomenon that is not covered in this literature review (Matt et al. 2011). Indoor concentrations of all SHS constituents depend on smoking frequency, dilution volume, and ventilation rate (Singer et al. 2002). Further, as SHS ages, the concentration of its constituents is affected by chemical transformations. The high temperatures reached during the combustion of a cigarette induces pyrolytic reactions as well as the formation of reactive radical species (Borgerding, and Klus 2005; Schramm et al. 2011). These reactive radical species may react with various components present in SHS and lead to the formation of new components or increase the concentration of others (Schramm et al. 2011), causing the composition of SHS to evolve until all reactive species are quenched or consumed (Baker, and Bishop 2004). For example, it has been suggested that gas-phase nitrosation of nicotine and/or nicotine breakdown products in aging SHS may increase the concentration of nitrosamines in SHS (Schick, and Glantz 2007). This is based on unpublished research from Philip Morris Tobacco Company which shows that NNK can form in sidestream smoke after it has been released into the air, with increases as much

as 50% to 200% per hour during the first 6 h after cigarettes are extinguished in a chamber study and increases for the first 2 h after cigarettes are extinguished in real offices (Schick, and Glantz 2007). SHS constituents in outdoor SHS are typically lower than indoor SHS because of larger dilution volumes and ventilation rates along with meteorological factors such as wind speed, temperature, and relative humidity.

HEALTH EFFECTS OF SECONDHAND SMOKE EXPOSURE

Molecular, animal and epidemiologic studies have provided compelling evidence that exposure to SHS causes disease and death in humans. These health effects include, and are not limited to, cancer, cardiovascular disease, and respiratory diseases. The evidence and underlying mechanisms implicating SHS exposure in the formation of these diseases will be discussed in this subsection of the *Literature Review*. As done before, the literature search was conducted using Google Scholar search engine and PubMed and MEDLINE databases as well as relevant references from the 2006 and 2010 Surgeon General Reports (USDHHS 2006, 2010).

Secondhand Smoke and Cancer

The International Agency for Research on Cancer (IARC) has identified 69 carcinogens in tobacco smoke that are carcinogenic to laboratory animals, of which 15 are rated as carcinogenic in humans (group 1 carcinogens) (IARC 2004). These 15 are benzo[a]pyrene, N-nitrosonornicotine (NNN), NNK, 2-naphthylamine, 4-aminobiphenyl, formaldehyde, benzene, arsenic, beryllium, nickel, chromium, cadmium, Po-210, vinyl chloride, and ethylene oxide (Hoffmann, Hoffmann, and El-Bayoumy 2001; IARC 2004; USDHHS 2006). There are no published studies on the concentration of some of the 69 carcinogens in sidestream smoke and SHS (Hoffmann et al. 2001). However, it is very likely that all of these carcinogens are present

in sidestream smoke and SHS (USDHHS 2006). It should be noted that sidestream condensate has been found to be approximately three times more toxic per gram and two to six times more tumorigenic per gram than mainstream condensate (Schick, and Glantz 2005).

The 2004 Surgeon General's report, *The Health Consequences of Smoking: A Report of the Surgeon General* (USDHHS 2004), concluded that there is sufficient evidence to infer a causal relationship between smoking and cancers of the lung, larynx, oral cavity, pharynx, esophagus, pancreas, bladder, kidney, cervix, and stomach, and acute myeloid leukemia. The 2004 report also found evidence suggesting a causal relationship between smoking and colorectal and liver cancers (USDHHS 2004). While it is not clear that all of these cancers are causally associated to SHS exposure because of the substantially lower carcinogenic dose from SHS (USDHHS 2006), IARC concluded that SHS causes lung cancer (IARC 2004). This conclusion was based on 50 epidemiologic studies of involuntary smoking and lung cancer risk in never smokers. These studies are further strengthened by biochemical data demonstrating carcinogen uptake in non-smokers exposed to SHS (Hecht 2004). The 2006 Surgeon General report suggested that uptake of NNK by non-smokers exposed to SHS provides a biochemical link between SHS exposure and lung cancer risk (USDHHS 2006).

Lung cancer in never smokers is an important public health issue. Approximately 10% to 15% of all lung cancers arise in never smokers, making lung cancer in never smokers one of the leading causes of cancer-related mortality (Jemal et al. 2008; Samet et al. 2009; Thun et al. 2006). In a review of six cohort studies: Nurses' Health Study; Health Professionals Follow-Up Study; California Teachers Study; Multiethnic Cohort Study; Swedish Lung Cancer Register in Uppsala/Orebro region; and First National Health and Nutrition Examination Survey Epidemiologic Follow-Up Study, Wakelee and colleagues reported age-adjusted lung cancer

rates among never smokers age 40 – 79 years to range from 14.4 to 20.8 per 100, 000 person-years in women and 4.8 to 13.7 per 100, 000 person years in men (Wakelee et al. 2007). There is strong evidence for a causal relationship between lung cancer in non-smokers and SHS. In a pooled analysis of two large case-control studies of lung cancer among never smokers from metropolitan areas in the United States, Germany, Italy, Sweden, United Kingdom, France, Spain, and Portugal, Brennan and colleagues found odds ratios (OR) for ever exposure to spousal smoking of 1.18 (95% CI = 1.01 – 1.37) and long term exposure of 1.23 (95% CI = 1.01 – 1.51) (Brennan et al. 2004). A clear dose-response consistent with a causal association was observed (Brennan et al. 2004). Stayner and colleagues conducted a meta-analysis of data from 22 studies from multiple locations worldwide of workplace exposure to SHS and lung cancer (Stayner et al. 2007). They reported relative risks (RR) of 1.24 (95% CI = 1.18 – 1.29) among workers exposed to SHS. They also reported a 2-fold increase in risk (RR = 2.01; 95% CI = 1.33 – 2.60) for workers classified as highly exposed to SHS (Stayner et al. 2007). Vineis and colleagues reported lung cancers in never- and ex-smokers in a large prospective study in 10 European countries [(European Prospective Investigation into Cancer and Nutrition) (N = 520,000)] and estimated the proportion of lung cancer attributable to SHS was between 16% (hazard ratio (HR): 1.34; 95% CI 0.85 – 2.13) and 24% (1.65; 95% CI = 1.04 – 2.63) (Vineis et al. 2007).

A growing number of studies have shown that SHS exposure is associated to breast cancer in women. The California Environmental Protection Agency (Cal/EPA) reviewed 26 published reports, of which three were meta-anlyses, investigating the association between SHS exposure and breast cancer. Their meta-analysis indicated relative risks ranging from OR 1.68 (95% CI = 1.31 – 2.15) for 14 of the studies that allowed analysis by menopausal status to 2.20 (95% CI = 1.69 – 2.87) for studies with the best exposure assessment (Miller et al. 2007). Based

on these significant relative risks and low likelihood of bias and confounding factors explaining the associations, Cal/EPA concluded that regular SHS exposure is causally related to breast cancer diagnosed in younger, primarily premenopausal women (Miller et al. 2007). A case-control study by Rollison and colleagues did not find evidence to support a causal association between SHS exposure and breast cancer of women in Delaware (Rollison et al. 2008). A large prospective study within the Million Women Study in the UK and a meta-analysis by Pirie and colleagues also found no significant association between breast cancer and SHS exposure during childhood or as an adult (Pirie et al. 2008). Despite these studies showing no significant association between breast cancer and SHS exposure, it has been suggested that evidence from epidemiologic studies of secondhand smoke used in Cal/EPA's 2005 report for breast cancer in younger, primarily premenopausal women was stronger than for lung cancer in the 1986 Surgeon General's report (Johnson, and Glantz 2008).

SHS may also be a risk factor for other cancers such as bladder and pancreatic cancers. One prospective study conducted by Alberg and colleagues investigated the association between bladder cancer and household exposure to SHS in two cohorts from Maryland, in 1963 (n = 45, 749; 93 cases) and 1975 (n = 48, 172; 172 cases) (Alberg et al. 2007). They reported that current household SHS exposure was associated to bladder cancer risk in the 1963 cohort (RR = 2.3, 95% CI = 1.0 – 5.4) but not in the 1975 cohort (RR = 0.9, 95% CI = 0.4 – 2.3) among nonsmoking women (Alberg et al. 2007). In a meta-analysis of three cohort and five case-control studies investigating SHS and bladder cancer risk, Van Hemelrijck and colleagues found no evidence for an association between SHS and bladder cancer (RR = 0.99, 95% CI = 0.86 – 1.14) (Van Hemelrijck et al. 2009). Bao and colleagues prospectively examined 86,673 women for 24 years in the Nurses' Health Study to investigate the association between SHS and pancreatic

cancer (Bao et al. 2009). Their results showed that maternal smoking significantly increased the risk of pancreatic cancer (RR = 1.42, 95% CI = 1.07 – 1.89) and no association was found between the risk and paternal smoking or adult exposure at home or at work, suggesting an association between SHS *in utero* or in early life may be associated to pancreatic cancer (Bao et al. 2009). Further, Peppone and colleagues reported that among never smokers, individuals with past SHS exposure were diagnosed with colorectal cancer at a significantly younger age compared to the unexposed (Peppone et al. 2008). More studies are required to characterize the association between the risk of non-lung cancers and SHS exposure.

The currently known underlying mechanisms by which carcinogens in tobacco smoke elicit carcinogenesis are well documented in the 2010 Surgeon General report (USDHHS 2010). Although the carcinogenicity of sidestream smoke and SHS has been less extensively studied relative to mainstream smoke, the mechanisms of cancer induction from exposure to SHS and mainstream smoke are probably similar because the same carcinogens are present in both (USDHHS 2006). As mentioned previously, the major difference is the lower carcinogenic dose from inhaling SHS compared with active smoking (USDHHS 2006). A genotoxic mode of action for some of the carcinogens has been elucidated, which relies on their ability to covalently bind DNA forming DNA adducts (Besaratinia, and Pfeifer 2008; Hecht 2003). DNA adduct formation is central to cancer induction and carcinogenesis. In order to covalently bind to DNA, most carcinogens in tobacco smoke must undergo metabolic activation processes (USDHHS 2010) that form reactive electrophilic species. These reactions are generally catalyzed by cytochrome P450 (P450) enzymes in phase I reactions. Several P450s are inducible by components of tobacco smoke, including P450s 1A1 and 1B1 which play an important role in the metabolic activation of PAHs (USDHHS 2010) and P450s 1A2, 2A, 2B1, and 3A which play a role in

NNK metabolism (Hecht 1998). Metabolic detoxification processes catalyzed by phase II enzymes such as glutathione-S-transferases (GSTs), epoxide hydrolase, and sulfatases, excretes carcinogen metabolites in generally less toxic breakdown products or conjugates. These detoxification processes compete with activation processes and variations in the balance of these competing processes among persons likely affect cancer susceptibility (USDHHS 2006). Some carcinogens such as ethylene oxide can form DNA-adducts through direct reaction with DNA without the need for metabolic activation (USDHHS 2010).

Direct repair, base excision repair, nucleotide excision repair, mismatch repair, and double-strand break repair are five of the main mechanisms of DNA repair (Houtgraaf, Versmissen, and van der Giessen 2006) along with apoptosis (programmed cell death) to rid cells of DNA adducts. Formation of DNA adducts can initiate carcinogenesis because persistent DNA adducts can be misinstructional during DNA replication, thus giving rise to mutations which may lead to loss of normal functions in control of cellular growth, ultimately resulting in cellular proliferation and cancer (Besaratinia, and Pfeifer 2008; Luch 2005; USDHHS 2010). Cigarette smoke activates epidermal growth factor receptors (EGFR) and cyclooxygenase (COX-2) which are both known to play vital roles in cell proliferation and transformation (USDHHS 2010). Recent studies have shown that activating mutations in the EGFR tyrosine kinase domain occur much more frequently in lung cancers in non- and never-smoking patients and that these mutations occur more often in adenocarcinomas (Mok et al. 2009). Interestingly, adenocarcinoma is the major form of lung cancer observed among never smokers (Brenner et al. 2010; Powell et al. 2003) and NNK has been known to induce it in lab animals (Schuller, and Cekanova 2005). SHS also contains cocarcinogens and tumor promoters that are not carcinogenic but enhance the carcinogenicity of SHS carcinogens through stimulation of cell

proliferation as well as epigenetic factors which hypermethylate genes leading to their silencing (USDHHS 2010).

Secondhand Smoke and Cardiovascular Diseases

Secondhand smoke increases the risk of cardiovascular disease by ~30% (Barnoya, and Glantz 2005; Glantz, and Parmley 1991; Taylor, Johnson, and Kazemi 1992) and accounts for at least 30,000 deaths annually in the United States (Adhikari et al. 2008). In a review of SHS and cardiovascular disease, Barnoya and Glantz concluded that the effects of brief (minutes to hours) SHS are substantial and rapid and are often as large, averaging 80% to 90%, as chronic active smoking (Barnoya, and Glantz 2005). The effects of SHS on the cardiovascular system can be summarized as: platelet activation; endothelial dysfunction; inflammation and infection; atherosclerosis; increased oxidative stress; decreased energy metabolism; increased insulin resistance; and outcome measures such as increased infarct size, decreased heart rate variability, increased arterial stiffness, and increased risk of coronary events (Barnoya, and Glantz 2005). The Institute of Medicine (IOM) concluded that on the basis of the available studies of chronic exposure to SHS and cardiovascular disease, there is scientific consensus that there is a causal relationship between SHS exposure and cardiovascular disease (IOM 2009). A number of chemicals in SHS which exceed 10 μg per cigarette have been found to be cardiotoxic. These include carbon monoxide, nicotine, formaldehyde, benzene, 1,3-butadiene, acrolein, carbon disulfide, and 3-vinylpyridine (IOM 2009).

Studies on platelet activity provided the first mechanistic evidence explaining why SHS increases the risk of heart disease or death (Barnoya, and Glantz 2005). Exposure to SHS has been shown to activate blood platelets making them more adhesive and thereby increasing the likelihood of a thrombus (USDHHS 2006). Circulating activated platelets further lead to the

formation of atherosclerotic lesions (Huo et al. 2002) and damage to the lining of the coronary arteries (USDHHS 2006). SHS promotes atherosclerosis by oxidizing low-density lipids (LDLs) in non-smokers which may in turn become lodged in the arterial wall, attracting macrophages, lymphocytes, and subsequently paracrinine factors that will lead to platelet accumulation and thus initiate the creation of foam cells, the first step in developing an atherosclerotic plaque (Campbell, Moffatt, and Stamford 2008; Vardavas, and Panagiotakos 2009). Markers of platelet activation such as fibrinogen, which is associated to higher risk of heart disease, and thromboxane (Topol, and Califf 2007) are elevated after exposure to SHS (Barnoya, and Glantz 2005; Jefferis et al. 2010; Schmid et al. 1996). SHS-activated platelets also damage the endothelium, a vital layer of the arterial wall (Barnoya, and Glantz 2005).

Arteries are lined by a layer of cells known as the endothelium, the first layer in the arterial bed that is in contact with blood (USDHHS 2006) and hence with toxins circulating in blood. By secreting nitric oxide and endothelin, the endothelium controls vasodilation and vasoconstriction, respectively (Barnoya, and Glantz 2005). The endothelium plays a central role in vascular homeostasis and the pathogenesis of cardiovascular disease (Widlansky et al. 2003). Endothelial dysfunction refers to the alterations in the endothelium that may contribute to the development and clinical expression of atherosclerosis (Levine, Keaney, and Vita 1995). SHS has been known to induce endothelial dysfunction, which can be manifested clinically within 15 to 30 minutes of SHS exposure as well as following chronic SHS exposure (Barnoya, and Glantz 2005; Glantz, and Parmley 2001; Jefferis et al. 2010). In one study Giannini and colleagues observed significant endothelium-dependent brachial artery dilation, one measure of endothelium dysfunction, in 18 healthy young never smokers, following 20 minutes exposure to SHS (Giannini et al. 2007). In another study, endothelial function was assessed by reactive hyperemia

peripheral arterial tonometry index (RH-PAT) in 18 non-smoking male following a 1–hr SHS exposure (Bonetti et al. 2010). Bonetti and colleagues reported a significant reduction in RH-PAT index following SHS exposure, indicating deterioration of peripheral microvascular endothelial function (Bonetti et al. 2010). Another study reported a significant increase in endothelial progenitor cells (EPC), plasma vascular endothelial growth factor and completely abolished EPC chemotaxis during 24 h following a 30-min SHS exposure, suggesting that SHS not only affects the vascular endothelium, but also the function of EPCs (Heiss et al. 2008). A longitudinal study of adolescents reported decreased flow-mediated dilation of the brachial artery in subjects chronically exposed to SHS (Kallio et al. 2010).

Human and animal data support the conclusion that SHS exposure increases inflammation, which is another potential mechanism by which SHS causes cardiovascular disease (Barnoya, and Glantz 2005). Atherosclerosis is itself regarded as an inflammatory disease, with progressive stages of atherosclerosis associated with the enhanced activation of T-cells, inflammatory cytokines, and platelet aggravation (Barnoya, and Glantz 2005; Vardavas, and Panagiotakos 2009). SHS exposure has been shown to stimulate human fibroblasts to express several chemokines which may play a role in the formation of atherosclerotic lesions (Vardavas, and Panagiotakos 2009). Several studies have reported associations between inflammatory markers and SHS. In a cross-sectional study of 5029 men and women, Jefferis and colleagues measured several circulating markers of inflammation such as C-reactive protein (CRP), fibrinogen, factor VIII, von Willebrand factor (VWF), and tissue plasminogen activator (tPA) that were positively associated with blood cotinine levels (Jefferis et al. 2010). Flouris and colleagues reported significant increases in tumor necrosis factor-alpha (TNF-α) and interleukin-4 following 1-h exposure to SHS (Flouris et al. 2009). Another study showed that after exposure

for about 30 minutes a day for a week, subjects had higher white blood cell counts and CRP (Panagiotakos et al. 2004). Yuan *et al*. concluded from a mouse model that long-term exposure to SHS creates a state of permanent inflammation (Yuan et al. 2007). Further chronic infections such as chronic obstructive pulmonary disease (COPD), recurrent urinary tract infection, and chronic bronchitis, which are associated to SHS exposure, have been proposed to contribute to atherosclerosis just as inflammation does (Barnoya, and Glantz 2005; Gupta 1999).

SHS is a source of free radical and reactive oxygen species (ROS) that are destructive to heart muscle cell membrane, other processes within the cell (USDHHS 2006), and depletion of antioxidants, thus creating oxidative stress. This SHS-induced oxidative stress is attained by direct delivery of ROS in SHS to the vascular system and consumption of antioxidants used to protect against endogenous ROS produced in the respiratory process (Barnoya, and Glantz 2005). Products of lipid oxidations such as isoprostanes have been used to show SHS induces oxidative stress in non-smokers (Barnoya, and Glantz 2006; Kato et al. 2006). Further, a mouse model has demonstrated that a 30-minute SHS exposure leads to oxidative DNA damage in the myocardium assessed by increased levels of 8-hydroxydeoxyguanosine (8-OHdG) (Howard, Briggs, and Pritsos 1998; USDHHS 2006).

Epidemiologic data have provided compelling evidence that SHS is a risk factor for cardiovascular disease. A number of published meta-analyses have yielded relative risks between 1.2 and 1.3 (Barnoya, and Glantz 2005). In a pooled analysis of 29 studies, Barnoya and Glantz computed a relative risk 1.31 (95% CI = 1.21 – 1.41). In a prospective study of 13,443 English and Scottish participants, Hamer and colleagues used objectively measured SHS exposure to compute hazard ratios for death by cardiovascular disease as 1.21 (95% CI = 0.85 – 1.73) (Hamer et al. 2010). Hill *et al.* conducted a cohort study to determine the risk of cardiovascular

disease among lifelong non-smokers exposed to SHS at home (Hill et al. 2007). They reported relative risk estimates for all cardiovascular diseases as 1.19 (95% CI = 1.04 – 1.38) among men and 1.01 (95% CI = 0.88 – 1.16) among women from their 1981 – 1984 cohort, and 1.25 (95% CI = 1.06 – 1.47) among men and 1.35 (95% CI = 1.11 – 1.64) among women from their 1996 – 1999 cohort (Hill et al. 2007).

Recently, comparisons between prevalence of cardiovascular disease before and after the implementation of smoking bans have provided even more evidence of the association between SHS and cardiovascular disease. Reduction in acute myocardial infarctions have reportedly reduced by 11% in Italy (Barone-Adesi et al. 2006) and 40% in Montana (Sargent, Shepard, and Glantz 2004). The study in Helena, Montana was the first of such studies, in which the association of a newly enacted smoking ban with admissions for myocardial infarction from within Helena (intervention) and from outside Helena, where there was no smoke-free ordinance, was investigated (Sargent et al. 2004). Seo and colleagues also reported a decline in acute myocardial infarction admissions to hospitals in Indiana following the implementation of smoking bans there (Seo, and Torabi 2007). In a pooled analyses, Lightwood and colleagues computed random effects estimate of the rate of acute myocardial infarction hospitalization 12 months after implementation of smoking bans as 0.83 (95% CI = 0.80 – 0.87) (Lightwood, and Glantz 2009).

Even as the evidence that SHS causes cardiovascular disease continues to accumulate, the current evidence shows consistently and convincingly that SHS has significant effects on the cardiovascular system, effects that are on average 80% to 90% as large as those from active smoking (Barnoya, and Glantz 2005).

Secondhand Smoke and Respiratory Diseases

The 1986 and 2006 Surgeon General reports concluded, and was reemphasized in the 2010 report, that the current scientific evidence is sufficient to infer a casual association between a number of respiratory outcomes and exposure to SHS (USDHHS 1986, 2006, 2010). These include associations between parental smoking and lower respiratory illnesses in infants and children, middle ear disease in children, and cough, phlegm, wheeze, and breathlessness among school-aged children (USDHHS 2004, 2006). The reports also documented associations between maternal smoking during pregnancy and persistent adverse effects on lung function across childhood; exposures to SHS after birth with lower level of lung function during childhood; and other effects such as odor annoyance and nasal irritation (USDHHS 2004, 2006, 2010).

There are several constituents of SHS that are selectively toxic to the respiratory track. These include acrolein, which is toxic to cilia and impairs lung function; formaldehyde, which acts as a respiratory track irritant and is toxic to cilia; nitrogen oxides, which act as oxidants; cadmium promotes emphysema and causes oxidative injury; and hydrogen cyanide, which affects cells through oxidative metabolism (USDHHS 2010). Further, the smallest particles present in SHS, less than 2.5 μm, can penetrate to and be deposited deep in the lung. Ultrafine particles, <0.1 μm, have been shown to induce oxidative stress in Clara cells of the respiratory track in allergic lung inflammation (Alessandrini et al. 2010).

Asthma, a chronic respiratory disease of the airways, has been causally associated to SHS exposure. A number of studies have identified significant associations between parental smoking and development of asthma in children (Cook, and Strachan 1997; Jaakkola, and Gissler 2004). One study showed that the prevalence of asthma in children increased with the number of household smokers (Cook, and Strachan 1997). Others have shown that the risk for asthma and

wheezing in children is linked to both prenatal and childhood exposures to SHS (Wang et al. 2008). Several mechanisms have been suggested to explain the increased risk of childhood asthma from prenatal and childhood exposures to SHS. These include: the impairment of fetal airway development; induction of bronchial hyperreactivity; impairment of neural control of airways; altered immune responses; and modification of the balance of immune cells in the airways (USDHHS 2006). Based on a mouse model, the developing fetus has been shown to be extraordinarily sensitive to cigarette smoke, exhibiting increased lung inflammation, atopy, and airway resistance, and induction of allergic asthma after postnatal exposures to allergens (Singh et al. 2009). Epidemiologic studies have also provided evidence that SHS exposure is a risk factor for new-onset asthma among adults and exacerbates pre-existing adult asthma (Eisner 2008). Several adult deaths have been attributed to SHS exposure (Invernizzi et al. 2008; Stanbury et al. 2008).

There is growing evidence that SHS is a potential cause of obstructive lung disease. While these diseases generally result from long-term processes, studies on short-term exposures have provided evidence supporting the association between SHS and obstructive lung diseases. Flouris and colleagues reported significant decrements on lung function and increases in inflammatory cytokines after a 1-hr SHS exposure (Flouris et al. 2009). The cytokines remained elevated for at least three hours following SHS exposure (Flouris et al. 2009). Pro-inflammatory cytokines have been linked to the development and/or exacerbation of chronic lung disease (Chung 2001). Eisner and colleagues have reported several studies that have demonstrated associations between biological markers of SHS exposure and greater chronic obstructive pulmonary disease (COPD) severity, exacerbation, and increased emergency department visits for COPD (Eisner et al. 2006; Eisner et al. 2010; Eisner et al. 2009a; Eisner et al. 2009b). One

study of healthy, never smoking flight attendants attributed long-term damage to their lungs to SHS exposures in cabin during flights (Arjomandi et al. 2009).

Several other studies have reported increased risk of chronic bronchitis from SHS exposure. A study in Canada reported a 50% odds of never-smokers and ex-smokers exposed to SHS having chronic bronchitis (Vozoris, and Lougheed 2008). This was consistent with the results of the 2005 Canadian Community Health Survey, which reported that home and vehicle SHS were significantly associated to chronic bronchitis in children and adolescents [odds ratio = 2.30 (95% CI = 1.46 – 3.63) and 2.25 (95% CI = 1.42 – 3.58), respectively] (Evans, and Chen 2009). A study of women exposed to a lifetime of SHS in Taiwan found a 3.65-fold (95% CI = 1.19 – 11.26) increase in chronic bronchitis due to SHS exposure (Wu et al. 2010). Other respiratory outcomes such as cough and sinusitis (Hammad et al. 2010) have been attributed to SHS as well as childhood exposures to SHS with early emphysema in adult non-smokers (Lovasi et al. 2009).

MARKERS OF SECONDHAND SMOKE EXPOSURE

Environmental Markers

Exposure to SHS can be estimated using environmental markers of tobacco smoke measured in air. Assessing SHS constituents in air has been difficult because few SHS-unique constituents have been detected. The National Research Council's (NRC) proposed criteria for valid markers of air SHS are: a) should be unique or nearly unique for SHS, b) should be easily detectable, c) should be emitted at similar rates for a variety of tobacco products, and d) should have a fairly constant ratio to other SHS components of interest under a range of environmental conditions (Benowitz 1999; NRC 1986). Nicotine and its related alkaloids are unique to SHS but

they appear to behave differently in the ambient environment than particulate matter or constituents of concern (Jenkins et al. 2000). Constituents such as carbon monoxide, nitrogen oxides, formaldehyde, and petroleum hydrocarbons are present in ambient environments from SHS but are frequently the principal result of other sources (Jenkins et al. 2000). Nicotine breakdown products of pyrolytic reactions and tobacco proteins such as pyridines, pyrrolidines, and nitriles may be SHS-specific and are potential markers of SHS (Jenkins et al. 2000). The currently favored environmental markers of SHS are solanesol and scopoletin, which are used as measures of particulate matter (Bayne, Dindal, and Guerin 1996; Douce, Clench, and Frost 2001), 3-ethenyl pyridine for volatile organic compounds (LaKind et al. 1999), and nicotine (Jenkins, Palausky, and Counts 1995; Jenkins et al. 2000). Particulate matter less than 2.5 μm ($PM_{2.5}$) although not specific to SHS has been used extensively as an environmental marker of SHS (Cameron et al. 2010) and has been shown to be highly correlated to air nicotine ($R^2 = 0.91$) (Jenkins et al. 1996). $PM_{2.5}$ and carbon monoxide were used as environmental markers of exposure in this doctoral research project.

Biological Markers of Exposure

While measurement of specific markers such as nicotine and non-specific proxies such as carbon monoxide and particulate matter are able to estimate SHS exposure, human exposure estimates may be highly imprecise due to factors such as room ventilation and proximity to smokers. The optimal assessment of exposure to tobacco smoke is through direct measurement of a component of tobacco smoke, a biologic marker or biomarker, in body fluids of an exposed individual (Benowitz 1999). Valid biomarkers of SHS should also satisfy the NRC's criteria listed above under "Environmental Markers" in addition to other issues such as how well the biomarker indicates long-term exposure to SHS as well as whether a biomarker predicts the

likelihood of SHS-related disease (Benowitz 1999). Salivary cotinine and urinary NNAL were used as biomarkers of SHS in this doctoral research project.

i. Cotinine

Nicotine, the principal tobacco alkaloid (about 95% of total alkaloid content and about 1.5% by weight in commercial cigarettes) is extensively metabolized to a number of metabolites by the liver. The most important of these metabolites is cotinine, which accounts for 70% to 80% of nicotine (Hukkanen, Jacob, and Benowitz 2005). Nicotine is converted to cotinine via two steps: 1) CYP450-mediated conversion to nicotine-1(5')-iminium ion, followed by, 2) cytoplasmic aldehyde oxidase-catalysed transformation to cotinine (Hukkanen et al. 2005). Cotinine has little or no known health effects in humans (Hukkanen et al. 2005).

Cotinine is at present, the most specific and most sensitive biomarker for exposure to nicotine from SHS (Benowitz 1999). Plasma, urinary, and salivary concentrations of cotinine have been used to assess population exposures to SHS (Akhtar et al. 2007; Farrelly et al. 2005). Cotinine levels can be used to distinguish active from passive smokers. Optimal serum cotinine cutpoints have been revised to 3.08 ng/mL and 2.99 ng/mL for adults and adolescents (Benowitz et al. 2009). Studies have also shown that cotinine correlates well with certain health endpoints of SHS such as asthma (Eisner et al. 2005). Recently, however, it was suggested that based on the discrepancy between relative cotinine levels and disease risk with SHS versus active smoking (cotinine levels in SHS exposed is <1% of smokers' levels while cardiovascular disease is 80% to 90% the size of smokers) that cotinine is not providing an accurate measure of exposure to the toxic constituents of tobacco smoke (Benowitz et al. 2010). Despite this new finding, and although limited by interindividual variability among human subjects due to differences such as metabolism and clearance, and its inability to characterize long-term exposure, cotinine levels

provide valid and quantitative measures of average ongoing human SHS exposure over time (Benowitz 1999).

ii. NNAL

As mentioned previously, SHS contains the carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Hoffmann, and Hecht 1990; Hoffmann et al. 2001). The overall pathway leading to the initiation of cancer has been demonstrated for tobacco-specific nitrosamines like NNK in humans, which is supported overwhelmingly by epidemiologic data (Hecht 1998). There is strong evidence that NNK is a causative agent in the formation of lung adenocarcinoma in smokers (Hoffmann, Rivenson, and Hecht 1996). Adenocarcinoma is now the most frequent type of lung tumor found in non-smokers (Hoffmann et al. 1996). NNK is mainly metabolized by keto reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) which is conjugated through the glucuronidation pathway to 4-(methylnitrosamino)-1-(3-pyridyl)-but-1-yl]-β-O-D-glucosiduronic acid (NNAL-Gluc) (Hecht 1996). NNAL is not a detoxified metabolite since it has similar carcinogenic activity to NNK (NNAL-GLuc is a detoxified metabolite) (Hecht 1998). Urine is the major route of excretion of NNK metabolites (Hecht 1998).

NNAL/NNAL-Gluc more directly reflect exposure to carcinogens in SHS than cotinine (Benowitz 1999). Further, the half-life of NNAL (10 – 16 days) is much longer than that of cotinine (16 hr), suggesting that NNAL is a better biomarker of long-term SHS exposure (Goniewicz et al. 2011). Recent studies have shown that urine cotinine levels underestimate exposure to NNK in passive versus active smokers (Benowitz et al. 2010). The ratio NNAL/cotinine has been shown to be much higher in passive smokers compared to active smokers (Goniewicz et al. 2011).

Biological Markers of Effect

Acute and short-term SHS exposure are associated to changes in endothelial vascular function (Argacha et al. 2008), increased circulating levels of cytokines (Flouris et al. 2009), and oxidative stress (Kato et al. 2006). Urinary Clara cell protein (CC16) was used to assess the acute effects of SHS in this doctoral thesis.

Clara cell protein (CC16)

The respiratory epithelium, a selectively permeable barrier separating the airways and airspaces from the submucosa and interstitium of the lungs and the pulmonary vasculature, acts as a barrier to the entry of potentially noxious agents such as bacteria, viruses, pollutants, and allergens (Morrison et al. 1999). The impermeable barrier is made possible by tight junctions, cell-cell, and cell extra-cellular matrix interactions (Holgate 2008). Although the exact mechanism is not clearly elucidated, evidence suggests that mainstream cigarette smoke increases the permeability of human airways (Olivera et al. 2007); the lung epithelium of smokers is more permeable than that of non-smokers (Jones et al. 1980). Cigarette smoke-induced alterations of pulmonary epithelial permeability are rapidly reversible (Mason et al. 1983). Olivera and colleagues demonstrated that smoke-induced loss of epithelial barrier function is a regulated process rather than a cytotoxic response and the activation of protein tyrosine kinases and Rho kinase and inactivation of myosin light chain kinase contribute to the increased airway permeability caused by mainstream cigarette smoke (Olivera et al. 2007). The airway epithelium is central to the pathogenesis of respiratory diseases such as asthma; the barrier function of the epithelium is impaired through defective tight junction formation potentially leading to penetration of toxic and damaging environmental agents in asthma (Holgate 2008).

One of the biomarkers used to assess epithelial permeability is the 16-kDa Clara cell specific protein (CC16, CC10 or CCSP). Clara cells, non-ciliated cells found predominantly in the respiratory and terminal bronchioles, are known for their high vulnerability to inhaled or systemic lung toxicants (Bernard 2008). CC16 is also found in prostate, endometrium, and kidney about 20 times lower than those present in the lung (Broeckaert et al. 2000b). CC16 is a short-lived protein that acts as an immunosuppressant and downregulates the activation of Th1 cell immune system, thus protecting from tissue injuries (Kotani et al. 2007). CC16 responds very quickly to permeability changes in the bronchoalveolar capillary barrier (Bernard 2008), hence its utility as a marker of epithelial damage. CC16 is normally secreted in large amounts at the surface of airways and leaks across the epithelium into the blood probably through passive diffusion due to the observed high concentration gradient between the epithelial lining fluid and blood (Broeckaert et al. 2000a; Hantson, Bernard, and Hermans 2008). Increased epithelial permeability may result in higher rates of passive diffusion and a transient increase in the concentration of CC16 protein in serum. Serum concentration of CC16 serves as a new sensitive marker to detect an increased permeability of the epithelial barrier, which is one of the earliest signs of air pollution-induced lung injury (Broeckaert et al. 2000a). While CC16 is not specific to a particular toxicant, it is specific to lung epithelium damage or dysfunction caused by toxic chemicals (Bernard 2008). Urinary CC16 has also been used because urine collection is less invasive, less complicated, and easily self-administered compared to blood sampling.

Human and animal data demonstrate the utility of CC16 as a marker of changes in lung epithelial permeability after exposure to several toxicants. Van Miert and colleagues measured up to five-fold increases in serum CC16 in rats following 1-h exposure to mainstream tobacco smoke at 2 and 4 h after exposure and a return to baseline concentrations at 24 h after exposure

(Van Miert, Dumont, and Bernard 2005). In an epidemiologic study, Jacquemin and colleagues reported significant associations between $PM_{2.5}$ from combustion sources and urinary CC16; urinary CC16 increased by 0.6% per 1 x 10^{-5} m^{-1} increase in same-day levels of $PM_{2.5}$ (Jacquemin et al. 2009). The association between ultrafine particles and increased urinary CC16 was also reported in a study of human subjects in Germany, Netherlands, and Finland (Timonen et al. 2004). Another study reported significant associations between short-term variations in ambient air pollution and increases in serum CC16 among elderly men in Oslo, Norway (Madsen et al. 2008). A study involving firefighters showed 328% average increase in serum CC16 after a fire in the absence of any functional sign of lung impairment (Bernard, Hermans, and VanHoute 1997). Broeckaert and colleagues observed significant increases in serum CC16 in cyclists who exercised for 2 h during episodes of photochemical smog (Broeckaert et al. 2000a). These studies confirm that CC16 can be appropriately used as a biomarker of toxicant-induced transient changes to lung epithelial permeability.

SMOKE-FREE AIR LAWS

The increasing and overwhelming body of evidence showing elevated disease risk among non-smokers exposed to SHS has led to the passage of clean indoor air acts that ban smoking in the indoor environment in workplaces and public places, including restaurants and bars. As of January 2011, 21, 850 municipalities are covered by a 100% smokefree provision in workplaces, and/or restaurants, and/or bars, by either a state, commonwealth, or local law, representing 79.4% of the US population (ANRF 2011). Further, 39 states and the District of Columbia have local laws in effect that require 100% smoke-free workplaces and/or restaurants and/or bars (ANRF 2011). The state of Georgia passed a state-wide partial smoking ban in 2005 banning

smoking in restaurants and bars that serve or employ minors (Georgia 2005). The Athens-Clarke County in the state of Georgia further implemented a 100% smoke-free ordinance in 2005 prohibiting smoking in all restaurants and bars but not in all workplaces (ACC 2005). Efforts to control SHS exposure are also seen on a global front. In March 2004, Ireland became the first country in the world to implement smoke-free indoor workplaces, restaurants, and bars (Anon 2004). New Zealand introduced a smoke-free bar and restaurant policy in December 2004 (Thomson, and Wilson 2006). Similar legislations and smoking restrictions of various degrees and jurisdictions are being passed in England, Scotland, Australia, Canada, and Hong Kong, among others (Arnott et al. 2007; Kohli 2006; Lal, and Siahpush 2008; Lam et al. 2002; Luk, Ferrence, and Gmel 2006). Smoke-free environments offer the greatest protection for smokers and non-smokers and this is most effectively achieved by enacting smoking bans (USDHHS 2000).

Smoke-free air laws have been very effective in reducing exposures to constituents of SHS such as particulate matter and carcinogenic PAHs in workplaces and establishments like restaurants and bars (Bondy et al. 2009; Howell 2005; Mulcahy et al. 2005; Repace 2004; Repace, Hyde, and Brugge 2006). The reduced exposures have also led to decreased acute myocardial infarction, angina, stroke, and asthma (Herman, and Walsh 2010; Marlow 2010; Sargent et al. 2004; Seo, and Torabi 2007). Other studies have shown significant improvements in respiratory symptoms, pulmonary function, and decreased levels of circulating markers of inflammation following implementation of smoke-free laws (Assadourian 2006; Eagan, Hetland, and Aarø 2006; Menzies et al. 2006). These improvements in health associated to smoke-free laws further support the associations between SHS and adverse health outcomes such as cardiovascular and respiratory diseases.

Smoke-free air laws and outdoor secondhand smoke exposure

While smoke-free air laws have been shown to have large positive effects on public health, indoor smoking bans seem to result in increased smoking outside establishments. The magnitude of outdoor SHS exposure and health risks are unknown as very few studies have characterized outdoor SHS exposure and associated health endpoints. One of the first outdoor SHS studies was conducted by Klepeis and colleagues using $PM_{2.5}$ as a proxy for SHS (Klepeis, Ott, and Switzer 2007). The overall average SHS respirable PM concentration for the visits to public places during smoking was about 30 $\mu g/m^3$ (Klepeis et al. 2007). Average concentrations over the duration of a cigarette and within 0.5 m exceeded 200 $\mu g/m^3$ (Klepeis et al. 2007). Proxies of SHS exposure such as CO and $PM_{2.5}$ may provide some indication as to the extent of SHS in the outdoor setting but provide little information on the systemic exposure of non-smokers to constituents of SHS. Another study was done recently in which the authors assessed SHS exposure using $PM_{2.5}$ inside and outside establishments, reporting that average outdoor $PM_{2.5}$ with smoking was significantly higher than background level and significantly and positively correlated with number of lit cigarettes (Kaufman et al. 2010). These results are very similar to the results of the study presented in Chapter 3. Significant associations between outdoor $PM_{2.5}$ and CO and the number of smokers outside restaurants and bars are reported in that study. Another study by Hall and colleagues measured significant gains in salivary cotinine in participants exposed to SHS outside of restaurants and bars in Athens, GA, with average increase in salivary cotinine reported as 162%, 102%, and 16% in subjects at the bar, restaurant, and control site where no smokers were reported (Hall et al. 2009). This was the first study, based on an exhaustive literature search, which utilized biomarkers to characterize outdoor exposure to SHS.

Human health risk assessment and policy making are hindered by the current lack of data characterizing human exposure to and associated health outcomes of outdoor SHS. There is therefore a need to both characterize the exposure levels of outdoor SHS as well as determine whether these levels are associated to health outcomes.

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CHAPTER 3

PARTICULATE MATTER ($PM_{2.5}$) AND CARBON MONOXIDE FROM SECONDHAND SMOKE OUTSIDE BARS AND RESTAURANTS IN DOWNTOWN ATHENS, GEORGIA[1]

[1] St.Helen, G., D. B. Hall, L. H. Kudon, J. Pearce, S. Baptiste, S. Ferguson, T. Green, and L. P. Naeher. 2011. Accepted by *Journal of Environmental Health*

ABSTRACT

Objectives: To measure particles ≤ 2.5 μm in aerodynamic diameter ($PM_{2.5}$) and carbon monoxide (CO) in outdoor waiting areas and patios of restaurants and bars in downtown Athens, Georgia where indoor smoking is banned and to investigate whether the measured concentrations are directly associated with number of cigarettes lit in these settings.

Methods: Real-time $PM_{2.5}$ and CO were monitored on four summer weekend afternoons/evenings in outdoor waiting areas or patios at five locations in Athens, including two restaurants, two bars, and a control site (i.e., sidewalk with no smokers present). In addition, smokers and pedestrians present or passing and motorized vehicles passing each sampling location were counted. The effects of smokers, pedestrians, and vehicles on $PM_{2.5}$ and CO were estimated through linear mixed effects regression models which accounted for heterogeneity from sample to sample and autocorrelation through time.

Results: $PM_{2.5}$ levels were 63.9 ± 50.2 $\mu g/m^3$ and 51.0 ± 51.1 $\mu g/m^3$ at the two bars and 39.7 ± 21.0 $\mu g/m^3$ at one restaurant and were significantly higher than levels at the control location (all p-values > 0.001). $PM_{2.5}$ at the control and the other restaurant were 20.4 ± 3.4 $\mu g/m^3$ and 16.6 ± 7.9 $\mu g/m^3$, respectively. Carbon monoxide levels outside the restaurant and bar sites did not differ significantly from the control and ranged from 1.2 to 1.6 ppm. The number of smokers had a significant positive effect on log(CO) ($\delta = 0.0121$, $p = 0.032$) as well as on log($PM_{2.5}$) ($\delta = 0.0575$, $p<0.001$) while the effects of pedestrians and vehicles on log(CO) and log($PM_{2.5}$) were not statistically significant.

Conclusions: The results of this study indicate that: (1) SHS leads to significant increases in $PM_{2.5}$ outside of restaurants and bars; and, (2) although CO can be used as a proxy for SHS in these outdoor environments, its levels remain relatively low.

INTRODUCTION

Secondhand smoke is the mixture of smoke given off at the burning end (sidestream smoke) of tobacco products and the mainstream smoke exhaled by smokers (USDHHS 2006). Molecular, animal, and epidemiologic studies have provided compelling evidence that exposure to SHS causes disease and premature death in humans (USDHHS 2006). Health effects include cancer, respiratory and cardiovascular diseases in the general population as well as lower respiratory tract infections, asthma exacerbation, and cognitive decline in children (Alipour, Deschamps, and Lesage 2006; Cook, and Strachan 1997; IARC 2004; Jaakkola, and Gissler 2004; Jaakkola 2002; Janson 2004; Law, and Wald 2003; Rizzi et al. 2004; Trimble et al. 2005; Vineis, and Grp 2005; WHO 1999; Yolton et al. 2005).

This increasing body of scientific evidence showing elevated disease risk among non-smokers exposed to SHS has led to the passage of indoor smoking bans and restrictions in workplaces and public places, including restaurants and bars. As of November 2009, 17,068 municipalities in the United States are covered by a 100% smoke-free provision in workplaces, and/or restaurants and/or bars, by a state, commonwealth, or local law, covering 71.0% of the US population. Thirty-eight US states and the District of Columbia have local laws in effect that require 100% smoke-free workplaces, and/or restaurants and/or bars (ANRF 2009a). Although the state of Georgia does not have a 100% smoke-free law, a state-wide partial smoking ban was passed in July, 2005 banning smoking in restaurants and bars that serve or employ minors. The Athens-Clarke County in the state of Georgia, where the University of Georgia is located, passed its 100% smoke-free law in 2005 prohibiting smoking in all restaurants and bars but not in all workplaces (ANRF 2009b). Efforts to control SHS exposure are also seen globally. In March 2004, Ireland became the first country to implement smoke-free indoor workplaces, restaurants,

and bars (Anon 2004). Similar legislations and smoking restrictions of various degrees and jurisdictions are being passed in New Zealand, England, Scotland, Australia, Canada, and Hong Kong, among others (Arnott et al. 2007; Kohli 2006; Lal, and Siahpush 2008; Lam et al. 2002; Luk, Ferrence, and Gmel 2006). Smoke-free environments offer the greatest protection for smokers and non-smokers and this is most effectively achieved by enacting smoking bans (USDHHS 2000).

In response to indoor smoking bans and restrictions, smokers are moving outdoors to sidewalks and outdoor seating areas to smoke. The potential increase in SHS exposure to non-smokers in these outdoor settings is therefore a growing public health concern. Studies in the literature have focused almost exclusively on indoor SHS levels and associated adverse health endpoints. Thus, empirical data on the magnitude of exposure and extent of the effects of street level or outdoor SHS on human health is limited and this hinders risk assessment and policy making. Recently, there has been a growing interest in characterizing SHS exposure outdoors (Hall et al. 2009; Klepeis, Ott, and Switzer 2007). Hall and colleagues used salivary cotinine to assess SHS exposure outside of restaurants and bars in the same city as the current study. While use of salivary cotinine is very specific to tobacco smoke and noninvasive, the analysis may be expensive and time-consuming. Klepeis and colleagues assessed outdoor SHS exposure by measuring particulate matter (PM) of various sizes in public outdoor locations and in a residential patio. The study by Klepeis and colleagues begins to provide valuable data on typical outdoor versus indoor SHS levels, effects of wind, and proximity to smokers on SHS concentrations based on PM measurements. However, PM levels outside establishments are not specific to tobacco smoke and may be influenced by traffic emissions (Canepari et al. 2008; Han

et al. 2005). Other sources of PM have to be accounted for when using PM as a proxy for outdoor SHS.

PM has commonly been used as a non-specific proxy for SHS and constitutes a significant part of mainstream and sidestream smoke (Jenkins et al. 1996; Miller, and Nazaroff 2001). Its utility is cheap and not time-consuming. Confidence in the use of PM as a proxy for outdoor SHS is improved when the association between PM and smokers as well as other sources of PM, such as traffic, are quantified. This holds true for all other non-specific environmental proxies of SHS such as carbon monoxide (CO). The objectives of our study were, therefore, to measure the concentrations of particulate matter less than 2.5 μm in aerodynamic diameter ($PM_{2.5}$) and carbon monoxide (CO) outside of bars and restaurants in downtown Athens, Georgia and to investigate whether the measured concentrations are directly associated with the number of cigarettes lit (as a proxy for SHS) in these settings.

METHIODS

Study Locations

The study was conducted in downtown Athens, Georgia, a city of about 102,000 people, during two weekends in July of 2006. A convenience sample of establishments was selected for the project, including two family restaurants, three bars, and a control site. Bar and restaurant sites were selected based on our previous observations of their patronage and significant number of outdoor smokers at these sites. The bar and restaurant sites varied from being fully open-air to being partially enclosed on two or three sides by walls and a roof. A description of the sites is given in Table 1. An open-air control site (SW) was located on a side-walk on the north campus of the University of Georgia, separated from site R2 by a four-lane street. No smokers were

expected at the control site and therefore, it provided information on typical street-level $PM_{2.5}$ and CO concentrations in a smoke-free outdoor location downtown. Study visits were designed so we could measure the average concentration of $PM_{2.5}$ and CO emitted from burning tobacco products during normal bar and restaurant operating hours, which also included peak business times. We obtained verbal approval from the location owners or managers.

Downtown air pollution sampling

Real-time $PM_{2.5}$ and CO were the two constituent pollutants of SHS that were monitored during this study. Air pollution sampling was conducted from 3:00 PM to 2:30 AM on Fridays and Saturdays of the two weekends; each day was treated as a separate sampling period (Table 3.1). Sites B1, R1, R2, and SW were monitored during all four sampling periods. However, site B3 was replaced with site B2 during sampling periods two to four due to bar B3's closure. Real-time $PM_{2.5}$ was measured using six laser photometer aerosol monitors (DustTrak—TSI Inc., Model 8520, Shoreview, MN). Monitor inlets were set to record at respiratory height (approximately 1.5 to 2 m) concentrations of $PM_{2.5}$ at 30 s intervals. Real-time CO levels were recorded using a Langan CO monitor (Langan Product Inc., Model T15v, San Francisco, CA) set to record concentrations at 30 s intervals (range of CO sensor: 0 to 200 ppm). Particle and CO monitors were collocated in a central area on the premises but away from the direct path of employees and patrons to prevent damage to the monitors or inconvenience to the employees and patrons. Langan CO monitors were placed at the same height as the inlet tube of the DustTraks. Monitors were within 0.3 m to 5 m radius from smokers and pedestrians and within 3 to 15 m from the street (Table 3.1). Langan monitors were calibrated at the University of Georgia Air Quality Lab (UGA AQL) two days before the first field visits using >99.999% N_2 (zero CO gas) and 100 ppm CO gas. DustTraks were calibrated by the manufacturer less than a year prior to the

study. For consistency, every location was equipped with the same DustTrak and Langan CO monitors during all sampling periods. After each day of sampling all data were downloaded to a computer in the UGA AQL.

Due to over-reporting of $PM_{2.5}$ by DustTraks (Schmidt-Ott, and Ristovski 2007; Volckens et al. 1999; Yanosky, Williams, and MacIntosh 2002), $PM_{2.5}$ values were reduced by multiplying by a well accepted correction factor of 0.32 (Trent 2006). Also, to determine the variation in $PM_{2.5}$ readings of the DustTrak monitors and to increase comparability, side-by-side measurements were carried out in the UGA AQL by allowing the DustTraks to run for over 40 hrs (or until the batteries died) immediately following the site sampling phase of the study. DustTraks had a 9.76% coefficient of variation (CV). In order to correct for the large CV, the ratios between real-time $PM_{2.5}$ of DustTrak B1 and DustTraks B2, R1, R2, and SW, respectively were computed and were as follows: 1.203, 1.049, 0.972, and 1.193. DustTrak B1 was selected as the reference because the median concentration of its reported $PM_{2.5}$ was closest to the median of $PM_{2.5}$ from all five DustTraks combined. (DustTrak are identified by the site IDs where they were used). The ratios were used to adjust reported real-time $PM_{2.5}$ of DustTraks B2, R1, R2, and SW to DustTrak B1 measurements and thus correct for the relatively high CV.

Smoking, pedestrian, and traffic count

In addition to area monitoring of $PM_{2.5}$ and CO, the number of smokers, pedestrians, and vehicles at or passing by each site were counted continuously and the cumulative count for each five minute period was recorded. Counts were reset at the beginning of each five minute period. The smoker count included every individual with a lit tobacco product who was walking past, sitting, or standing in the seating/standing area or outdoor patio of the locations. The pedestrian count included both nonsmoking and smoking individuals at or passing by each location.

Smokers and pedestrians were within a 5 meter radius from $PM_{2.5}$ and CO monitors. The traffic count included every motorized vehicle driving past or with an idle engine in front of the locations. Vehicle, smoker, and pedestrian counts were not obtained for location SW due to our limited number of technicians. However, SW was not expected to have any significant smoking based on our previous observations and would have similar vehicle counts as site R2. Further, no vehicle count was obtained for site B1, a large open-air patio located on the second floor of a bar, because vehicles were not visible from its vantage point. This site was selected because it is a very popular location with college students and locals. All of these data were recorded by teams of two technicians at each site who were all trained together before the study began.

Statistical Analysis

Data first were aggregated into fifteen-minute averages for pollutants and sums over the same fifteen-minute period for smoker, pedestrian, and vehicle counts. Three sets of analyses were performed on the data. In each case, the pollutant was modeled on the natural log scale because the measurements displayed non-constant variance and skewed-right distributions. In the first set of models, we investigate the effect of smoker and pedestrian count on pollutant concentrations. We used data from sites B1, B2, B3, R1, and R2 for which we had smoker and pedestrian counts; the variable "vehicles" was excluded from this analysis. In order to investigate possible effects of car traffic, a second set of models used data from all samples that had data for vehicles and the response variables; we included "vehicles" as an explanatory variable in the analysis. Finally, a third set of models was fit to compare average pollutant levels in sites B1, B2, B3, R1, and R2 with those of the control location, SW. Comparisons between the mean pollutant levels at the test sites and the control site (SW) were made using linear mixed effect

models. Bonferroni's method was applied to control the error rate and did not include any covariate effects.

All models were linear mixed effect models and can be written as:

$$y_{ijt} = \alpha + b_{ij} + \beta_{ij}t + \gamma_{ij}t^2 + \delta smokers_{ijt} + \theta pedest_{ijt} + \phi cars_{ijt} + e_{ijt} \qquad (\textit{Equation 3.1})$$

where, y_{ijt} represents the response, log(CO) or log($PM_{2.5}$), for the j^{th} sample within the i^{th} site measured at the t^{th} time point. In addition, the model contains fixed effects, α (an intercept), β_{ij} (linear effect of time, with a different effect for each site/sampling period combination), γ_{ij} (quadratic effect of time, with a different effect for each site/sampling period combination), δ (linear effect of smokers), θ (linear effect of pedestrians), and ϕ (the linear effect of cars). The intercept is allowed to vary from sample to sample through the random effect, b_{ij}. Finally, the error term e_{ijt} is assumed to follow an autoregressive moving average (ARMA) to account for autocorrelation through time, which is present in these data. The selection of the order of the ARMA model was done by choosing the model with the smallest value of Akaike's information criterion (AIC).

The focus of interest in these models is on the coefficients δ, θ, and ϕ. Estimated values of these parameters quantify the degree of association between the pollutants and smokers, pedestrians, and cars, respectively, above and beyond the effects of other variables in the model after accounting for heterogeneity from sample to sample and autocorrelation through time in the time series that are being analyzed. Finally, models of the form *Equation 3.1* are reported here, but lagged values of the explanatory variables also were checked to see if they were significant predictors. That is, other terms like $\phi smokers_{ij,\,t-1}$ were added to the model All models were fit using the NLME library of functions in S-PLUS, Version 7.0 for Windows (Insightful Corporation, Seattle, WA). Statistical tests were considered significant at $\alpha = 0.05$.

RESULTS

We measured SHS exposure in outdoor seating/standing areas and an open-air patio of three bars and two restaurants. Table 3.2 presents the descriptive statistics for all sites monitored. We measured significantly higher average $PM_{2.5}$ at the two bars, B1 and B2, and at one restaurant, R1, compared to the control site, SW (all p-values < 0.001) (see Table 3.3). The average $PM_{2.5}$ recorded outside of the two bars, B1 and B2, over all sampling periods monitored were 63.9 ± 50.2 $\mu g/m^3$ and 51.0 ± 51.1 $\mu g/m^3$, respectively (mean corrected $PM_{2.5}$ ± SD). We recorded an average $PM_{2.5}$ concentration of 39.7 ± 21.0 $\mu g/m^3$ at the restaurant site, R1. The average $PM_{2.5}$ recorded at the control site, SW, was 20.4 ± 3.4 $\mu g/m^3$ compared to 16.6 ± 7.9 $\mu g/m^3$ at R2. Carbon monoxide levels outside the restaurant and bar sites did not differ significantly from the control and ranged from 1.2 to 1.6 ppm.

Figure 3.1 shows the graphs of $PM_{2.5}$ and CO vs. number of smokers from all sites and sampling times, excluding the control site, SW, as well as graphs for bars only and restaurants only. The graphs show a linear increase in $PM_{2.5}$ and CO concentrations as the number of smokers increase. This linear trend is more pronounced between $PM_{2.5}$ and smokers (Figure 3.1(a) $R^2 = 0.605$) than CO and smokers (Figure 3.1(b) $R^2 = 0.286$). The dependent relationship between real-time smoker count and real-time $PM_{2.5}$ is further displayed in Figure 3.2.

Table 3.4 presents the results of the analyses using *Equation 3.1*, in which the coefficients δ, θ, and ϕ quantify the degree of association between the responses, log(CO) and log($PM_{2.5}$), and the number of smokers, pedestrians, and vehicles, respectively. In the first analysis, where we included smoker and pedestrian counts as covariates and excluded vehicle count (model 1 in Table 3.4), an increase in number of smokers outside the sites is significantly associated with log($PM_{2.5}$) ($\delta = 0.0481$, $p < 0.001$) and log(CO) ($\delta = 0.0104$, $p = 0.039$). An

increase in the number of pedestrians at the sites has a positive effect on log($PM_{2.5}$) (θ = 0.0043, $p < 0.001$) but the effect was an order of magnitude smaller than the effect of smokers on log($PM_{2.5}$). The number of pedestrians had no significant effect on log(CO). In the second analysis (model 2 in Table 3.4), the number of vehicles is included as an explanatory variable to investigate the possible effects of vehicle traffic. The number of smokers had a significant positive effect on log(CO) (δ = 0.0121, p = 0.032) as well as on log($PM_{2.5}$) (δ = 0.0575, $p<0.001$). The effects of pedestrians and vehicles on log(CO) and log($PM_{2.5}$) were not statistically significant in this analysis (Table 3.4). Finally, lagged values of smokers, pedestrians, and cars were non-significant in all of the models, so those results are omitted.

DISCUSSION

Numerous studies have been conducted to characterize the exposure to and effects of SHS in the workplace and indoor environment (Siegel, and Skeer 2003). However, empirical data on the magnitude of exposure and extent of the effects of outdoor SHS on human health is limited. While Klepeis and colleagues used particulate matter (PM) of various sizes as proxies for SHS (Klepeis et al. 2007), the effects of number of smokers and pedestrians on PM and CO as well as the contribution from other sources such as vehicles were not presented.

In this study, we utilized airborne particulate matter with aerodynamic sizes ≤ 2.5 μm ($PM_{2.5}$) and carbon monoxide (CO) as environmental markers of SHS. These two pollutants are accepted proxies for SHS exposure (Liu et al. 2009; Repace, Al-Delaimy, and Bernert 2006; Travers et al. 2004) because of their ease of measurement and correlation to other SHS contaminants. Although $PM_{2.5}$ is not unique to SHS, it is emitted at high levels from tobacco combustion and is measurable above background levels even at high ventilation and low

smoking rates (Leaderer 1990). In addition, $PM_{2.5}$ has been associated with many health effects that SHS has been attributed to (Pope et al. 2001), which increases its public health relevance as an SHS indicator. CO is also a non-specific SHS marker and is a significant component of incomplete combustion. Further, ambient $PM_{2.5}$ and CO are regulated by the US Environmental Protection Agency. [National Ambient Air Quality Standard: $PM_{2.5}$ 15.0 $\mu g/m^3$ annual average and 35 $\mu g/m^3$ not to be exceeded more than one 24-hr period a year; CO 9 ppm average over 8-hr period and 35 ppm not to be exceeded more than one 1-hr period a year (EPA 2008)].

$PM_{2.5}$ levels outside of the bars, sites B1 and B2, and one restaurant, site R1, were two to three times the levels observed at the control site, SW. Levels measured at SW provide an estimate of background and traffic-generated pollution in the absence of smokers. None of the sites had statistically significant higher CO when compared to the control site, which seems to indicate that outdoor CO pollution from SHS is not significant. The above background and traffic-generated $PM_{2.5}$ at the three locations compared to the control site may be directly attributable to the higher number of smokers observed at these sites (see Table 3.2). Figure 3.1(a) shows that $PM_{2.5}$ and number of smokers at all sites increased linearly ($R^2 = 0.605$) and supports the conclusion that outdoor $PM_{2.5}$ levels is directly proportional to the number of smokers present there. Figure 3.1(b) shows a weaker positive linear relationship between outdoor CO and number of smokers overall ($R^2 = 0.286$). Although there is evidence of a positive linear trend between smokers and CO outside the bars where CO measured was higher (Figure 3.1(d) $R^2 = 0.498$), a linear relationship between smokers and CO may be less pronounced at low CO levels. Our analyses presented as models 1 and 2 in Table 3.3 also show that $PM_{2.5}$ and CO outside restaurants and bars originate from smokers. In the first analysis, number of smokers ($\delta = 0.0481$, $p < 0.001$) and pedestrians ($\theta = 0.0043$, $p < 0.001$) had highly significant positive

effects on log($PM_{2.5}$). While we saw a significant effect of pedestrian/customer count on log($PM_{2.5}$), the effect of the number of smokers at the sites was an order of magnitude greater than the effect of pedestrians on log($PM_{2.5}$). This clearly shows the strong positive effect of increasing number of smokers at a site on $PM_{2.5}$. The number of smokers had a weaker but positive effect on log(CO) ($\delta = 0.0104$, $p = 0.039$) in this same analysis.

In the second analysis, we tested the effect of vehicle traffic on the pollutant levels by excluding sites with no vehicle count (site B1) and including "vehicles" as an explanatory variable. The effect of the number of smokers on log($PM_{2.5}$) remained highly significant ($\delta = 0.0576$, $p<0.001$). However, neither number of pedestrians/customers nor vehicle traffic had statistically significant effects on log($PM_{2.5}$). The number of smokers also had a statistically significant effect on log(CO) ($\delta = 0.0121$, $p = 0.032$) in contrast to pedestrian and vehicle counts. As seen in Table 3.4 (model 2), the effect of number of smokers was over four times greater on log($PM_{2.5}$) than on log(CO). This shows the higher sensitivity of $PM_{2.5}$ as an environmental marker of SHS compared to CO. These results clearly indicate that $PM_{2.5}$ and CO in outdoor seating/standing areas and outdoor patios of restaurants and bars originated primarily from SHS. Based on the results of our study, the pollutant of concern seems to be $PM_{2.5}$. The approximately 12 hr average levels of $PM_{2.5}$, especially at bars, exceed the 24-hr EPA protective standard of 35 $\mu g/m^3$ even when DustTrak readings are corrected for their over-reporting of $PM_{2.5}$ (Table 3.2). (*Note: The EPA standard is over 24 hours and the current data were averaged over about 12 hours*). Health effects such as coronary heart disease and lung cancer can potentially be observed at chronic exposure to $PM_{2.5}$ at levels that were measured during this study (Repace 2000). Carbon monoxide levels at no point reached close to or exceeded the EPA standard.

The $PM_{2.5}$ levels that we measured on the sidewalk (SW) (20 $\mu g/m^3$) were comparable or lower than levels reported in traffic-related studies in the US (23 to 32 $\mu g/m^3$) (Riediker et al. 2003) and in the UK (34 to 39 $\mu g/m^3$). In contrast $PM_{2.5}$ levels measured at sites R1, B1, and B2 (40 to 64 $\mu g/m^3$) were comparable to traffic-related $PM_{2.5}$ in some developing countries such as Buenos Aires, Argentina (44 $\mu g/m^3$) (Bogo et al. 2003) and in-vehicle $PM_{2.5}$ in Mexico City (61 to 71 $\mu g/m^3$) (Gómez-Perales et al. 2004). In a study similar to the current study, Klepeis and colleagues reported average $PM_{2.5}$ concentrations outside restaurant, bar, and pub patios and airport sidewalk as high as 64 $\mu g/m^3$ also (Klepeis et al. 2007). In comparison, a recent study of indoor SHS in three Pennsylvania casinos reported $PM_{2.5}$ levels of 106 $\mu g/m^3$ (range 84 – 133 $\mu g/m^3$) inside the casinos compared to 18 $\mu g/m^3$ outdoors (Repace 2009). Other studies have reported $PM_{2.5}$ levels as high as 179 and 190 $\mu g/m^3$ in restaurants and bars before implementation of indoor smoking restrictions (Brauer, and Mannetje 1998; Repace, Hyde, and Brugge 2006).

The findings of this study are subject to a number of limitations. First, the venues sampled were not necessarily representative of venues throughout Athens, Georgia. Some sites were fully open-air while others were open on only one side facing the road and had a roof. However, they did provide us with a range of venue types, sizes, and patronage. Second, in addition to traffic and SHS, $PM_{2.5}$ and CO are also influenced by cooking, especially grilling. While we did not observe any grilling in the vicinity of the locations that we sampled, we did not account for $PM_{2.5}$ and CO that may have originated from the restaurant kitchens. Third, use of 0.32 as a correction algorithm, or any other in the literature, may not be fully accurate for our purposes due to differences in temperature, humidity, outdoor vs. indoor, and aerosol sources. Finally, we did not collect meteorological data such as wind speed, temperature, and humidity,

all factors that determine how quickly air pollutants such as particulates and CO are dispersed and therefore their air concentrations. These factors may also vary from site to site thus making site comparisons challenging. Accounting for these meteorological variables would have strengthened the results of this study.

The results of this study indicate that: (1) SHS leads to significant increases in $PM_{2.5}$ outside of restaurants and bars; and, (2) although CO can be used as a proxy for SHS in conjunction with $PM_{2.5}$ in these outdoor environments, its levels remain relatively low.

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TABLES AND FIGURES

TABLE 3.1. Description of secondhand smoke (SHS) monitoring locations

Site ID	Description	Sampling Period(s) (Study days)	Distance from road (m)	Number of Tables/Benches	Seating/standing capacity
B1	(Bar 1) Bar with outdoor patio on backside of building on second floor; away from the street. Enclosed by two walls of adjacent buildings, open at one end, and had no roof.	4 (days 1 to 4)	15	6	150
B2	(Bar 2) Bar with open-air standing area next to street sidewalk. No walls and roof.	3 (days 2 to 4)	3	0	>50
B3	(Bar 3) Bar with open-air standing area next to sidewalk. Enclosed by two walls of adjacent buildings, had a roof, and no wall at front facing sidewalk.	1 (day 1)	3	0	>50
R1	(Restaurant 1) Family-oriented restaurant with open-air seating area next to side walk. Enclosed by three walls, had a roof, but had no wall facing sidewalk.	4 (days 1 to 4)	3	5	20
R2	(Restaurant 2) Family-oriented restaurant with open-air seating area next to Sidewalk. No walls or roof. On opposite side of site SW.	4 (days 1 to 4)	3	5	20
SW	Sidewalk next to four-lane bi-directional highway on UGA[†] north campus with no buildings; Control Site	4 (days 1 to 4)	3	0	>50

[†]University of Georgia, Athens, GA

TABLE 3.2. Descriptive statistics of data collected summarized over 15 minute intervals

Site ID	Statistics†	$PM_{2.5}$ ($\mu g/m^3$)	$PM_{2.5}$ ($\mu g/m^3$)‡	CO (ppm)	Smokers	Pedestrians	Vehicles
B1	N	154	154	92	154	146	
	Mean	199.7	63.9	1.5	6	41	
(4 sampling days)	Min	50.6	16.2	0.6	0	0	N/A
	Max	639.7	204.7	2.8	18	177	
	SD	157	50.2	0.5	6	44	
B2	N	92	92	136	136	136	136
	Mean	159.4	51.0	1.6	5	32	21
(3 sampling days)	Min	51.4	16.4	1	0	3	9
	Max	847.7	271.3	3.4	20	128	42
	SD	159.6	51.1	0.5	5	30	7
B3	N	47	47	47	47	47	47
	Mean	94.2	30.1	1.2	3	17	19
(1 sampling day)	Min	52.7	16.9	0.9	0	2	5
	Max	202.4	64.8	1.9	12	55	31
	SD	40.7	13.0	0.2	4	12	5
R1	N	184	184	176	183	183	183
	Mean	124.2	39.7	1.4	2	27	28
(4 sampling days)	Min	47.5	15.2	0.9	0	6	11
	Max	361.6	115.7	2.9	6	63	51
	SD	65.5	21.0	0.3	2	12	8
R2	N	185	185	185	185	185	185
	Mean	51.9	16.6	1.3	2	33	44
(4 sampling days)	Min	24.6	7.9	0.1	0	6	13
	Max	139.6	44.7	3.7	6	80	85
	SD	18.5	5.9	0.6	1	17	17
SW	N	185	185	44			
	Mean	63.7	20.4	1.3			
(4 sampling days)	Min	33	10.6	0.9	N/A	N/A	N/A
	Max	138	44.2	5.3			
	SD	10.7	3.4	0.2			

†Descriptive statistics are based on 15-minute summarization of data; N represents the number of 15-min intervals; ‡Correction factor of 0.32 applied to DustTrak reading
N/A = Not available

TABLE 3.3. Pair-wise comparisons between pollutant levels at non-control bar and restaurant sites and the control site

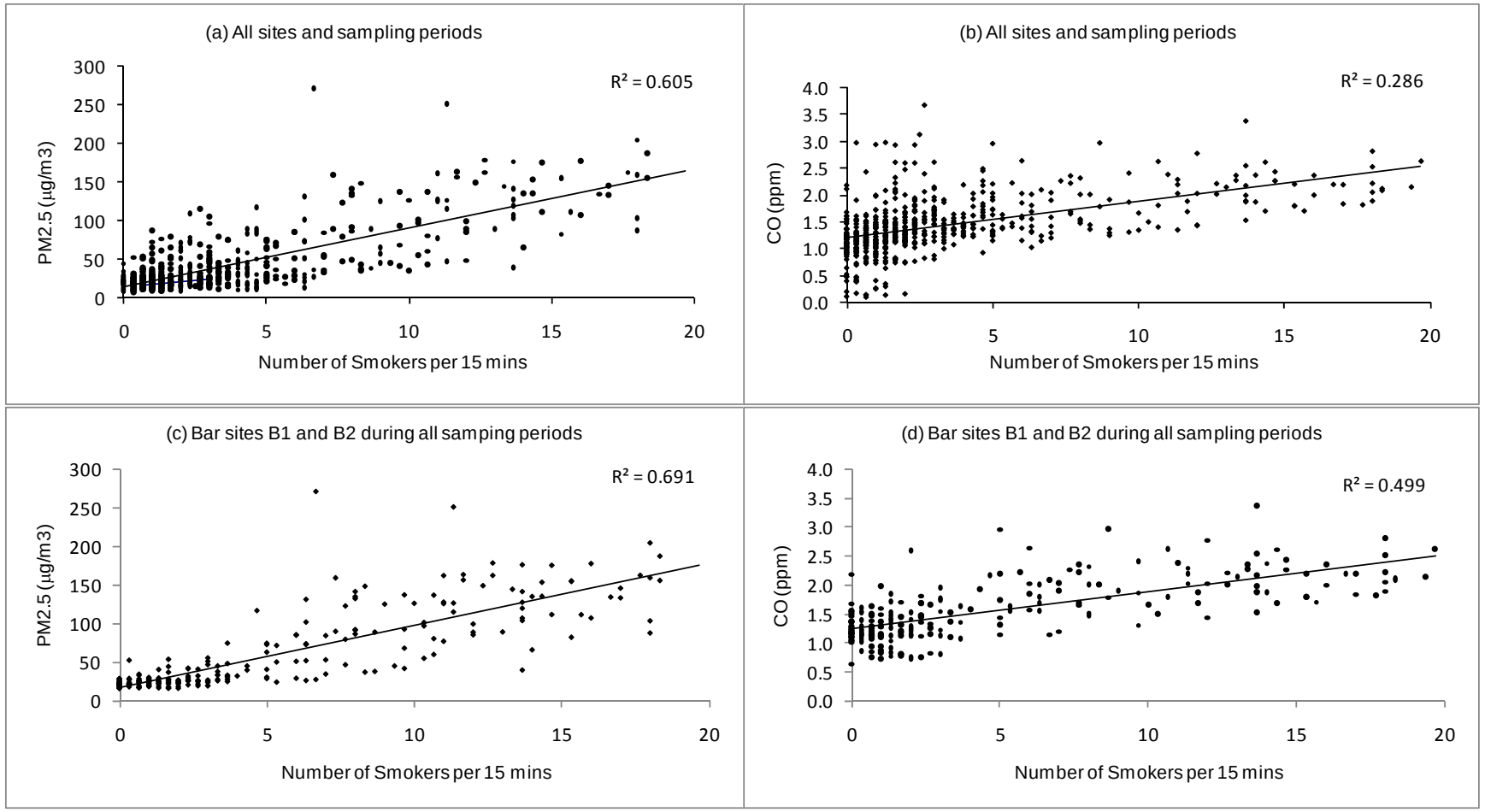
Site	Log (CO)			Log ($PM_{2.5}$)		
	Difference from SW (control site)	**Std. Error**	**p-value**	**Difference from SW (control site)**	**Std. Error**	**p-value**
B1	0.059	0.205	0.777	0.709	0.099	<0.001†
B2	0.208	0.187	0.293	0.562	0.122	<0.001†
B3	0.101	0.025	0.695	0.303	0.156	0.074
R1	0.065	0.177	0.723	0.568	0.099	<0.001†
R2	0.121	0.177	0.51	-0.252	0.099	0.024

†Statistically significant difference at 0.01 (Bonferroni)

TABLE 3.4. Summaries of models fit to data. Model 1 was fit using data for all sites except the control site, SW, and vehicle count is excluded from among the explanatory variables. Model 2 was fit to data from all sites except sites B1 and SW which did not have vehicle count data. Vehicles is included as an explanatory variable

Model	**Explanatory Variable**	**Log(CO)**		**Log($PM_{2.5}$)**	
		Parameter Estimate	**p-value**	**Parameter Estimate**	**p-value**
1	Smokers	0.0104	0.039‡	0.0481	<0.001‡
	Pedestrians	-0.0002	0.858	0.0043	<0.001‡
2	Smokers	0.0121	0.032‡	0.0576	<0.001‡
	Pedestrians	-0.0007	0.472	0.0021	0.173
	Vehicles	-0.0008	0.546	-0.0027	0.727

‡ Statistically significant at $\alpha = 0.05$



(Figure 3.1 continued on Page 80)

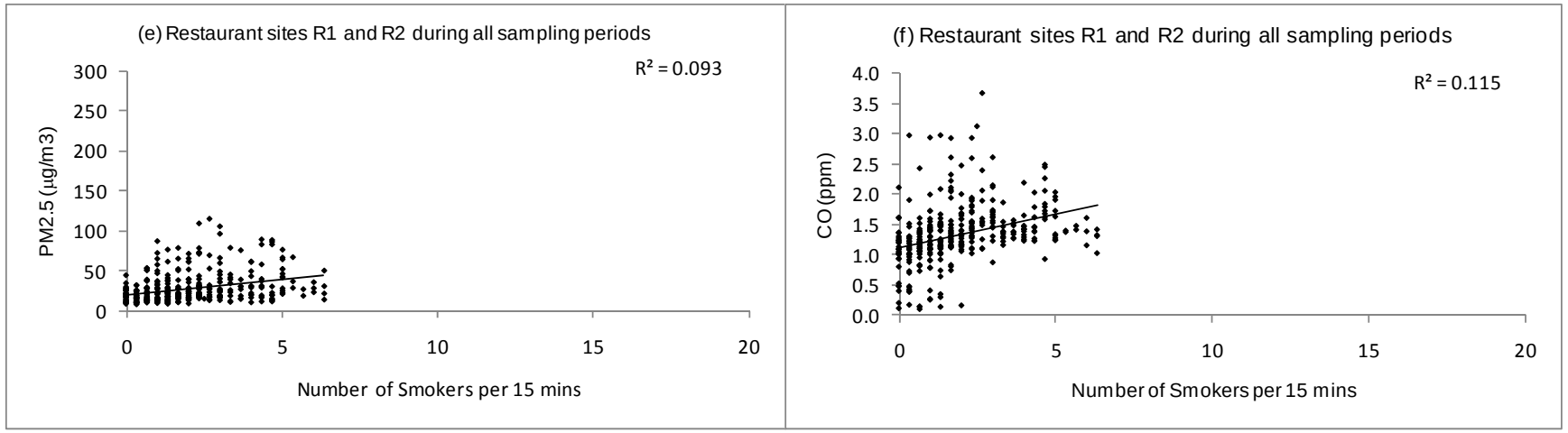


FIGURE 3.1 Average particulate matter less than 2.5 μm ($PM_{2.5}$) and carbon monoxide (CO) versus number of smokers. (a) and (b): Average particulate matter less than 2.5 μm ($PM_{2.5}$) and carbon monoxide (CO), respectively, versus number of smokers per 15 minutes in outdoor standing/seating areas or patio of all bar and restaurant sites over all sampling periods. (c) and (d): Average $PM_{2.5}$ and CO, respectively, versus number of smokers per 15 minutes in outdoor patio of bar (B1) and standing area of bar (B2) combined over all sampling periods. (e) and (f): Average $PM_{2.5}$ and CO, respectively, versus number of smokers per 15 minutes at outdoor seating areas of restaurants R1 and R2 over all sampling periods.

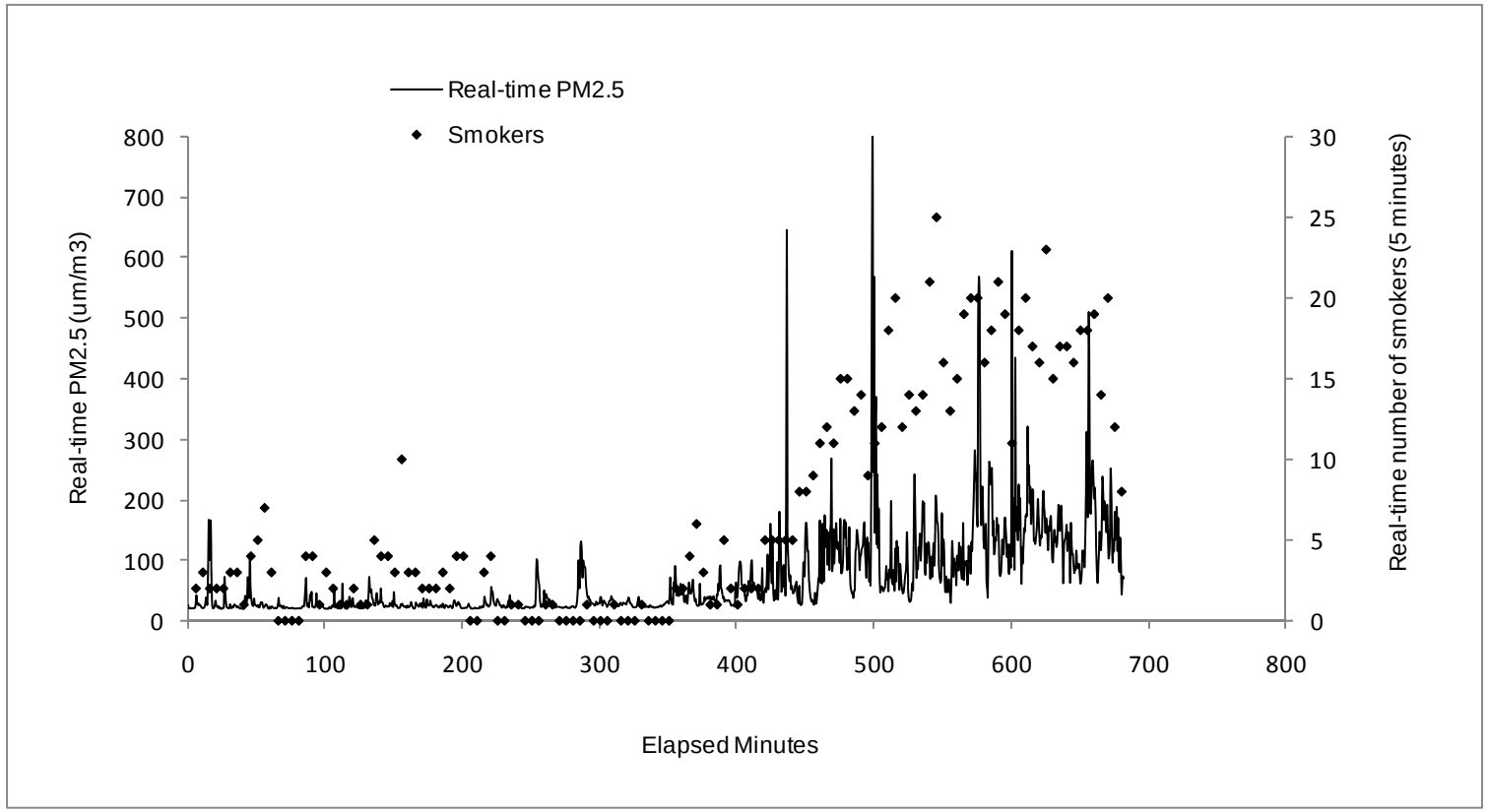


FIGURE 3.2. Real-time monitoring of secondhand smoke in an outdoor patio of a bar (B1) during the second sampling day. This graph is typical of all three sampling days at site B1. The graph shows the total number of smokers in 5-min increments and the real-time 30-sec particulate matter less than 2.5 μm ($PM_{2.5}$) measured simultaneously. Sampling periods ran from 3:00 PM to 2:30 AM.

CHAPTER 4

EXPOSURE TO SECONDHAND SMOKE OUTSIDE OF A BAR AND A RESTAURANT IN ATHENS, GEORGIA LEADS TO INCREASES IN TOBACCO EXPOSURE BIOMARKERS IN NON-SMOKERS[1]

[1] St.Helen, G., J.T. Bernert, D.B. Hall, C.S. Sosnoff, Y. Xia, J.R. Balmes, J.E. Vena, J.S. Wang, N.T. Holland, and L.P. Naeher. To be submitted to *Environmental Health Perspective*

ABSTRACT

Objectives: To characterize the exposure of non-smokers exposed to secondhand smoke (SHS) outside a restaurant and a bar in Athens, Georgia using salivary cotinine and urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).

Methods: Twenty-eight subjects were assigned to outdoor patios of a restaurant and a bar and an open-air location with no smokers (control) on three weekend days in a crossover study; subjects visited each site once and stayed for 3 hours. Saliva and urine samples were collected before, immediately following visits (post-exposure), and next morning, and analyzed for cotinine and total NNAL, respectively. Mixed-effects models were fit and changes in biomarkers were contrasted between locations. A second set of models was used to determine the effect of cigarette count on elevated biomarker levels.

Results: Significant increases in salivary cotinine were measured immediately following visits to the restaurant and bar sites compared to the control ($F=76.72$, $p<0.001$). Differences in geometric means (plus 95% confidence interval) for post minus pre-exposure salivary cotinine at the bar, restaurant, and control were 0.115 (0.105,0.126), 0.030 (0.028,0.031), and -0.004 ng/mL, respectively and these levels were similar to next-day minus pre-exposure measures. Post- minus pre-exposure creatinine-corrected urinary NNAL were non-significant. Next-day minus pre-exposure concentrations were higher following visits to the bar and restaurant sites compared to the control ($F=6.16$, $p=0.005$). Next-day minus pre-exposure changes at the bar, restaurant and control were 1.858 (0.897,3.758), 0.615 (0.210,1.761), and -0.007 pg/mg creatinine, respectively.

Conclusion: Salivary cotinine and urinary NNAL increased significantly in non-smokers following brief outdoor SHS exposure indicating that they are at risk for health effects associated with carcinogenic tobacco-specific nitrosamines such as NNAL.

INTRODUCTION

The US Surgeon General released a landmark report in 1986 that was the first of its kind to identify a chronic disease risk from exposure to tobacco smoke for individuals other than smokers (USDHHS 1986). Secondhand smoke (SHS) or environmental tobacco smoke (ETS), as it was referred to in the 1986 report, is defined as a combination of smoke emitted from a burning tobacco product between puffs (sidestream smoke) and the smoke exhaled by the smoker (mainstream smoke). The 1986 report presented evidence that the chemical composition of sidestream smoke is qualitatively similar to mainstream smoke inhaled by active smokers and that both sidestream and mainstream smoke were carcinogens (USDHHS 1986). An update of the 1986 report was released 20 years later in the 2006 US Surgeon General report, concluding that SHS causes premature death and disease in children and adults who do not smoke; exposure to SHS has immediate adverse effects on the cardiovascular system and causes coronary heart disease and lung cancer; and, there is no risk-free level of exposure to SHS (USDHHS 2006).

Scientific evidence continues to show that SHS exposure is causally associated with lung cancer in never- or non-smokers (Vineis et al. 2007; Wakelee et al. 2007), breast cancer in non-smoking, premenopausal younger women (Miller et al. 2007), as well as a risk factor for other cancers such as bladder and pancreatic cancers (Alberg et al. 2007; Bao et al. 2009; Van Hemelrijck et al. 2009). Secondhand smoke has been shown to increase the risk of cardiovascular disease by ~30% (Barnoya, and Glantz 2005) and accounts for at least 30,000 deaths annually in the United States (Adhikari et al. 2008). Other studies further support the causal link between SHS exposure and respiratory diseases such as asthma in children (Jaakkola, and Gissler 2004), chronic bronchitis (Vozoris, and Lougheed 2008), and sinusitis (Hammad et

al. 2010), and as a potential cause of obstructive lung disease in non-smokers (Eisner et al. 2010; Flouris et al. 2009).

The increasing and overwhelming body of evidence showing elevated disease risk among non-smokers exposed to SHS has led to the passage of smoking bans in workplaces and public places, including restaurants and bars. As of January 2011, 21,850 municipalities are covered by a 100% smoke-free provision in workplaces, and/or restaurants, and/or bars, by either a state, commonwealth, or local law, representing 79.4% of the US population (ANRF 2011). Smoke-free air laws have been very effective in reducing exposures to constituents of SHS (Bondy et al. 2009) as well as decreasing acute myocardial infarction, angina, stroke, and asthma (Herman, and Walsh 2010; Marlow 2010; Sargent, Shepard, and Glantz 2004; Seo, and Torabi 2007). The state of Georgia passed a state-wide smoking ban in 2005 in restaurants and bars that serve or employ minors (Georgia 2005). Athens-Clarke County in Georgia further implemented an ordinance in 2005 prohibiting smoking in all restaurants and bars but not in all workplaces (ACC 2005).

While smoke-free air laws have been shown to have large positive effects on public health, indoor smoking bans seem to result in increased smoking outside establishments, in outdoor seating areas or at their entrances. The magnitude of outdoor SHS exposure and associated health risks are relatively unknown as very few studies have characterized outdoor SHS exposure. An exhaustive literature search shows that three published studies have measured SHS outside establishments, all of which used environmental proxies of SHS (Kaufman et al. 2010; Klepeis, Ott, and Switzer 2007; St.Helen et al. 2011). Systemic human exposure estimates from environmental markers reported in these studies are subject to factors such as proximity to smokers and meteorological conditions and may be highly imprecise (Benowitz 1999). We also

previously conducted the first biomonitoring of non-smoking subjects exposed to outdoor SHS using salivary cotinine (Hall et al. 2009). Cotinine is the primary proximate metabolite of nicotine.

Secondhand smoke contains carcinogenic tobacco-specific nitrosamines (TSNA) such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Hoffmann, and Hecht 1990). Uptake of NNK by non-smokers exposed to SHS has been identified as a biochemical link between SHS exposure and lung cancer risk (USDHHS 2006). NNK is mainly metabolized by keto reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and conjugated to 4-(methylnitrosamino)-1-(3-pyridyl)-but-1-yl]-β-O-D-glucosiduronic acid (NNAL-Gluc) (Hecht 1996). Although cotinine is appropriate as a biomarker of SHS exposure (Benowitz 1999), it may not always be an accurate measure of exposure to some toxicants in tobacco smoke such as NNK (Benowitz et al. 2010), and thus underestimate SHS health risks. Further, the ratio urinary NNAL to urinary cotinine has been shown to be much higher in passive smokers compared to active smokers (Benowitz et al. 2010). There are currently no studies reporting NNAL levels or ratio of NNAL to cotinine in non-smokers exposed to outdoor SHS.

Therefore, the objectives of this study were to: a) characterize the exposure of non-smokers to SHS, in outdoor seating areas and outside a restaurant and a bar in Athens, Georgia where only indoor smoking is banned using salivary cotinine; b) characterize their uptake of TSNA using urinary NNAL; and c) determine their ratio of urinary NNAL to salivary cotinine.

METHODS

Study Location

The study was conducted during three weekends in August and September of 2010 in Athens, GA, a city with an estimated population of 102,000 and with a local county ordinance banning smoking in restaurants and bars and most workplaces. In order to characterize human exposure to outdoor SHS, three locations were selected: outdoor seating or standing areas of a bar, a family restaurant, and an open air seating area outside the Environmental Health Science (EHS) building at the University of Georgia (UGA). Descriptions of the study sites are presented in Table 4.1. Previous data show relatively high SHS outside the bar site selected, hence its inclusion (Hall et al. 2009; St.Helen et al. 2011). Although lower SHS was previously measured at family restaurants in Athens (Hall et al. 2009; St.Helen et al. 2011), a restaurant site was added because restaurants may serve as potential SHS exposure sources to children and individuals who do not frequent bars. An open-air seating area outside the EHS building was selected as the control site because no smokers were present during study times. The study was designed as a crossover study in which participants visited each site once over three weekends, one site per study day.

Subject Recruitment and Selection

Subjects were University of Georgia college and graduate students. Participation was limited to self-reported, healthy non-smokers aged 21 to 40 years and enrollment was directed towards a target population size of 24. A questionnaire was administered to potential study participants to determine eligibility. Questions include current and past smoking status and current SHS exposure at home, work or elsewhere. If the individual did not smoke, did not use nicotine in any alternative form (i.e., smokeless tobacco, nicotine replacement therapy, etc.), did

not report respiratory illnesses such as asthma, and was not or could not be pregnant, they were considered eligible to participate. Respondents who met the eligibility requirements attended personal information sessions in which the study and protocol were discussed and concerns or questions were addressed. Respondents who chose to participate signed consent forms and gave their preference of study day, Friday or Saturday. Subjects were assigned to study sites in this crossover study based on a replicated Latin square in which each subject participated at different sites on the three occasions (i.e., once each at the bar, restaurant, and control sites). Twenty-eight subjects were enrolled in the study and were financially compensated for their participation. This study was reviewed and approved by the Institutional Review Boards at UGA and the Centers for Disease Control and Prevention (CDC).

Site Visits

Participants were called by telephone the day before each study day to remind them of assigned study location and time. Participants were also encouraged to stay hydrated and avoid urinating at least 2 h before study times. Participants arrived at the EHS building about 1 h before site visits and were briefed again on study protocol when at study sites and proper saliva and urine sample collection. Pre-exposure saliva and urine samples were collected as described below in *Biological Sample Collection* and participants were then transported, if assigned to the restaurant and bar sites, on a designated non-smoking EHS van. Restaurant and control sites were visited at 6:00 pm to 9:00 pm and the bar site at 11:00 pm to 2:00 am on study days. These times represent peak business hours for restaurants and bars in Athens, respectively and possibly higher smoking activity than other times. Participants remained at each study site for the full 3 h except for necessary bathroom breaks and were encouraged to stand or seat in close proximity to smokers, which ranged from about 0.5 m to 5 m at any given time. Participants ate dinner while

they were at the restaurant and control sites while they ate dinner prior to visits to the bar site. One assigned subject at each location took the cumulative 10-min smoker (cigarette) and pedestrian counts for the 3-h visit. The cigarette count included every lit tobacco product from persons who were walking past, sitting, or standing in the seating/standing area or outdoor patio of the locations. The pedestrian count included both nonsmoking and smoking individuals at or passing by each location. Following the 3-h visit, participants at the control site returned to the EHS building and those at the restaurant and bar sites were transported on the EHS van. Post-exposure saliva and urine samples were collected within 30 minutes of subjects leaving the study sites and a questionnaire assessing participants' exposures to SHS for the 48-h period prior to site visits was given. Participants were then given materials for next-day sample collection in a biohazard bag, with instruction to keep all materials away from SHS or smokers and to freeze samples immediately after collection.

Biological Sample Collection

Participants provided saliva and urine samples, immediately pre- and post site visits and first-morning void on the next day. Hereafter, same-day post-exposure will be referred to as post-exposure and next-day post-exposure as next-day. Urine and saliva samples were taken at roughly the same times. Saliva samples were collected using Salivettes (Sarstedt, Newton, NC, USA) with the cotton swab inserts. Samples were collected by gently chewing on the cotton swab for about 2 min. The swab was returned to its container and immediately placed in -20 ^{o}C freezer without further treatment until analysis. To avoid contamination with prostatic secretions, male participants provided urine samples in three portions: 1) 75 mL portion in a 200 mL cup pre-marked at the 75 mL level; 2) 25 mL in a 50 mL conical tube labeled '1' pre-marked at 25 mL level (referred to as 75 – 100 mL portion); and 3) remaining urine in a second 50 mL conical

tube labeled ‘2’ (referred to as post-100 mL portion). Female participants provided urine samples in one portion in a 200 mL cup of which an aliquot was taken for NNAL analysis. NNAL was measured in the 75 mL portion of urine samples from males. Both male and female urine samples were analyzed for Clara cell protein (CC16) in a concurrent study that will be separately reported (St Helen et al. 201x). Pre- and post-exposure saliva and urine samples were collected at the EHS building. Next-day samples were collected at the participants’ homes and were kept frozen until delivery by the participants to EHS on each Monday following the study weekend. Samples were stored in -80 ^{o}C freezer at the Air Quality Lab (AQL) in EHS until shipment on dry ice to the CDC six weeks after collection for analyses of salivary cotinine and urinary creatinine and NNAL as well as external labs for other biomarker analyses.

Biomarker Analysis

Salivary cotinine was measured by high-performance liquid chromatography atmospheric-pressure chemical ionization tandem mass spectrometry (LC APCI MS/MS) using a method that has been described elsewhere in detail (Bernert et al. 2000). Briefly, the saliva sample was equilibrated with a trideuterated cotinine internal standard for 15 min, extracted with methylene chloride, dried, reconstituted in water, and analyzed on an AB Sciex API 4000 tandem mass spectrometer with the heated nebulizer installed. Cotinine concentrations were quantified by comparison with standards using least squares linear regression. The limit of detection (LOD) was 15 pg/mL. All analytical runs included a blank and two quality control (QC) samples.

Total NNAL measurements were made by using a method that has been previously described (Xia, and Bernert 2010). In these analyses, the urine samples were hydrolyzed with β-glucoronidase and total NNAL was measured. Total NNAL is constituted of free NNAL and

NNAL-Gluc. Briefly, $^{13}C_6$-NNAL was added as an internal standard followed by a preliminary separation and sample clean-up using specially developed molecularly imprinted polymer columns, and analysis by high-performance liquid chromatography coupled with electrospray-ionization tandem mass spectrometry. The LOD for these analyses was 0.6 pg/mL. Creatinine in urine was measured by a commercially available automated colorimetric enzymatic method (Roche Creatinine Plus) implemented on a Hitachi Modular P analyzer. Urinary NNAL measurements were made in groups of samples containing appropriate blank and known QC materials. All results were from analytical runs determined to be in statistical control by use of standard quality assurance procedures (Bernert et al. 2000).

Statistical Analysis

Due to the approximate log-normal distribution of salivary cotinine and urinary NNAL data, these values were log-normalized. In order to include non-detects (zero concentration), a small number (10^{-7}) was added to all concentrations before log-transformation. These include log(pre-exposure), log(post-exposure), and log(nextday-exposure). When adjusting urinary NNAL concentrations for creatinine, the logs of the ratios of urinary NNAL to urinary creatinine were used. Geometric means and 95% confidence intervals (CI) were computed for pre-, post-, and next-day variables by exponentiating the means of the logs of the variables and corresponding 95% CI; 10^{-7} was then subtracted from all reported means. Differences in geometric means and 95% CI of pre-exposure, post-exposure, and next-day-exposure biomarker data were computed and used to represent changes in biomarker data following exposure to SHS. When concentrations were below the limit of detection (LOD), the concentrations reported from the analytical procedures were used.

Analyses of the changes in biomarker data following 3-hr site visits (post-exposure) and next-day relative to pre-exposure levels proceeded from a statistical model for the replicated Latin square design employed in this study. For response y_{ijk} measured on the k^{th} subject on the j^{th} measurement occasion (day) under the i^{th} exposure location, Equation 4.1 was assumed. In this study design, participants were assigned only one of two weekend days (Friday or Saturday), therefore, day was nested in week ($w_{(j)l}$) as shown in Equation 4.1.

$$y_{ijk} = \mu_i + w_{(j)l} + s_k + e_{ijk} \qquad \text{Equation 4.1}$$

Here, μ_i represents the mean response for the i^{th} exposure location, and $w_{(j)l}$ and s_k are mean zero, constant variance, normal random effects for day nested in weeks and subjects, respectively. The response variables were *log(post)-log(pre)* and *log(nextday)-log(pre)*. F tests were conducted of no overall effect of exposure location as well as F tests of pair-wise contrasts between the control, restaurant and bar locations, respectively, adjusted by Tukey's method for multiple comparisons. In order to test for differences in biomarker responses across gender and race, these two variables were introduced into Equation 4.1 as covariates (fixed effects) in a separate analysis and pair-wise contrasts were made between levels of gender and race, respectively. Spearman rank correlation coefficients between changes in cotinine, NNAL, and creatinine-corrected NNAL were computed. Finally, ratios between urinary NNAL and salivary cotinine as well as creatinine-corrected NNAL and cotinine concentrations were computed and non-parametric Kruskal-Wallis analysis of variance was used to compare these ratios by exposure location.

Finally, since non-detects and below LODs made up 28.9% and 14.9%, respectively, of 242 urine samples analyzed for NNAL, Friedman's non-parametric Chi-square test was used to verify the results of the mixed-effects model described in Equation 4.1 where location-type was

the independent variable. In short, pre-, post-, and next day urinary NNAL were treated as repeated measures on participants, urinary NNAL concentrations were ranked, and location-type was introduced as the independent variable of interest while controlling for variability from week to week. Analyses were carried out using SAS v. 9.1 (SAS Institute, Inc. Cary, NC, USA). All statistical tests were considered significant at $\alpha = 0.05$.

RESULTS

Twenty-eight participants (18 females) were initially enrolled in this study. Seventeen of the subjects were white (11 female), seven were black (three females), three were Asians (all female) and one described race as other (female). All participants were within 21 to 37 years. While four subjects reported smoking by others near or at their residences or workplaces, most were not routinely exposed to SHS. Subjects were asked to avoid all SHS as much as possible three days prior to each study weekend. The range of baseline pre-exposure salivary cotinine concentrations confirmed their relatively low pre-study SHS exposure (0.011 – 0.480 ng/mL). Biomarker data from one male participant were excluded from all statistical analyses after baseline pre-exposure salivary cotinine concentration was found to be 5.25 ng/mL, 11 times higher than the next maximum (0.480 ng/mL). This participant lived with a smoker. Weeks 2 and 3 locations for two subjects were changed from the pre-assigned locations due to personal scheduling conflicts. Data from these participants were omitted from mixed-effects models because their new location assignment violated the Latin square (crossover) design. However, biomarker data for these two participants were used in computation of descriptive statistics. In all, there were eight complete Latin squares in which groups of three subjects (24 subjects in

total) were assigned to three study locations for three weekend days (12 subjects on Fridays and 12 subjects on Saturdays).

Geometric means of pre-exposure, post-exposure, and next-day salivary cotinine are given in Tables 4.2. Eleven of 242 samples did not have enough saliva for analysis (n = 1 pre-exposure, n = 7 post-exposure, and n = 3 next-day) and one sample was below the LOD (LOD = 0.015 ng/mL). The difference in geometric means of post- and pre-exposure and next-day and pre-exposure are also presented in Table 4.2. Statistically significant higher salivary cotinine was measured in female subjects both post- versus pre-exposure ($t = 3.10$, $p = 0.004$) and next-day versus pre-exposure ($t = 2.47$, $p = 0.018$) compared to male subjects (biomarker data for males and females are not presented separately). Comparisons by race were non-significant.

Significant differences in salivary cotinine both post- versus pre-exposure ($F = 76.72$, $p<0.001$) and next-day versus pre-exposure ($F = 40.99$, $p <0.001$) were observed across the exposure locations selected for all subjects and across gender. The tests of exposure location effect as well as pair-wise comparisons between locations are presented in Table 4.3. Differences in geometric means of post- and pre-exposure and next-day and pre-exposure are also presented in Figures 4.1A and 4.1B, respectively [presented as differences in geometric means and 95% CI]. Mean post- minus pre-exposure salivary cotinine was significantly higher when subjects were outside the bar location [0.115 (0.105, 0.126) ng/ml] compared to the control [-0.004 (-0.005, -0.003) ng/ml] ($t = 12.36$, $p<0.001$) as well as when subjects were outside the restaurant [0.030 (0.028, 0.031) ng/ml] compared to the control ($t = 5.32$, $p<0.001$). Post-versus pre-exposure changes in salivary cotinine were higher following visits to the bar location compared to the restaurant ($t = 6.72$, $p<0.001$). Similar results were obtained when we looked at next-day versus pre-exposure (Table 4.3, Figure 4.1B). Further, post-exposure salivary cotinine and next-

day salivary cotinine concentrations were not significantly different among subjects ($F = 0.87$, $p = 0.426$). This is further illustrated in Figures 4.2A – 4.2C, where median and first and third quartiles of salivary cotinine concentrations are presented. Compared to baseline salivary cotinine levels (pre-exposure on first sampling day), week-3 pre-exposure salivary cotinine was significantly higher ($t = 3.25$, $p = 0.006$). Week-2 pre-exposure salivary cotinine was not significantly different from baseline pre-exposure levels ($t = 1.51$, $p = 0.416$) or week 3-pre-exposure salivary cotinine levels ($t = 1.75$, $p = 0.262$).

Of 27 subjects, NNAL was measured above the LOD (LOD = 0.6 pg/mL) in 9 (33.3%) subjects, below the LOD in 10 (37.0%), and not detected in 8 (29.6%) subjects at baseline. Overall, of 242 urine samples collected during the study period from 27 subjects (81 pre-, 81 post-, and 80 next-day samples, one subject did not provide a next-day urine sample), urinary NNAL was measured over the LOD in 56.2% (n = 136), below the LOD in 14.9% (n = 36), and not detected in 28.9% (n = 70) (Table 4.4). Because of the low concentrations involved following SHS exposure at these locations, only total NNAL concentrations, i.e., concentrations measured following β-glucuronidase hydrolysis, are presented. Geometric means for pre-exposure, post-exposure, next-day urinary NNAL, and differences between these variables are given in Table 4.5. Creatinine-corrected variables are also presented in Table 4.5. When compared by gender, females had higher post-exposure versus pre-exposure NNAL (uncorrected NNAL, $p = 0.016$; creatinine-corrected NNAL, $p = 0.026$; data not presented). No significant differences were observed when comparing next day versus pre-exposure by gender (uncorrected NNAL, $p = 0.191$; creatinine-corrected NNAL, $p = 0.167$). Differences in uncorrected and creatinine-corrected NNAL changes were not observed by race (all p-values > 0.400).

In addition, there were no significant differences between post-exposure versus pre-exposure urinary NNAL in subjects who were at the bar or restaurant sites compared to the control site ($F = 1.62$, $p = 0.210$) (Table 4.3). When adjusted for creatinine, these differences remained non-significant ($F = 2.05$, $p = 0.142$). Next-day versus pre-exposure changes in urinary NNAL were significantly different among the exposure locations (uncorrected: $F = 6.30$, $p = 0.004$; and creatinine –corrected: $F = 6.16$, $p = 0.005$). Changes in uncorrected urinary NNAL were significantly higher following visits to the bar location ($t = 3.42$, $p<0.001$) and restaurant location ($t = 2.60$, $p = 0.006$) compared to the control site. These changes remained significant when corrected for creatinine (bar vs. control, $t = 3.41$, $p < 0.001$; restaurant vs. control, $t = 2.48$, $p = 0.009$). Significant differences in next day versus pre-exposure urinary NNAL were not observed when bar was compared to restaurant visits (Table 4.3). Changes in unadjusted and creatinine-adjusted urinary NNAL are presented in Figure 4.1C and 4.1D, respectively. Figures 4.2D – 4.2F show the change in urinary NNAL at the different sampling times. NNAL increased significantly from post-exposure to next-day following visits to the bar and restaurant sites compared to the control (unadjusted: $F = 7.53$, $p = 0.002$); creatinine-adjusted: $F = 7.26$, $p = 0.002$). Compared to baseline urinary NNAL levels, pre-exposure NNAL did not increase significantly in the second and third weeks (uncorrected: $F = 0.25$, $p = 0.778$; creatinine-corrected: $F = 1.85$, $p = 0.169$). When pre-, post-, and next-day NNAL were analyzed as repeated measures by the non-parametric Friedman's Chi-square test, location-type had a significant effect on the distribution of urinary NNAL (uncorrected: $\chi^2 = 7.16$, $p = 0.028$; creatinine-corrected: $\chi^2 = 13.9$, $p = 0.001$).

Both salivary cotinine and urinary NNAL biomarker levels showed a clear exposure-dependent effect with cigarette count. The average sums of cigarettes smoked at the site for the

3-h sampling period over the entire study were: bar, 144.5 ± 39.9; restaurant, 33.5 ± 28.0; and none at the control (presented as mean of sums ± SD) (Table 4.1). Models in which cigarette count was used as the independent variable instead of location-type were consistent with the results presented above where location-type was the independent variable. Post- vs. pre-exposure salivary cotinine and next-day vs. pre-exposure salivary cotinine changes were significantly associated with cigarette count ($p<0.001$). Just as with location-type, cigarette count was not associated with post- vs. pre-exposure changes in urinary uncorrected and creatinine-corrected NNAL but was significantly associated with next-day vs. pre-exposure changes in unadjusted NNAL ($F = 6.06$, $p = 0.018$) and creatinine-corrected NNAL ($F = 6.01$, $p = 0.018$).

Spearman rank correlation coefficients (ρ) between post-exposure minus pre-exposure and next-day minus pre-exposure salivary cotinine and urinary NNAL (unadjusted and creatinine-adjusted) are presented in Table 4.6. Post- minus pre-exposure salivary cotinine was not significantly correlated to post- minus pre-exposure urinary NNAL (uncorrected and creatinine-corrected). Next day minus pre-exposure salivary cotinine was significantly correlated to next day minus pre-exposure urinary NNAL (uncorrected, $\rho = 0.49$; creatinine-corrected $\rho = 0.60$) and creatinine-corrected post- minus pre-exposure creatinine-corrected urinary NNAL ($\rho = 0.33$).

Geometric means and 95% CI for the ratios between urinary NNAL measured in pg/mL (either uncorrected or corrected to urinary creatinine concentration in pg/mL) and salivary cotinine measured in ng/mL are given in Table 4.7. Ratios computed are for biomarker levels at pre-exposure, post-exposure, and next-day sampling times, respectively. There was no significant difference in pre-exposure urinary NNAL:salivary cotinine ratios when location type, gender, and race were considered, respectively (uncorrected and creatinine-corrected NNAL). A

marginally non-significant difference in post-exposure ratios (uncorrected NNAL) was observed by location-type ($\chi^2 = 5.76$, $p = 0.056$), with lower ratios following the restaurant and bar sites compared to the control site. This was non-significant when corrected for creatinine ($\chi^2 = 4.29$, $p = 0.117$). Post-exposure ratios did not differ significantly by gender and sex. Location and gender differences were not observed when next day ratios were considered, but there were significant differences across race in creatinine-corrected NNAL:salivary cotinine ratios ($\chi^2 = 13.44$, $p = 0.004$).

DISCUSSION

In this study we have investigated the uptake of tobacco-specific compounds in 27 non-smokers following exposure to SHS outside a restaurant and a bar in Athens, GA five years after the implementation of an indoor smoking ban at these establishments. We observed significant increases in cotinine measured in saliva collected both immediately post 3-hr site visits and next-day and significant increases in NNAL (uncorrected and creatinine-corrected) measured in urine collected at first-morning void (next-day). The changes in salivary cotinine and urinary NNAL measured after visits to bar and restaurant locations were significantly higher than what was observed following visits to the control site, where no smokers were present, with a clear exposure-response with the number of cigarettes smoked at each location.

Although it is recognized that there is no risk-free level for SHS very few studies have characterized outdoor SHS. However, with the passage of indoor smoking bans and growing numbers of smokers outside establishments, in outdoor seating areas, and their entrances, public health concerns are justifiable. Therefore, characterization of outdoor SHS and human exposures is needed for human health risk assessment. One of the first outdoor SHS studies reported in the

literature was conducted by Klepeis and colleagues using particulate matter ($PM_{2.5}$) as a proxy for SHS (Klepeis et al. 2007). Another study was done recently in which the authors assessed SHS exposure using $PM_{2.5}$ inside and outside establishments, reporting that average outdoor $PM_{2.5}$ with smoking was significantly higher than background level and significantly and positively correlated with number of lit cigarettes (Kaufman et al. 2010). We also reported the results of a study conducted in Athens, GA in 2006 in which 12-hr real-time $PM_{2.5}$ and carbon monoxide (CO) were used to assess outdoor SHS at restaurants and bars (St.Helen et al. 2011). The number of cigarettes counted at each site during that study had a significant positive effect on log(CO) (estimate = 0.012, $p = 0.032$) as well as on log($PM_{2.5}$) (estimate = 0.058, $p<0.001$). The effects of pedestrians and vehicles on these SHS proxies were not statistically significant. While these studies provide information on SHS in ambient air, they cannot accurately predict human SHS systemic exposure or uptake because of factors such as proximity to smokers and air exchange rates. The use of biological markers specific to tobacco smoke is a method to overcome these limiting factors.

Cotinine has been proposed as a very sensitive and specific biological marker of SHS exposure (Benowitz 1999). Cotinine has an average half-life of 16 hr, so cotinine is eliminated from the body within 3–4 days following the last exposure (Benowitz 1996). Thus, use of cotinine as a biomarker of recent SHS exposure is appropriate in this study since we are interested in changes over less than 24 hr. Salivary cotinine was used in the current study instead of the commonly used serum cotinine because of its ease of collection. Salivary cotinine levels are typically 15-30% higher than serum cotinine (Bernert et al. 2000) and previous studies have shown that serum and salivary cotinine levels are highly correlated with a 1.1 – 1.4 saliva to blood ratio (Curvall et al. 1990; Jarvis et al. 1984). To the best of our knowledge, we previously

reported the first biological assessment of outdoor SHS (Hall et al. 2009). In that study, we used salivary cotinine to characterize uptake of SHS constituents following a 6-hr visit to outdoor patios and seating areas of restaurants and bars. Geometric mean changes in salivary cotinine following visits to outdoor patios of bars and restaurants were 0.114 ng/ml and 0.039 ng/ml, respectively, levels that were significantly higher than at the control (0.006 ng/ml) (Hall et al. 2009). The levels of salivary cotinine reported in the current study are very similar to the levels reported by Hall and colleagues although site visit duration was 3 hr compared to 6 hr in the previous study. The results of these two studies are comparable despite longer exposure times in the first study because the potentially highest SHS exposure times had been indentified from the previous study. This may indicate that while duration at the sites contributes significantly to exposure magnitude, shorter durations with high smoking activity may also lead to significant SHS exposure. We observed a clear exposure-response between cigarettes counted at the sites and increased salivary cotinine, showing that changes in salivary cotinine are attributed to SHS exposure outside these establishments.

Salivary cotinine was measured on both the day of SHS exposure and the following day. The significant increases in post-exposure and next-day salivary cotinine relative to pre-exposure levels are indicative of recent exposures to SHS outside the restaurant and bar locations, unlike at the control site where no smokers were present (Figures 4.2A – 4.2C). We did not observe significant differences between next-day salivary cotinine concentrations and what we measured at the end of site-visits (post-exposure).

We observed significantly higher salivary cotinine in female subjects. For example, following visits to the bar site, post-exposure minus pre-exposure changes in salivary cotinine among female subjects was 0.123 (0.109 – 0.137) ng/mL (n = 16) compared to 0.099 (0.079 –

0.122) ng/mL (n = 9) among males (geometric means, 95% confidence intervals). One recent study, among others, has shown that females eliminate cotinine faster than males (Bernert et al. 2009), which may explain the higher levels of cotinine in saliva from females. We also observed higher salivary cotinine increases in females when next-day/pre-exposure salivary cotinine was considered. Conversely, we did not find significant differences in changes in salivary cotinine following SHS exposure by race. Differences in nicotine metabolism by race have been previously established (Benowitz, Hukkanen, and Jacob 2009) so it seems that this result may be more a reflection of the small study size and imbalance in size of subgroups of race enrolled in the study.

We also report changes in urinary NNAL following outdoor SHS exposure which based on an exhaustive literature search is the first study to do so. NNAL is the metabolite of NNK, both of which are systemic pulmonary carcinogens specific to tobacco smoke (Hecht 2003). There is strong evidence that NNK is a causative agent in the formation of lung adenocarcinoma in smokers (Hoffmann, Rivenson, and Hecht 1996), which is now the most frequent type of lung tumor found in non-smokers (Hoffmann et al. 1996). Total NNAL more directly reflects exposure to carcinogens in SHS than cotinine (Benowitz 1999) and has been used to characterize human exposure to carcinogenic tobacco-specific nitrosamines among adult non-smokers with regular exposure to SHS (Anderson et al. 2001) as well as among elementary school-aged children with exposure to SHS (Hecht et al. 2001). In addition, epidemiologic studies linking lung cancer to SHS have been strengthened by the detection of NNAL in non-smokers (USDHHS 2006).

We observed significant increases in total uncorrected and creatinine-corrected NNAL measured in next-day first-void urine samples following both bar and restaurant site visits

compared to the control site visit (Table 4.3, Figures 4.1C and 4.1D). We did not observe significant increases in total NNAL measured in immediate post-exposure urine following visits to the bar and restaurant compared to visits to the control site. The non-significant changes in post-exposure urinary NNAL are most likely due to the longer elimination half-life of NNAL, which averages 10 – 16 days (Goniewicz et al. 2009). Next-day sample collection was added to the study design because of this longer half-life. Although the concentrations of total NNAL reported in the current study were consistently low, NNAL was detected above the LOD in 66 of 80 next-day samples (83%) compared to 38 of 81 (47%) post-exposure and 32 of 81 (40%) of pre-exposure urine samples. Our results clearly demonstrate that non-smokers with even brief durations of SHS (3 h) are exposed to detectable levels of tobacco-specific nitrosamines, albeit at low concentrations. We observed significantly higher post- versus pre-exposure urinary NNAL among female subjects compared to males; on the other hand, next day versus pre-exposure among females and males were not significantly different. Differences in urinary NNAL were also not observed across race. These results are largely consistent with a recent study of controlled exposure to sidestream smoke by Bernert and colleagues (Bernert et al. 2009).

Short-term (post- minus pre-exposure) and longer-term (next-day minus pre-exposure) salivary cotinine were much more correlated to longer-term urinary NNAL than short-term urinary NNAL. Lower correlations with short-term NNAL are most likely due to the non-significant changes in short-term NNAL concentrations observed. While significant, the moderate correlations observed between salivary cotinine and NNAL show that salivary cotinine from outdoor SHS exposure may not always be highly predictive of urinary NNAL concentrations. The explanation for the moderate correlations can potentially include differences in metabolic rates of these compounds as well as different behaviors of the parent compounds,

nicotine and NNK, in the ambient atmosphere. Unpublished research from Philip Morris Tobacco Company shows that NNK can form in sidestream smoke after it has been released into the air, with increases as much as 50% to 200% per hour during the first 6 h after cigarettes are extinguished in a chamber study and increases for the first 2 h after cigarettes are extinguished in real offices (Schick, and Glantz 2007) while air nicotine concentrations decrease. The extent to which this applies in outdoor locations is uncertain and needs further study but cannot be ruled out.

A study by Benowitz and colleagues recently showed that urinary cotinine levels underestimate exposure to NNK in passive versus active smokers (Benowitz et al. 2010) and therefore may underestimates disease risk. While cotinine concentrations in saliva, the medium used in this study, and urine may differ due to different elimination rates, the concentrations of cotinine in saliva and urine are not drastically different. Thus the conclusion by Benowitz and colleagues can potentially apply to salivary cotinine. The ratio urinary NNAL:urinary cotinine is higher in passive smokers compared to active smokers (Benowitz et al. 2010). We present the first set of data on the uncorrected and creatinine-corrected urinary NNAL:salivary cotinine ratios following outdoor SHS exposure. We did not observe significant differences in pre-exposure and next-day NNAL:cotinine ratios, respectively, across study location. However, our data seem to suggest that post-exposure NNAL:cotinine ratios were marginally lower following visits to bar and restaurant sites compared to the control location. Table 4.7 also shows a clear trend in the ratios following SHS exposure, with low ratios immediately post-exposure and higher ratios at pre-exposure and next-day time points. The low post-exposure ratios indicate significant increases in salivary cotinine immediately following end of SHS exposure while NNAL concentrations remained unchanged. It seems low versus high NNAL:cotinine ratios in

passive smokers would indicate time from most recent exposure, where low ratios would be indicative of more recent SHS exposure. This warrants further investigation as a tool in epidemiologic studies to characterize passive smokers by time from most recent SHS exposure.

The exposure of non-smokers to SHS continues to represent an important public health concern although recent efforts to reduce SHS exposure such as indoor smoking bans have had a major effect. A study consisting of a series of National Health and Nutrition Examination Surveys (NHANES) investigating the trends in serum cotinine levels among non-smokers in the U.S. population over a 14-year period from 1988 through 2002 reported an approximate 70% decline in serum cotinine levels over that period (Pirkle et al. 2006). Average serum cotinine concentration among U.S. non-smokers aged 20 years or older in NHANES 2005 – 2006 was 0.047 (0.042 – 0.053) ng/mL (geometric mean and 95% CI), which would be roughly equivalent to 0.071 (0.063 – 0.079) ng/mL salivary cotinine based on Equation 4.2 (Bernert et al. 2000).

$$\log_{10}(\text{salivary cotinine}) = 0.962817 \times \log_{10}(\text{serum cotinine}) + 0.127478 \qquad \text{Equation 4.2}$$

In comparison, baseline geometric mean (with 95% CI) salivary cotinine levels in the current study was 0.039 (0.032 – 0.047) ng/mL, values that are below the national average.

Concentrations of urinary NNAL measured following visits to the bar site in this study were about two to five times lower than studies reporting urinary NNAL measured in non-smokers exposed to indoor SHS during one work-shift at workplaces such as restaurants, bars, and other hospitality workplaces and in chronically exposed hospital workers. These studies report urinary NNAL ranging from 3.8 pg/mg creatinine to 12.3 pg/mL (Jensen et al. 2010; Parsons et al. 1998; Stark et al. 2007). One of these studies measured urinary NNAL in non-smokers before and 24 h after a 4 h visit to a casino (Anderson et al. 2003). Mean changes in total urinary NNAL reported in that study was 3.8 pg/mg creatinine. Our reported NNAL levels

are at least two orders of magnitude lower than what has been measured in active smokers (Byrd, and Ogden 2003; Stepanov, and Hecht 2005). In spite of these low exposure levels, SHS is estimated to cause 3,000 lung cancer deaths (USDHHS 2006) and greater than 30,000 coronary heart disease deaths per year in the United States (Adhikari et al. 2008). In addition, our reported NNAL concentrations measured after a 3-h visit outside the bar and restaurant locations raise concerns about bar and restaurant employees who are potentially exposed to SHS for longer hours and more frequently during the week. Due to the relatively long elimination half-life of NNAL in urine, longer and more frequent exposures to SHS may lead to higher systemic exposures and therefore elevated health risks.

The current study has several strengths and limitations. Among the strengths, we used a replicated Latin square design which is a crossover design in which subjects served as their reference. This is especially important since biomarker responses have such high inter-individual variation. The study design is considered robust to high variability among participants. Further, this study is the first to report NNAL and urinary NNAL:salivary cotinine ratios for non-smokers exposed to outdoor SHS, results that could potentially have public health implications and applications in epidemiologic studies, respectively. On the other hand, one of the limitations of the study is the absence of objective proxies of SHS such as $PM_{2.5}$, CO or air nicotine. This was considered infeasible because of the attention that use of the monitors generates from patrons and the sensitivity of establishment owners to tobacco smoke-related research. Instead we used cigarette count to assess SHS at each site, which showed an exposure-response with biomarkers. Further, we did not collect variables such as temperature and wind-speed at the study sites that would more accurately characterize exposure considering that the sites are different. For example, the bar patio was partially enclosed, which may have led to less SHS dilution and

greater SHS exposure than at the restaurant site. The bar site was chosen because it represents one of the highest sources of outdoor SHS in Athens, GA. Finally, there is a possibility of carryover effect from week to week in biomarker response. This was more important with NNAL due to its half-life that is longer than the one week washout period. However, we did not observe significantly higher pre-exposure NNAL in weeks two and three compared to baseline pre-exposure at week one. In addition, we investigated the changes between post-exposure and pre-exposure and next-day and pre-exposure respectively; these response variables are less likely to be affected by carryover effects.

CONCLUSIONS

Our results indicate that both salivary cotinine and urinary NNAL increased significantly in an exposure-dependent manner due to inhalation of SHS outside of restaurants and bars in Athens, GA. Although the concentrations reported for urinary total NNAL are relatively low, measurement of NNAL clearly indicates that non-smokers exposed to brief periods of SHS outside establishments, especially bars, may be exposed to measurable concentrations of carcinogenic tobacco-specific nitrosamines such as NNK.

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TABLES AND FIGURES

TABLE 4.1 Description of study sites

Site	**Bar**	**Restaurant**	**Control**
Description	Bar with outdoor patio on second floor; partially enclosed by two walls of adjacent buildings, open at one end, and has no roof	Family restaurant with large open-air patio	Open air seating area
Location	Downtown Athens, Georgia; five minutes from University of Georgia's Environmental Health Science (EHS) Building	Athens west; ten minutes from EHS	Outside EHS building
No. of Tables	6	17	5
Approximate outdoor Area (m^2)	176	549	N/A
‡Cigarette Count			
Mean ± SD	144.5 ± 39.9	33.5 ± 28.0	0
Min - Max	86 - 202	12 - 86	0
†Pedestrians Count			
Mean ± SD	67 ± 25	32 ± 10	5 ± 3
Min - Max	45 - 109	12 - 41	1 - 7

‡Cigarette count statistics computed from 3-h sums of 10-minute cigarette count
†Pedestrian count statistics computed from 10-minute averages of 10-min pedestrian/patron count; N/A = not applicable

TABLE 4.2 Salivary cotinine descriptive statistics

Location		**Pre-exposure**	**Post-exposure**	**Next day**	**Post minus Pre**	**Next minus Pre**
Control	n	26	26	26	26	26
	Range	0.019, 0.480	0.021, 0.434	0.024, 0.359	-0.046, 0.020	-0.294, 0.133
	GM	0.049	0.044	0.053	-0.004	0.005
	95% CI	(0.037, 0.063)	(0.034, 0.058)	(0.041, 0.070)	(-0.005, -0.003)	(0.003, 0.006)
Restaurant	n	27	24	25	24	25
	Range	0.011, 0.165	0.036, 0.188	0.029,0.181	-0.037, 0.125	-0.051, 0.110
	GM	0.046	0.075	0.069	0.030	0.023
	95% CI	(0.036, 0.058)	(0.064, 0.089)	(0.058, 0.082)	(0.028, 0.031)	(0.022, 0.024)
Bar	n	27	25	26	25	26
	Range	0.015, 0.356	0.094, 0.407	0.035, 0.444	0.026, 0.214	-0.039, 0.398
	GM	0.045	0.161	0.165	0.115	0.120
	95% CI	(0.035, 0.059)	(0.140, 0.184)	(0.136, 0.200)	(0.105, 0.126)	(0.102, 0.141)

Data in ng/mL; 95% CI is 95% confidence interval of geometric means; n is number of subjects

TABLE 4.3 Test of effect of exposure location and pair-wise comparisons between location types

Analyte	Response variable	Test		Estimate	F or t-value[‡]	p-value	Adjusted p-value
Cotinine	log(post) – log(pre)	Fixed effect	Site-type	n/a	76.72	<0.001	n/a
		Comparisons	Restaurant vs. Control	0.264	5.32	<0.001	<0.001
			Bar vs. Control	0.596	12.36	<0.001	<0.001
			Bar vs. Restaurant	0.334	6.72	<0.001	<0.001
NNAL		Fixed effect	Site-type	n/a	1.62	0.210	n/a
NNAL[cc]		Fixed effect	Site-type	n/a	2.05	0.142	n/a
Cotinine	log(nextday) - log(pre)	Fixed effect	Site-type	n/a	40.99	<0.001	n/a
		Comparisons	Restaurant vs. Control	0.180	2.75	0.004	0.008
			Bar vs. Control	0.570	8.83	<0.001	<0.001
			Bar vs. Restaurant	0.390	6.05	<0.001	<0.001
NNAL		Fixed effect	Site-type	n/a	6.30	0.004	n/a
		Comparisons	Restaurant vs. Control	1.458	2.60	0.006	0.012
			Bar vs. Control	1.915	3.42	<0.001	0.001
			Bar vs. Restaurant	0.456	0.83	0.411	
NNAL[cc]		Fixed effect	Site-type	n/a	6.16	0.005	n/a
		Comparisons	Restaurant vs. Control	1.370	2.48	0.009	0.001
			Bar vs. Control	1.884	3.41	<0.001	0.001
			Bar vs. Restaurant	0.553	0.95	0.348	

NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; [cc]Creatinine-corrected urinary NNAL; n/a = not applicable
[‡]F value applies to fixed effects test while t-values applies to pair-wise comparisons

TABLE 4.4 Number of urine samples in which NNAL was detected

NNAL Detection (LOD = 0.6 pg/mL)	**†Baseline** (n = 27)	**Pre-exposure** (n = 81)	**Post-exposure** (n = 81)	**Next-day** (n = 80)	**Total** (‡N = 242)
Above LOD	9 (33.3%)	32 (39.5%)	38 (46.9%)	66 (82.5%)	136 (56.2%)
Below LOD	10 (37.0%)	21 (25.9%)	14 (17.3%)	1 (1.3%)	36 (14.9%)
Non-detects	8 (29.6%)	28 (34.6%)	29 (35.8%)	13 (16.3%)	70 (28.9%)

Note: NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. Analytical instruments report NNAL concentrations in both above and below LOD samples. Non-detects were treated as 0 pg/mL. ‡1 subject did not return next-day urine sample. †Baseline represents pre-exposure urinary NNAL from first week of study.

TABLE 4.5 Urinary NNAL and creatinine-corrected NNAL descriptive statistics

Location	**Variable**	**Pre-exposure** (n = 27)	**Post-exposure** (n = 27)	**Next day** (n = 27)	**Post minus Pre**[a] (n = 27)	**Next day minus Pre**[a] (n = 27)
Control	Range	0, 11.300	0, 6.900	0, 6.300	-4.400, 1.800	-5.100, 10.000
	GM	0.033	0.050	‡0.038	0.018	‡0.005
	95% CI	(0.005, 0.203)	(0.008, 0.302)	(0.005, 0.263)	(0.003, 0.099)	(0.000, 0.060)
Control[cc]	Range	0, 4.061	0, 2.724	0, 3.099	-2.325, 2.314	-1.790, 2.143
	GM	0.038	0.057	‡0.030	0.020	‡-0.007
	95% CI	(0.007, 0.191)	(0.012, 0.285)	(0.005, 0.198)	(0.004, 0.093)	
Restaurant	Range	0, 10.900	0, 2.100	0, 7.300	-9.400, 2.100	-8.800, 18.900
	GM	0.041	0.008	0.774	-0.034	0.733
	95% CI	(0.007, 0.239)	(0.001, 0.047)	(0.268, 2.234)	(-0.192, -0.006)	(0.261, 1.996)
Restaurant[cc]	Range	0, 15.875	0, 1.921	0, 10.501	-14.413, 1.377	-13.791, 10.501
	GM	0.056	0.013	0.671	-0.043	0.615
	95% CI	(0.011, 0.274)	(0.002, 0.069)	(0.221, 2.035)	(-0.204, -0.009)	(0.210, 1.761)
Bar	Range	0 , 10.200	0, 3.600	0, 10.400	-12.300, 3.100	-7.200, 9.900
	GM	0.037	0.109	2.407	0.072	2.370
	95% CI	(0.007, 0.206)	(0.023, 0.503)	(1.068, 5.425)	(0.017, 0.297)	(1.061, 5.219)
Bar[cc]	Range	0, 95.500	0, 3.749	0, 6.113	-96.500, 5.150	-92.426, 6.113
	GM	0.039	0.182	1.898	0.143	1.858
	95% CI	(0.007, 0.229)	(0.044, 0.755)	(0.904, 3.986)	(0.037, 0.526)	(0.897, 3.758)

Data in pg/mL or pg/mg creatinine; NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; [a]Differences between geometric means of post and pre and next-day and pre-exposure concentrations; 95% CI is 95% confidence interval of geometric means; ‡n = 26 subjects, otherwise n = 27; [cc]Creatinine-corrected urinary NNAL

TABLE 4.6 Spearman rank correlation coefficients between changes measured in salivary cotinine and urinary NNAL. Post-pre represents the difference between immediate post-3h SHS exposure and immediate pre-exposure concentrations while next day-pre represents the difference between next morning first-void concentrations and immediate pre-exposure concentrations.

	Cotinine Post – Pre	Cotinine Next day – Pre	NNAL Post-Pre	NNAL Next day – Pre	NNAL[cc] Post – Pre	NNAL[cc] Next day – Pre
Cotinine	1	**0.78**	0.12	**0.42**	0.21	**0.48**
Post – Pre		<0.001	0.323	<0.001	0.068	<0.001
		73	75	74	75	74
Cotinine		1	0.22	**0.49**	**0.33**	**0.60**
Next day – Pre			0.057	<0.001	0.004	<0.001
			76	76	76	76
NNAL			1	**0.47**	**0.75**	**0.36**
Post – Pre				<0.001	<0.001	0.001
				80	81	80
NNAL				1	**0.46**	**0.80**
Next day – Pre					<0.001	<0.001
					80	80
NNAL[cc]						**0.53**
Post – Pre						<0.001
						80
NNAL[cc]						1
Next day – Pre						

NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; [cc]Creatinine-corrected urinary NNAL concentrations; Post – Pre is post-exposure minus pre-exposure; Next day – Pre is next day minus pre-exposure; Numbers in cells are given as Spearman correlation, p-values, and n (number of samples)

TABLE 4.7 Urinary NNAL to salivary cotinine ratio among study participants by study location and gender

Group		†NNAL to Cotinine Ratio			Creatinine-corrected †NNAL to Cotinine Ratio		
		Pre-exposure	**Post-exposure**	**Next day**	**Pre-exposure**	**Post-exposure**	**Next day**
Control	n	17	17	16	17	17	16
	GM	19.6	21.3	23.1	15.7	17.9	16.2
	95% CI	(10.8, 35.6)	(13.5, 33.5)	(13.8, 38.6)	(9.5, 25.8)	(12.9, 24.8)	(10.1, 25.9)
	Range	2.3, 148.7	2.1, 103.0	1.7, 91.3	4.0, 79.6	5.0, 40.7	2.0, 51.6
Restaurant	n	18	11	23	18	11	23
	GM	15.6	10.3	21.2	15.3	12.0	19.2
	95% CI	(8.3, 29.5)	(6.1, 17.6)	(15.6, 28.8)	(8.6, 27.2)	(7.7, 18.7)	(13.5, 27.3)
	Range	2.4, 320.6	1.7, 25.0	6.2, 88.0	2.2, 466.9	2.3, 25.6	4.4, 126.5
Bar	n	18	19	25	18	19	25
	GM	13.4	5.0	21.1	14.2	6.9	16.1
	95% CI	(7.7, 23.2)	(3.2, 7.8)	(16.3, 27.2)	(7.1, 28.5)	(4.7, 10.2)	(12.5, 20.8)
	Range	1.7, 141.7	1.0, 19.0	3.6, 102.9	2.9, 1340.3	1.3, 26.4	5.8, 59.9
Females	n	31	32	42	31	32	42
	GM	18.5	11.1	20.7	18.0	11.4	18.1
	95% CI	(11.9, 28.7)	(7.8, 15.8)	(16.3, 26.3)	(12.3, 26.2)	(8.5, 15.3)	(14.2, 23.1)
	Range	1.7, 320.6	1.2, 50.0	1.7, 88.0	2.9, 466.9	2.3, 34.7	2.0, 126.5
Males	n	22	15	22	22	15	22
	GM	12.9	8.0	23.4	11.7	10.5	15.6
	95% CI	(7.8, 21.3)	(4.0, 15.9)	(17.3, 31.6)	(6.5, 21.1)	(6.5, 16.8)	(11.4, 21.3)
	Range	2.4, 148.7	1.0, 103.0	8.0, 102.9	2.2, 1340.3	1.3, 40.7	4.2, 59.9
All Subjects	n	53	47	64	53	47	64
	GM	15.9	10.0	21.6	15.0	11.1	17.2
	95% CI	(11.5, 22.0)	(7.3, 13.7)	(18.0, 26.0)	(10.9, 20.8)	(8.7, 14.1)	(14.2, 20.8)
	Range	1.7, 320.6	1.0, 103.0	1.7, 102.9	2.2, 1340.3	1.3, 40.7	2.0, 126.5

Note: GM = geometric mean; CI = confidence interval; †NNAL concentration measured in pg/ml or pg/mg creatinine and cotinine in ng/mL; NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

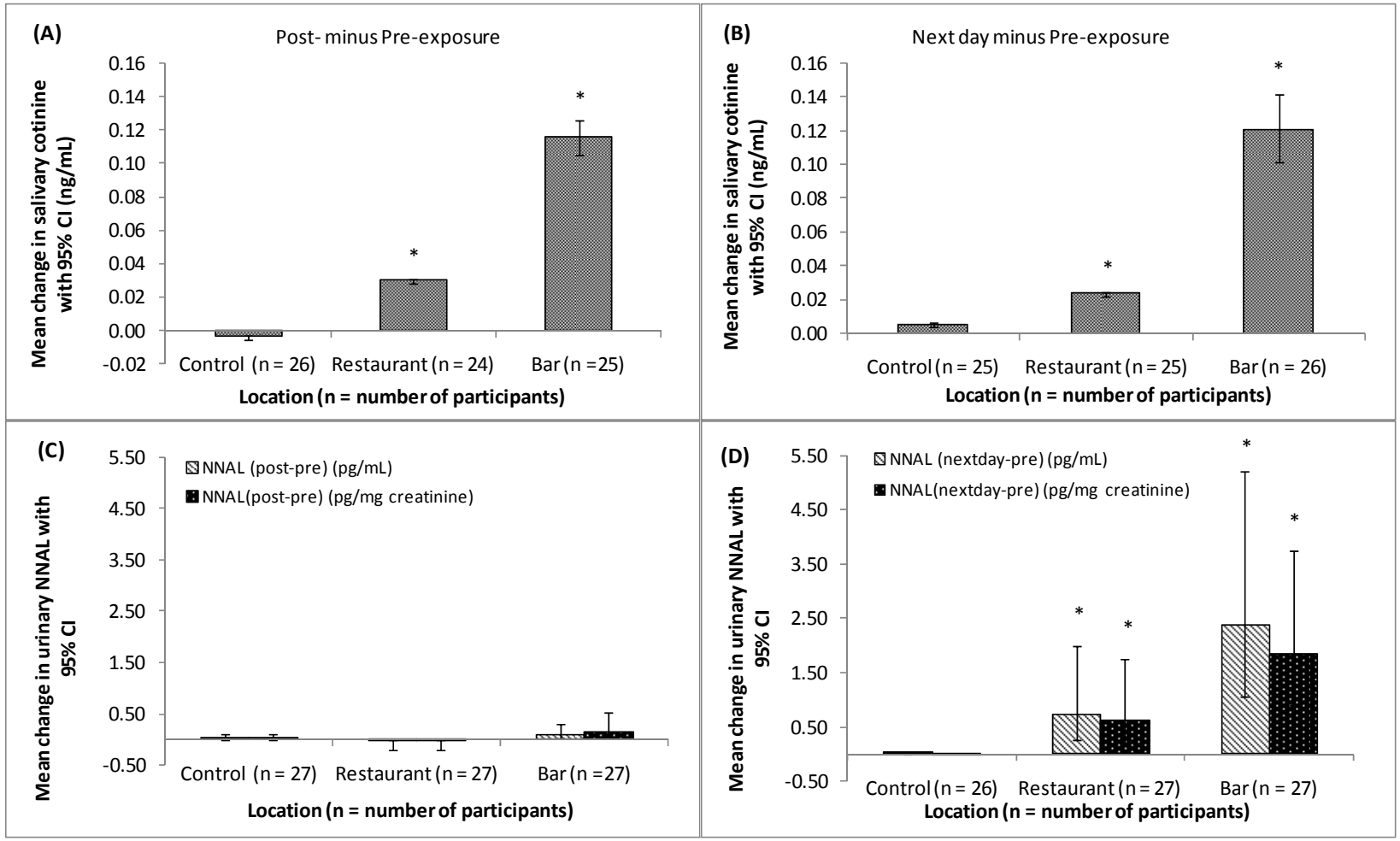

FIGURE 4.1 Saliva cotinine and urinary NNAL changes following 3-hr exposure to secondhand smoke outside a restaurant and bar where outdoor smoking is allowed and open-air control location with no smokers. (A) Salivary cotinine post-exposure minus pre-exposure (B) Salivary cotinine next day minus pre-exposure (C) Uncorrected and creatinine-corrected post-exposure minus pre-exposure urinary NNAL (D) Uncorrected and creatinine-corrected next day minus pre-exposure urinary NNAL. Bars and error bars represent differences in geometric means and 95% confidence intervals. NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. * Statistically higher than control at $\alpha = 0.05$ level of significance.

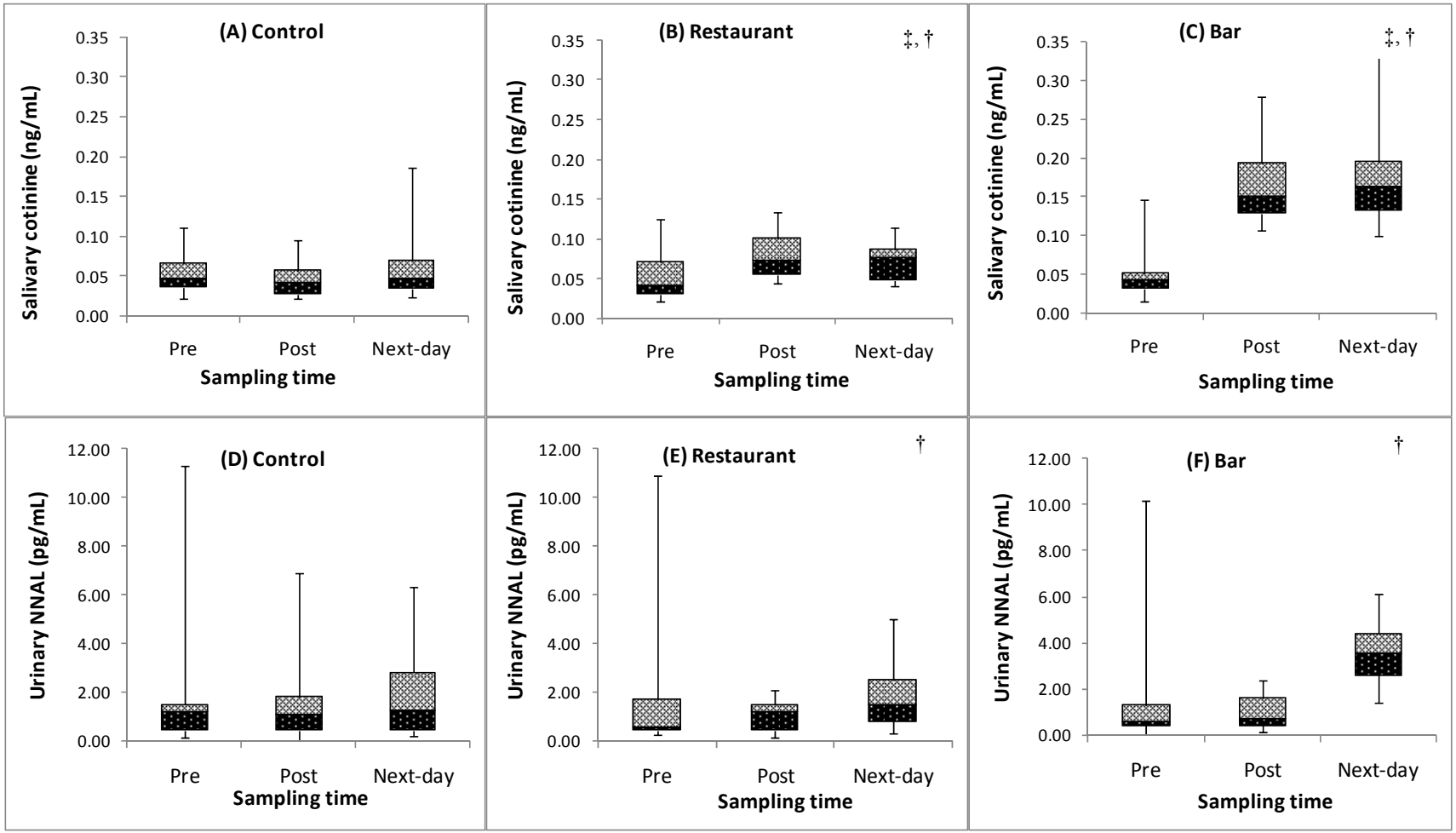

FIGURE 4.2 Salivary cotinine (ng/mL) and urinary NNAL (pg/mL) measured immediate pre-exposure, immediate post 3 hr- site visits, and first void next-day (morning) samples from n = 27 subjects. Box-plots show first quartile, median, third quartile, and 95th and 5th percentiles as upper and lower tails, respectively. ‡Post-exposure minus pre-exposure at site statistically higher than control; †Next day minus pre-exposure at site statistically higher than control. NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

CHAPTER 5

UTILITY OF URINARY CLARA CELL PROTEIN (CC16) TO DEMONSTRATE INCREASED LUNG EPITHELIAL PERMEABILITY IN NON-SMOKERS EXPOSED TO OUTDOOR SECONDHAND SMOKE[3]

[3] St.Helen, G., N.T. Holland, J.R. Balmes, D.B. Hall, J.T. Bernert, J.E. Vena, J.S. Wang, and L.P. Naeher. To be submitted to *Journal of Exposure Science and Environmental Epidemiology*

ABSTRACT

Objectives: To assess the utility of urinary Clara cell protein (CC16) as a biomarker of increased lung epithelial permeability in non-smokers exposed to outdoor secondhand smoke (SHS).

Methods: Twenty-eight healthy non-smoking adults between 21 and 37 years were assigned to outdoor patios of a restaurant and a bar where non-participants smoked and an open-air control with no smokers on three weekend days in a crossover study; subjects visited each site once for three hours. Number of lit cigarettes at the study sites was recorded as a measure of outdoor SHS. Urine samples were collected at baseline, immediately post-exposure, and next-morning, and analyzed for CC16. Mixed-effects models were fit with log(post)-log(pre) and log(nextday)-log(pre) creatinine-adjusted CC16 concentrations as the response, respectively, and location-type or cigarette count as the predictor in separate models. Models were fit to all subjects and stratified by gender.

Results: Urinary CC16 was higher in males (n=9) compared to females (n=18) at all measurement occasions ($p<0.002$). Changes in urinary CC16 from pre-exposure to post-exposure and next-day were not significantly different across locations. However, there was a tendency of increasing urinary CC16 from pre-exposure to post-exposure with increasing SHS among females. Cigarette count had a significant effect on post-exposure to pre-exposure urinary CC16 ratios among females ($p=0.048$).

Conclusion: Possible effect of prostatic CC16 on urine samples variability may limit the use of urinary CC16 as a biomarker of outdoor SHS and other air-pollution induced lung epithelial changes in men. However, this study suggests that urinary CC16 may be a useful biomarker of increased lung epithelial permeability among female non-smokers; further work will be required to evaluate its applicability to males.

INTRODUCTION

The exposure of non-smokers to secondhand smoke (SHS) continues to represent an important public health concern although recent public health efforts to reduce SHS exposure have had an important effect (Pirkle et al. 2006; USDHHS 2006). SHS, also referred to as environmental tobacco smoke (ETS), is a combination of smoke emitted from a burning tobacco product between puffs (sidestream smoke) and the smoke exhaled by the smoker (mainstream smoke) (USDHHS 1986). Tobacco smoke is a complex mixture of over 7,000 chemicals including hundreds that are hazardous and at least 69 known carcinogens (IARC 2004; USDHHS 2010). Scientific evidence continues to show that SHS exposure is causally associated with lung cancer in never- or non-smokers (Vineis et al. 2007; Wakelee et al. 2007), breast cancer in non-smoking, premenopausal younger women (Miller et al. 2007), as well as a risk factor for other cancers such as bladder and pancreatic cancers (Alberg et al. 2007; Bao et al. 2009; Van Hemelrijck et al. 2009). SHS has been shown to increase the risk of cardiovascular disease by ~30% (Barnoya, and Glantz 2005) and accounts for at least 30,000 deaths annually in the United States (Adhikari et al. 2008). Other studies further support the causal link between SHS exposure and respiratory diseases such as asthma in children (Jaakkola, and Gissler 2004), chronic bronchitis (Vozoris, and Lougheed 2008), and cough and sinusitis (Hammad et al. 2010), and as a potential cause of obstructive lung disease in non-smokers (Eisner et al. 2010; Flouris et al. 2009).

While smoke-free air laws have been shown to have large positive effects on public health (Herman, and Walsh 2010; Marlow 2010; Sargent, Shepard, and Glantz 2004; Seo, and Torabi 2007), indoor smoking bans seem to result in increased smoking outside establishments or at their entrances. The magnitude of outdoor SHS exposure and associated health risks are

unknown as very few studies have characterized outdoor SHS exposure. To the best of our knowledge three published studies have measured SHS outside establishments using environmental markers of SHS (Kaufman et al. 2010; Klepeis, Ott, and Switzer 2007; St.Helen et al. 2011) and one used cotinine as a biomarker of exposure (Hall et al. 2009). Further, no studies have assessed immediate health endpoints of exposure to outdoor SHS. Biomarkers are useful tools that can serve as early indicators of adverse effects before onset of symptoms following exposures to environmental pollutants (Andersson 2010). Evaluation of these biomarkers are often done in transitional epidemiologic studies which bridge the gap between laboratory experimentation and population-based epidemiology (Hulka 1991).

The respiratory epithelium, a selectively permeable barrier separating the airways and airspaces from the submucosa and interstitium of the lungs and the pulmonary vasculature, acts as a barrier to the entry of potentially noxious agents such as bacteria, viruses, pollutants, and allergens (Morrison et al. 1999). There are several constituents of SHS that are selectively toxic to the respiratory tract, including acrolein, formaldehyde, and nitrogen oxides (USDHHS 2010). Although the exact mechanism is not clear, evidence suggests that cigarette smoke increases the permeability of human airways (Olivera et al. 2007), changes that are rapidly reversible (Mason et al. 1983). Serum concentration of the 16-kDa Clara cell specific protein (CC16, CC10 or CCSP) has been proposed as a sensitive marker to detect increased permeability of the epithelial barrier, which is one of the earliest signs of air pollution-induced lung injury (Broeckaert et al. 2000a). CC16 responds very quickly to permeability changes in the bronchoalveolar capillary barrier (Bernard 2008), hence its utility as a marker of epithelial damage. CC16 is secreted by Clara cells, non-ciliated cells found predominantly in the respiratory and terminal bronchioles (Bernard 2008) but also in prostate, endometrium, and kidney at levels 20 times lower than those

in the lung (Broeckaert et al. 2000b). CC16 is normally secreted in large amounts at the surface of airways and leaks across the epithelium into the blood probably through passive diffusion due to the observed high concentration gradient between the epithelial lining fluid and blood (Broeckaert et al. 2000a; Hantson, Bernard, and Hermans 2008). Increased epithelial permeability may result in higher rates of passive diffusion and a transient increase in the concentration of CC16 protein in serum and subsequently in urine following glomerular filtration.

Serum and/or urinary CC16 have been used to evaluate the impact of several air pollutants such as ozone and photochemical smog (Arjomandi et al. 2008; Broeckaert et al. 2000a) and particulate matter less than 2.5 μm ($PM_{2.5}$) (Jacquemin et al. 2009) in humans as well as mainstream tobacco smoke in rats (Van Miert, Dumont, and Bernard 2005). The utility of CC16 in any biological media as a biomarker of increased epithelial permeability following exposure to outdoor SHS has not been investigated. Urine collection is less invasive than blood sampling and easily self-administered, making it particularly suitable for studies that require repeated sampling. Therefore, the objective of this study was to assess the utility of urinary CC16 as a biomarker of increased lung epithelial permeability in non-smokers exposed to SHS outside of a restaurant and a bar in Athens, Georgia under real-life conditions.

METHODS

Study Location

The study was conducted during three weekends in August and September of 2010 in Athens, GA, a city with an estimated population of 102,000 and where a local county ordinance bans smoking in restaurants and bars and most workplaces. This project was carried out as one

component of a larger study investigating outdoor SHS exposure and its effects through the use of biomarkers (St Helen et al. 201x). In order to assess the effect of outdoor SHS on lung epithelial permeability, three locations were selected: outdoor seating/standing areas of a bar and a family restaurant and an open-air seating area outside the Environmental Health Science (EHS) building at the University of Georgia (UGA). Descriptions of the study sites are presented in Table 5.1. Previous data show relatively high SHS at the bar site selected, hence its inclusion (Hall et al. 2009; St.Helen et al. 2011). Although lower SHS was previously measured outside family restaurants in Athens (Hall et al. 2009; St.Helen et al. 2011), restaurants may serve as potential sources of SHS exposure to children and individuals who do not frequent bars. An open-air seating area outside the EHS building was selected as the control site because no smokers were expected to be present during study times. The study was designed as a crossover study in which participants visited each site once over three weekends, one site per study day.

Subject Recruitment and Selection

Subjects were University of Georgia college and graduate students. Participation was limited to self-reported, healthy non-smokers aged 21 to 40 years and enrollment was directed towards a target population size of 24. An oral questionnaire was administered to potential study participants to determine eligibility. Questions include current and past smoking status and current SHS exposure at home, work or elsewhere. If the individual did not smoke, did not use nicotine in any alternative form (i.e. smokeless tobacco, nicotine replacement therapy, etc.), did not report respiratory illnesses such as asthma, and was not or could not be pregnant, they were considered eligible to participate. Respondents who met the eligibility requirements attended personal information sessions in which the study and protocol were discussed and concerns or questions were addressed. Respondents who chose to participate signed consent forms. Subjects

were assigned to study sites based on a replicated Latin square design in which each subject participated at different sites on the three occasions (i.e. once each at the bar, restaurant, and control sites). Twenty-eight participants were initially enrolled in the study but one subject with unusually high levels of salivary cotinine who lived with a smoker was excluded from the analyses. Participants were financially compensated for participating in the study. This study was reviewed and approved by the Institutional Review Boards at UGA and the Centers for Disease Control and Prevention (CDC), respectively.

Site Visits

Participants were called by telephone the day before each study day to remind them of assigned study location and time. Participants were also encouraged to stay hydrated and avoid urinating at least 2 hrs before study times. Participants arrived at the EHS building about 1 hr before site visits and were briefed on study protocol when at study sites as well as proper saliva and urine sample collection. Pre-exposure urine samples were collected as described below in *Biological Sample Collection* and participants were then transported, if assigned to the restaurant and bar sites, on a designated non-smoking EHS van. Restaurant and control sites were visited from 6:00 pm to 9:00 pm and the bar site from 11:00 pm to 2:00 am on study days. These times represent peak business hours for restaurants and bars, respectively, in Athens and may have higher smoking activity than other times. Participants remained at each study site for the full 3 hrs except for necessary bathroom breaks (about one break for no more than 5 min) and were encouraged to stand or seat in close proximity to smokers, which ranged from about 0.5 m to 5 m at any given time. Participants ate dinner while they were at the restaurant and control sites while they ate dinner prior to visits to the bar site. One assigned subject at each location took the cumulative 10-min cigarette and pedestrian counts for the 3-hr visit. The cigarette count included

every lit tobacco product from persons who were walking past, sitting, or standing in the seating/standing area or outdoor patio of the locations. The pedestrian count included both non-smoking and smoking individuals at or passing by each location. Following the 3-hr visit, participants at the control site returned to the EHS building and those at the restaurant and bar sites were transported on the EHS van. Post-exposure saliva and urine samples were collected within 30 min of subjects leaving the study sites and a questionnaire assessing participants' exposures to SHS for the 48-hr period prior to site visits was given. Participants were then given materials for next-day sample collection in a biohazard bag, with instruction to keep all materials away from SHS or smokers and to freeze samples immediately after collection.

Biological Sample Collection

Participants provided urine samples, immediately pre- and post-site visits and from the first-morning void on the next day. Hereafter, same-day post-exposure will be referred to as post-exposure and next-day post-exposure as next-day exposure. Male participants provided urine samples in three portions: 1) first 75 mL portion in a 200 mL cup pre-marked at the 75 mL level; 2) 25 mL in a 50 mL conical tube labeled '1' pre-marked at 25 mL level (referred to as 75-100 mL portion); and 3) remaining urine in a second 50 mL conical tube labeled '2' (referred to as post-100 mL portion). Because of prostatic CC16 secretions in male subjects, CC16 measured in post-100 mL urine samples is a more accurate reflection of CC16 originating from the respiratory tract than pre-100 mL portions (Andersson, Lundberg, and Barregard 2007). CC16 was analyzed in the 75-100 mL portion in males if they were unable to produce a post-100 mL urine sample. Female participants provided urine samples in one portion in a 200 mL cup. Pre- and post-exposure urine samples were collected at EHS. Next-day samples were collected at the participants' homes and were kept frozen until delivery to EHS on Monday following each study

weekend. Urine samples were stored in -80 ^{o}C freezer at the Air Quality Lab (AQL) in EHS until shipment for analysis. All samples were shipped on dry ice to the CDC six weeks after collection where they were aliquotted. One aliquot of urine samples from females and the 75-100 mL and post-100 mL portions of male samples were shipped on dry ice to the University of California, Berkeley for urinary CC16 and creatinine analyses. Pre-, post- and next-day exposure saliva samples were also collected together with urine samples. Salivary cotinine and urinary 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) were analyzed as biomarkers of tobacco smoke exposure in a concurrent study.

Biomarker Analysis

Urinary CC16 was determined by a commercially available ELISA kit (IBL-America, Minneapolis, MN). Analysis was conducted according to the manufacturer's protocol. In short, calibrators or samples were incubated with a polyclonal rabbit anti-human CC16 antibody coated in microtiter wells. After 1-hr incubation and a washing, polyclonal anti-human CC16 antibody labeled with biotin was added and incubated with captured CC16. After a thorough wash, streptavidin labeled with HRP was added. Following 1-h incubation and the last washing step, the remaining conjugate was allowed to react with the substrate H_2O_2- tetramethylbenzidine. The reaction was stopped by addition of acidic solution. Absorbance of the resulting yellow product was measured at 450 nm using Spectramax-M5 (Molecular Diagnostics, CA). The absorbance is proportional to the concentration of CC16. A standard curve was constructed by plotting absorbance values versus CC16 concentrations of calibrators. Concentrations of unknown samples were determined using this standard curve. This analytical method has a high sensitivity and reproducibility with the coefficient of variation (CV) 6.5%. Other laboratory quality controls included random repeats and internal controls. Creatinine concentrations were determined in

urine using commercially available ELISA (Oxford Biomedical Research, MI) with a coefficient of variability (CV) of 4.3%.

Statistical Analysis

Due to the approximate log-distribution of the urinary CC16 data, variables were normalized by taking the logs of the original concentrations. These include log(pre-exposure), log(post-exposure), and log(next-day). Variables were adjusted for urinary creatinine content by taking the logs of the ratios of urinary CC16 to urinary creatinine. Geometric means and 95% confidence intervals (CI) were computed for pre-, post-, and next-day variables by exponentiating the means of the logs of the variables and corresponding 95% CI. Geometric means of the ratios of creatinine-adjusted post-exposure to pre-exposure and next-day to pre-exposure and 95% CI were also computed as described above.

Analyses of the changes in urinary CC16 immediately following 3-hr site visits (post-exposure) and next-day relative to pre-exposure levels, respectively, proceeded from a statistical model for the replicated Latin square design employed in this study. For response y_{ijk} measured on the k^{th} subject on the j^{th} measurement occasion (day) under the i^{th} exposure location, Equation 5.1 was assumed. In this study design, participants were assigned only one of two weekend days (Friday or Saturday), therefore, day was nested in week ($w_{(j)l}$).

$$y_{ijk} = \mu_i + w_{(j)l} + s_k + e_{ijk} \qquad \text{Equation 5.1}$$

Here, μ_i represents the mean response for the i^{th} exposure location, and $w_{(j)l}$ and s_k are mean zero, constant variance, normal random effects for day nested in weeks and subjects, respectively. The response variables were *log(post)-log(pre)* and *log(nextday)-log(pre)*. This translates to the ratio of post-exposure to pre-exposure and next-day to pre-exposure urinary CC16, respectively. F tests were conducted of no overall effect of exposure location as well as F tests of pair-wise

contrasts between the control, restaurant and bar locations, adjusted by Tukey's method for multiple comparisons. In addition, analyses stratified by gender were carried out. In order to test for differences in biomarker responses across gender and race, these two variables were introduced into Equation 5.1 as covariates (fixed effects) in a separate analysis and pair-wise contrasts were made between levels of gender and race, respectively. The effect of total cigarettes lit outside the establishments on urinary CC16 changes was investigated using Equation 5.1, but cigarette count replaced location-type as the fixed independent variable. Analyses of cigarette count effect were also carried out using all subjects and stratified by gender. Spearman rank correlation coefficients between changes in creatinine-adjusted urinary CC16 and biomarkers of tobacco smoke (salivary cotinine and creatinine-adjusted urinary NNAL) were computed. Analyses were carried out using SAS v. 9.1 (SAS Institute, Inc. Cary, NC, USA). All statistical tests were considered significant at $\alpha = 0.05$.

RESULTS

Twenty-eight participants (18 females) were initially enrolled in this study. Seventeen of the subjects were white (11 female), 7 were black (3 females), 3 Asians (all female) and one female described her race as other. All participants were within 21 to 37 years. While four subjects reported smoking by others near or at their residences or workplaces, most were not routinely exposed to SHS. Subjects were asked to avoid all SHS as much as possible three days prior to each study weekend. The range of baseline pre-exposure salivary cotinine concentrations confirmed their relatively low pre-study SHS exposure (0.011-0.480 ng/mL). Biomarker data from one male participant were excluded from all statistical analyses after baseline pre-exposure salivary cotinine concentration was found to be 5.25 ng/mL, 11 times higher than the next

maximum (0.480 ng/mL). This participant lived with a smoker. Weeks 2 and 3 locations for two subjects were changed from the pre-assigned locations due to personal scheduling conflicts. Data from these participants were omitted from mixed-effects models because their new location assignment violated the Latin square (crossover) design. However, biomarker data for these two participants were used in computation of descriptive statistics. In all, there were eight complete Latin squares in which groups of three subjects (24 subjects in total) were assigned to three study locations for weekend days (12 subjects on Fridays and 12 subjects on Saturdays).

Of 81 urine samples (pre, post, and next-day over three sampling days) taken from the nine valid male subjects, 16% (n = 13) post-100 mL urine portions were not provided (n = 7 pre, n = 3 post, and n = 3 next-day). Therefore, CC16 was analyzed in the 75-100 mL portion of these samples. The levels fell within the range of observed concentrations measured in the post-100 mL portions. Geometric means of creatinine-adjusted pre-exposure, post-exposure, and next-day urinary CC16 are given in Table 5.2 and Figure 5.1. CC16 was detected in all urine samples (n = 241, one subject did not return a next-day sample). Baseline pre-exposure creatinine-adjusted urinary CC16 concentrations were not significantly different from pre-exposure urinary CC16 at weeks 2 and 3 ($F = 0.45$, $p = 0.639$). Geometric means and 95% CI of the ratios between post- and pre-exposure and next-day and pre-exposure are also presented in Table 5.2.

Creatinine-adjusted urinary CC16 concentrations were consistently several times higher in males compared to females, up to 10 times, at all measurement occasions (all p-values < 0.002) (Figure 5.1). Statistically significant higher post-exposure to pre-exposure creatinine-adjusted urinary CC16 ratios were observed among males compared to females ($t = 2.44$, $p = 0.019$). The ratios between next-day to pre-exposure creatinine-adjusted urinary CC16 were not

significantly different by gender ($t = 0.42$, $p = 0.674$). Comparisons of changes in urinary CC16 by race were non-significant.

The tests of location-type on the ratios post-exposure to pre-exposure and next-day to pre-exposure creatinine-adjusted urinary CC16 are presented in Table 5.3. In models where all subjects or males only, respectively, were considered post-exposure to pre-exposure and next-day to pre-exposure creatinine-adjusted urinary CC16 ratios were not significantly different across locations (all p-values > 0.50). When data from females only were fitted, differences in post-exposure to pre-exposure and next-day to pre-exposure creatinine-adjusted urinary CC16 ratios were non-significant across location type but the p-values were much smaller (post/pre, $p = 0.187$; next day/pre, $p = 0.121$). Table 5.3 also presents the results of analyses in which cigarette count was used as the independent fixed effect instead of location-type. A significant positive cigarette count effect on the post-exposure to pre-exposure creatinine-adjusted urinary CC16 ratios was observed when females only were considered ($p = 0.048$). The cigarette count effect was negative and non-significant when males were considered ($p = 0.635$). Cigarette count had a non-significant effect on next-day to pre-exposure creatinine-adjusted urinary CC16 ratios when all subjects, females only, or males only were analyzed, respectively. On exclusion of urinary CC16 concentrations measured in the 75-100 mL portions ($n = 13$) from males, the results of the above analyses were consistent.

Spearman rank correlation coefficients (ρ) between creatinine-adjusted changes in urinary CC16 and biomarkers of tobacco smoke exposure, salivary cotinine and creatinine-adjusted urinary NNAL, are presented in Table 5.4. Small but significant correlations were observed between post-exposure minus pre-exposure ($\rho = 0.25$, $p = 0.026$) and next-day minus pre-exposure ($\rho = 0.26$, $p = 0.018$) creatinine-adjusted urinary CC16 and post-exposure minus

pre-exposure creatinine-adjusted urinary NNAL. The correlations between changes in creatinine-adjusted urinary CC16 and next-day minus pre-exposure creatinine-adjusted urinary NNAL were non-significant. Changes in creatinine-adjusted urinary CC16 were not significantly correlated to changes in salivary cotinine concentrations.

DISCUSSION

In this study we have investigated the utility of urinary CC16 as a biomarker of increased lung epithelial permeability in 27 non-smokers exposed to SHS outside a restaurant and a bar in Athens, GA five years after the implementation of an indoor smoking ban at these establishments. Such small scale panel studies are critical in bridging the gap between laboratory experimentation and population-based epidemiology (Hulka 1991). When all subjects or males only were considered, we observed no significant differences in the ratios of post-exposure to pre-exposure and next-day to pre-exposure creatinine-adjusted urinary CC16 across locations. However, there was a tendency towards increased ratios of post-exposure to pre-exposure among female subjects with increasing outdoor SHS exposure across locations (Figure 5.2). In addition, we observed a significant positive effect of cigarette count on post-exposure to pre-exposure creatinine-adjusted urinary CC16 ratios among females ($p = 0.048$), suggestive of an increase in CC16 in urine over background concentrations with the number of cigarettes smoked at the locations. This is indicative of an effect of outdoor SHS on increasing lung epithelial permeability. This relationship was not observed for next-day to pre-exposure creatinine-adjusted urinary CC16 ratios, most likely due to changes in respiratory epithelial permeability being rapidly reversible once exposure is terminated (Mason et al. 1983).

The respiratory epithelium is the first line of defense against inhaled irritants. The impermeable barrier is made possible by tight junctions, cell-cell, and cell extra-cellular matrix interactions (Holgate 2008). Although the exact mechanism is not clearly elucidated, a study showed that mainstream cigarette smoke increases the permeability of human airways (Olivera et al. 2007); the lung epithelium of smokers is more permeable than that of non-smokers (Jones et al. 1980). Smoke-induced loss of epithelial barrier function is a regulated process rather than a cytotoxic response (Olivera et al. 2007), although SHS does contain constituents that have cytotoxic effects on pulmonary cells (USDHHS 2010). Gangl and colleagues showed recently that the transient loss in respiratory epithelial permeability induced by cigarette smoke may contribute to increased allergic inflammation and exacerbation of allergic disease due to increase allergen penetration (Gangl et al. 2009).

Validated biomarkers to assess pre-symptomatic changes such as altered lung epithelial function can play a critical role in identifying health effects of environmental agents. CC16 measured in serum has been proposed as one sensitive biomarker of increased lung permeability (Broeckaert et al. 2000a). Urinary CC16 has also been used to assess the impact of air pollution on lung epithelial barrier function (Timonen et al. 2004). There is currently no information on the utility of serum or urinary CC16 in assessing the health impact of real-life outdoor SHS levels which are typically lower than indoor environments. The use of urinary CC16 was considered more feasible in this study than the preferred serum CC16 because urine collection is noninvasive and easily done by subjects. However, measurement of CC16 in urine poses a few challenges. In addition to originating from the pulmonary tract, CC16 is also produced to a lesser extent in the prostate and washed out with urine. Andersson and colleagues showed that in order to eliminate or satisfactorily diminish CC16 in urine originating from the prostate, the first 100

mL should be discarded (Andersson et al. 2007). We successfully collected post-100 mL urine samples from male subjects but we also collected a 75-100 mL portion for CC16 analysis if male subjects did not produce >100 mL urine, as was the case with 13 of 81 samples. Further, CC16 elimination in urine is critically dependent on renal function and therefore spot urine samples, as collected in this study, have to be adjusted for urine flow. We collected first morning void spot urine samples (next-day), adjusted for urinary creatinine content to improve correlations with serum CC16 (Andersson et al. 2007). Spot urine samples collected pre- and post-exposure in late evening and night times were also adjusted for creatinine but the correlations of levels in these samples with serum CC16 are uncertain. A latency effect in CC16 concentrations in urine compared to serum is certainly present in all urine samples.

We observed significantly higher creatinine-adjusted urinary CC16 in males compared to females, up to 10 times higher per sampling occasion (all p-values < 0.002) (Table 5.2, Figure 5.1). Because serum CC16 concentrations from males and females have not been shown to be significantly different at baseline (Arjomandi et al. 2008; Shijubo et al. 1997) the most likely explanation for the observed large difference between male and female urinary CC16 levels in the present study is prostatic contamination even in the post-100 mL urine portions. The large gender difference in CC16 measured at pre-exposure , post-exposure, and next-day was observed with or without inclusion of the thirteen 75-100 mL urine portions from male subjects who did not produce >100 mL of urine. Stratification of statistical analyses by gender was therefore necessary.

We saw evidence of an association between outdoor SHS exposure at the locations and increasing lung epithelial permeability. When females only were considered, the ratio of post-exposure to pre-exposure creatinine-adjusted urinary CC16 trended upwards with low ratios

following visits to the control site and higher ratios following visits to the bar site but did not achieve significance (Figure 5.1). This increasing trend in post-exposure to pre-exposure creatinine-adjusted urinary CC16 observed in females across location was strengthened by the significant effect of total cigarettes lit outside the locations on that same ratio ($p = 0.048$).

We believe that a larger, more significant increase in urinary CC16 among females was not observed with increasing exposure to SHS across locations because there seems to be a natural decline in background urinary CC16 unrelated to SHS exposure over the approximately 3-hr period between pre- exposure and post-exposure sampling times that may mask SHS-induced urinary CC16 increases (Figure 5.1). A diurnal variation in urinary CC16 has previously been confirmed with low levels in the morning, high in afternoon and evening and low at night (Andersson et al. 2007). This explains the consistently lower levels observed in next-day samples collected in the morning. We did not see a significant difference in pre-exposure creatinine-adjusted urinary CC16 in samples collected at 6:00 p.m. and those collected at 11:00 p.m. throughout the study. This indicates that the decline in creatinine-adjusted CC16 over the 3-hr sampling period from pre- to post-exposure may not be due to a diurnal variation at these times. On the other hand, protein elimination in urine has been shown to decrease during periods of relaxation or physical inactivity (Poortmans, Rampaer, and Wolfs 1989) and that may be one likely explanation for the declining background urinary CC16 among female subjects as participants were generally inactive during site visits.

We did not observe a similar trend of increasing urinary CC16 across location or cigarette count when we looked at next-day to pre-exposure creatinine-adjusted urinary CC16 ratio among women. This is most likely explained by lung epithelial permeability being restored quickly to pre-SHS exposure activity once SHS-exposure is terminated (Mason et al. 1983). The behavior

of creatinine-adjusted CC16 from males at all sampling times was less predictable. This may be due to prostatic CC16 contamination as discussed earlier thus precluding inferences.

Small but significant correlations were observed between post-exposure minus pre-exposure and next-day minus pre-exposure creatinine-adjusted urinary CC16 and post-exposure minus pre-exposure creatinine-adjusted urinary NNAL (ρ = 0.25, and 0.26, respectively). Correlations of CC16 measures with salivary cotinine and with next-day minus pre-exposure NNAL were non-significant. This lends support to the hypothesis that cotinine does not provide an accurate measure of exposure to the toxic constituents of tobacco smoke (Benowitz et al. 2010). While NNAL is a potent lung carcinogen, cotinine is not known to have toxic effects on the human body (Hukkanen, Jacob, and Benowitz 2005). The significant correlations between post-exposure minus pre-exposure and next-day minus pre-exposure urinary CC16 and post-exposure minus pre-exposure creatinine-adjusted urinary NNAL versus the non-significant correlation with next-day minus pre-exposure creatinine-adjusted urinary NNAL indicate that the rapidly reversible changes in the respiratory epithelium were induced by SHS exposure occurring early during the 3-hr site visits. With a relatively long elimination half-life of 10-16 days (Goniewicz et al. 2009), the changes in urinary NNAL between urine collected at pre-exposure and immediately post-exposure would reflect either long-term SHS exposure or NNAL absorbed at the onset of SHS exposure during the 3-hr site visits. It is unlikely that short-term changes in urinary CC16 would be a result of respiratory epithelial changes from long-term SHS exposure. As noted already, lung epithelial permeability changes are rapidly reversible. The correlations between SHS biomarkers and CC16 need further investigation to prove the validity of our findings.

The current study has several strengths but also some limitations. Among the strengths, we used a replicated Latin square design which is a crossover design in which subjects served as their own control. This is especially important since biomarker responses such as urinary CC16 are subjected to high inter- and intra-individual variability. The design used makes the study robust to high variability in biomarker levels within and between participants. The use of the ratios between urinary CC16 concentrations at the various measurement occasions as the response variables and using each participant as his or her own control are laudable aspects of the statistical analysis and design, respectively. Further, this study is the first to use CC16, in any medium, to evaluate the effects of real-life outdoor SHS on lung epithelial permeability. On the other hand, one of the limitations of the study is the absence of objective measurements of SHS concentrations (e.g., air nicotine) or proxies such as $PM_{2.5}$ or CO. Measurement of these SHS constituents were infeasible. Instead we used cigarette count to assess outdoor SHS concentration at each site. Salivary cotinine and urinary NNAL measured in a concurrent study were used to ascertain SHS exposure. Further, we did not collect variables such as temperature and wind-speed at the study sites that would more accurately characterize exposure conditions at the different sites. Also, use of urinary CC16 proved problematic in males. It appears that we were unable to fully eliminate prostatic CC16 even after discarding the first 100 mL of urine. This raises questions about how effective a biomarker urinary CC16 is if so much urine has to be discarded before CC16 originating from the lungs can be characterized. Removing males from the statistical analyses reduced our study size and may have ultimately reduced the power to detect differences across the study locations selected.

CONCLUSION

Our results indicate a tendency towards increasing urinary CC16 with increasing SHS exposure outside the restaurant and bar sites among female subjects. We observed a significant positive effect of cigarette count on pre-exposure to post-exposure creatinine-adjusted urinary CC16 concentration among women, suggesting that outdoor SHS exposure increases lung epithelial permeability. The effect of outdoor SHS exposure on increasing lung epithelial permeability assessed by changes in urinary CC16 may be masked somewhat by physiological factors that may reduce elimination of CC16 in urine. Further, possible effect of prostatic CC16 on male urine samples variability may limit the use of urinary CC16 as a biomarker of outdoor SHS and other air-pollution induced lung epithelial changes in men. However, this study does suggest that urinary CC16 may be a useful biomarker of increased lung epithelial permeability among female non-smokers; further work will be required to evaluate its applicability to males.

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TABLES AND FIGURES

TABLE 5.1 Description of study sites

Site	Bar	Restaurant	Control
Description	Bar with outdoor patio on second floor; partially enclosed by two walls of adjacent buildings, open at one end, and has no roof	Family restaurant with large outdoor patio	Open air seating area
Location	Downtown Athens, Georgia; five minutes from University of Georgia's Environmental Health Science (EHS) Building	Athens west; ten minutes from EHS	Outside EHS building
No. of Tables	6	17	5
Outdoor Patio Area (m^2)	176	549	N/A
‡Cigarette Count			
Mean ± SD	144.5 ± 39.9	33.5 ± 28.0	0
Min - Max	86 - 202	12 - 86	0
†Pedestrians/Customers			
Mean ± SD	67 ± 25	32 ± 10	5 ± 3
Min - Max	45 - 109	12 - 41	1 - 7
Salivary cotinine (ng/mL)			
Post minus Pre	0.115 (0.105, 0.126)	0.030 (0.028, 0.031)	-0.004 (-0.005, 0.003)
Next day minus Pre	0.120 (0.1102, 0.141)	0.023 (0.022, 0.024)	0.005 (0.003, 0.006)
Urinary NNAL (pg/mL)			
Post minus Pre	0.072 (0.017, 0.297)	-0.034 (-0.192, -0.006)	0.018 (0.003, 0.099)
Next day minus Pre	2.370 (1.061, 5.219)	0.733 (0.261, 1.996)	0.005(0.000, 0.060)

‡Cigarette count statistics computed from 3-h sums of 10-minute cigarette count
†Pedestrian count statistics computed from 10-minute averages of 10-min pedestrian/patron count; N/A = not applicable

TABLE 5.2 Urinary Clara cell protein (CC16) descriptive statistics

Location	Group	Pre	Post	Next day	Post:Pre Ratio	Next:Pre Ratio
Control	All subjects	12.1	8.1	9.2[†]	0.67	0.77[†]
	(n = 27)	(7.5, 19.7)	(4.1, 15.8)	(4.9, 17.2)	(0.44, 1.02)	(0.53, 1.11)
	Females	7.4	3.9	5.6	0.53	0.76
	(n = 18)	(4.5, 12.1)	(2.0, 7.7)	(2.7, 11.5)	(0.30, 0.93)	(0.45, 1.28)
	Males	32.6	34.6	27.5[‡]	1.06	0.78[‡]
	(n = 9)	(14.0, 75.9)	(12.4, 96.6)	(9.4, 80.6)	(0.58, 1.95)	(0.49, 1.25)
Restaurant	All subjects	14.3	11.0	8.8	0.77	0.61
	(n = 27)	(7.9, 26.0)	(5.3, 22.8)	(3.9, 19.8)	(0.52, 1.14)	(0.36, 1.05)
	Females	8.7	4.9	4.7	0.57	0.54
	(n = 18)	(4.3, 17.7)	(2.4, 10.1)	(1.6, 13.5)	(0.35, 0.92)	(0.24, 1.20)
	Males	38.9	54.6	30.9	1.40	0.79
	(n = 9)	(15.7, 96.3)	(16.8, 178.0)	(12.3, 77.2)	(0.75, 2.62)	(0.46, 1.18)
Bar	All subjects	12.3	11.9	9.0	0.97	0.73
	(n = 27)	(6.1, 24.8)	(6.0, 23.5)	(5.2, 15.7)	(0.67, 1.39)	(0.46, 1.18)
	Females	6.9	6.5	5.9	0.94	0.86
	(n = 18)	(2.8, 17.0)	(3.1, 13.6)	(3.2, 10.8)	(0.56, 1.56)	(0.45, 1.63)
	Males	38.8	39.9	20.9	1.03	0.54
	(n = 9)	(17.2, 87.2)	(11.8, 134.3)	(7.0, 62.4)	(0.59, 1.79)	(0.25, 1.15)

Data are in ng/mg creatinine, geometric mean (95% confidence interval). [†]n = 26; [‡]n = 8. Pre is pre-exposure CC16, Post is post-exposure CC16, Next day is next day CC16, Post:Pre ratio is the ratio between post-exposure and pre-exposure creatinine-adjusted CC16, Next:Pre ratio is the ratio between next day CC16 and pre-exposure creatinine-adjusted CC16

TABLE 5.3 Models testing the effect of location-type or cigarette count on creatinine-corrected urinary Clara cell protein (CC16)

Main effect	Response variable	Data fitted	Estimate	F value	p-value
Location	log(post)-log(pre)	All subjects (n = 24)		0.63	0.539
		Females (n = 15)		1.80	0.187
		Males (n = 9)		0.44	0.653
	log(next day)-log(pre)	All subjects (n = 24)		0.74	0.482
		Females (n = 15)		2.30	0.121
		Males (n = 9)		0.58	0.577
Cigarettes	log(post)-log(pre)	All subjects (n = 24)	0.0009	1.77	0.191
		Females (n = 15)	0.0020	4.30	**0.048**
		Males (n = 9)	-0.0003	0.24	0.635
	log(next day)-log(pre)	All subjects (n = 24)	0.0004	0.23	0.633
		Females (n = 15)	0.0018	2.65	0.116
		Males (n = 9)	-0.0114	3.13	0.105

Location-type and cigarette count were entered in separate models as the independent variables. In addition, data were analyzed for all subjects, females only, or males only.

TABLE 5.4 Spearman rank correlation coefficients (ρ) between changes in Clara cell protein (CC16) and biomarkers of tobacco smoke, salivary cotinine and urinary NNAL (ρ, p-value, and number of samples)

Urinary CC16[cc]	**SHS Biomarkers**			
	Salivary Cotinine		**Urinary NNAL[cc]**	
	Post – Pre	Next day – Pre	Post – Pre	Next day – Pre
Post – Pre	0.02	-0.06	**0.25**	0.07
	0.886	0.618	0.026	0.524
	75	76	81	80
Next day – Pre	-0.07	-0.10	**0.26**	0.01
	0.574	0.399	0.018	0.916
	74	76	80	80

Post – Pre is post-exposure concentration minus pre-exposure concentration; Next day – Pre is next day concentration minus post-exposure concentration; NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; [cc]Creatinine-adjusted urinary NNAL concentrations

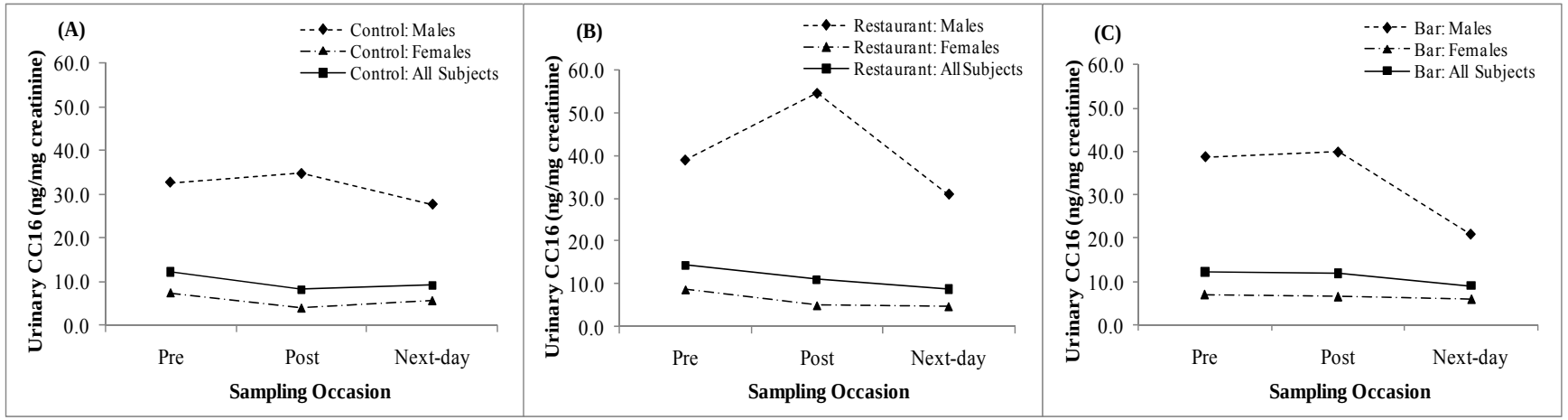

FIGURE 5.1 Pre-exposure, post- exposure, and next day creatinine-adjusted urinary Clara cell protein (CC16) measured in n = 27 subjects by study location. Data presented as geometric means. 95% confidence intervals are not included but can be found in Table 5.2. (A) Control, no exposure to second hand smoke; (B) subjects exposed outside of Restaurant; (C) subjects exposed outside of Bar.

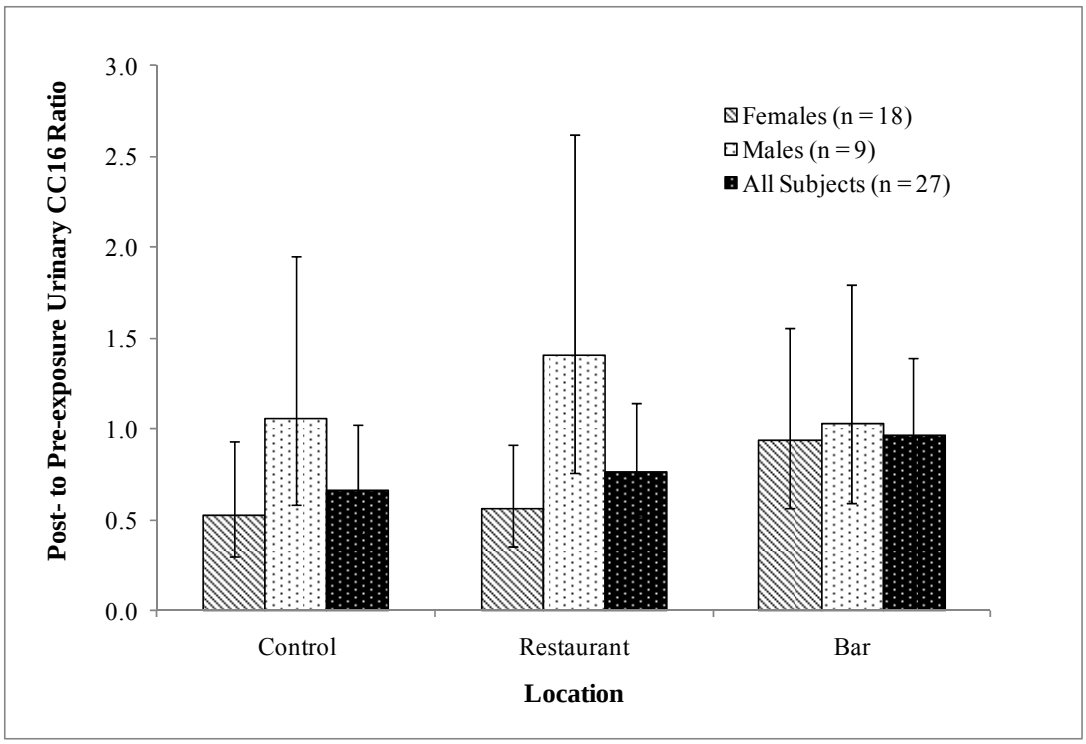

FIGURE 5.2 Ratio of post-exposure to pre-exposure creatinine-adjusted urinary Clara cell protein (CC16) in subjects following a 3-h visit to outdoor locations. Ratios below 1.0 indicate that pre-exposure CC16 levels were higher than post-exposure CC16 levels.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Secondhand smoke (SHS), also referred to as environmental tobacco smoke (ETS) and passive smoke, is defined as a combination of smoke emitted from a burning tobacco product between puffs (sidestream smoke) and the smoke exhaled by the smoker (mainstream smoke). Based on a tremendous accumulation of scientific evidence the 2006 US Surgeon General report concluded that SHS causes premature death and disease in children and adults who do not smoke; has immediate adverse effects on the cardiovascular system and causes coronary heart disease and lung cancer; and, there is no risk-free level of exposure to SHS (USDHHS 2006). Numerous other recent studies continue to provide compelling evidence associating SHS causally or as a risk factor for health outcomes such as breast cancer, pancreatic cancer, bladder cancer, cardiovascular diseases, and chronic obstructive respiratory diseases (Adhikari et al. 2008; Alberg et al. 2007; Bao et al. 2009; Barnoya, and Glantz 2005; Miller et al. 2007; Van Hemelrijck et al. 2009; Vineis et al. 2007; Vozoris, and Lougheed 2008; Wakelee et al. 2007).

With such overwhelming scientific evidence, public health efforts such as smoking bans in public places or indoor environments have been implemented and have been effective in reducing SHS exposure (Pirkle et al. 2006). However, exposure to SHS remains a public health concern, especially outside or near the entrances of establishments where indoor smoking only is prohibited. While two previously published studies have used environmental markers (Kaufman et al. 2010; Klepeis, Ott, and Switzer 2007) and one used salivary cotinine, a metabolite of nicotine, as a biological marker (biomarker) of outdoor SHS exposure (Hall et al. 2009) to

characterize outdoor SHS, information remains scarce on outdoor SHS exposure levels and associated health endpoints.

Therefore, the primary objectives of this dissertation were to characterize outdoor levels of SHS through environmental proxies of SHS and assess systemic exposure of non-smokers to constituents of outdoor SHS and associated health effects through the use of biomarkers. For the purposes of this dissertation, two major studies were conducted. The first study was conducted in 2006 in which environmental proxies of SHS, particulate matter less than 2.5 μm ($PM_{2.5}$) and carbon monoxide (CO), were used to characterize SHS outside restaurants and bars in Athens, Georgia. The details of this study are presented in Chapter 3. The second study was conducted in the summer of 2010 in which biomarkers of tobacco smoke were used to assess systemic exposure of non-smokers to outdoor SHS. In addition, the utility of Clara cell protein (CC16) as a biomarker of increased lung epithelial permeability following outdoor SHS exposure was investigated. The details of this study are presented in Chapters 4 and 5 of this dissertation. A brief summary of the findings and conclusions of these studies are presented below.

ENVIRONMENTAL MARKERS

The objective of this study was to measure $PM_{2.5}$ and CO in outdoor waiting areas and patios of restaurants and bars in downtown Athens, Georgia where indoor smoking is banned and to investigate whether the measured concentrations are directly associated with the number of cigarettes lit in these settings. $PM_{2.5}$ and CO, while they are proxies of the particulate and gaseous phases of SHS, respectively, they are not specific to SHS and have other potential sources such as fuel combustion in vehicles. Thus it was important to investigate, in addition to

the effect of smokers, the effect of vehicle emissions on outdoor $PM_{2.5}$ and CO levels at these outdoor locations.

Real-time $PM_{2.5}$ and CO were monitored on four summer weekend afternoons or evenings in outdoor waiting areas or patios at five locations in Athens, GA, including two restaurants, two bars, and a control site (i.e., sidewalk with no smokers present) for approximately 12 h per sampling period. In addition, total smokers and pedestrians present or passing and motorized vehicles passing each sampling location were counted. The effects of smokers, pedestrians, and vehicles on $PM_{2.5}$ and CO were estimated through linear mixed effects regression models which accounted for heterogeneity from sample to sample and autocorrelation through time.

Average $PM_{2.5}$ levels were 63.9 ± 50.2 $\mu g/m^3$ and 51.0 ± 51.1 $\mu g/m^3$ at the two bars and 39.7 ± 21.0 $\mu g/m^3$ at one restaurant and were significantly higher than levels at the control location (all p-values > 0.001). $PM_{2.5}$ at the control and the other restaurant were 20.4 ± 3.4 $\mu g/m^3$ and 16.6 ± 7.9 $\mu g/m^3$, respectively. Carbon monoxide levels outside the restaurant and bar sites did not differ significantly from the control and ranged from 1.2 to 1.6 ppm. The number of smokers had a significant positive effect on log(CO) (estimate = 0.0121, p = 0.032) as well as on log($PM_{2.5}$) (estimate = 0.0575, p<0.001) while the effects of pedestrians and vehicles on log(CO) and log($PM_{2.5}$) were not statistically significant.

The results of this study indicate that: (1) SHS leads to significant increases in $PM_{2.5}$ outside of restaurants and bars; and, (2) although CO can be used as a proxy for SHS in these outdoor environments, its levels remain relatively low. Although air concentrations of SHS constituents cannot accurately predict systemic exposure to these constituents this study shows that persons outside restaurants and bars may be exposed to significant levels of SHS. Exposure

levels may increase as the number of cigarettes smoked outside these establishments increase. While factors such as wind speed and meteorological conditions as well as proximity to smokers were not accounted for in this study, they will determine the extent to which non-smokers are exposed systemically.

SALIVARY COTININE AND URINARY NNAL

As stated previously, concentrations of SHS constituents measured in air do not accurately predict systemic exposure and health effects. Use of biomarkers is preferred. Therefore, the objectives of this second study were to characterize the exposure of non-smokers exposed to SHS outside a restaurant and a bar in Athens, Georgia using salivary cotinine, the primary proximate metabolite of nicotine, and urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a pulmonary carcinogen, and to determine the ratio of urinary NNAL to salivary cotinine in non-smokers exposed to outdoor SHS. This study is the first to present levels of urinary NNAL as well as the ratio between urinary NNAL to salivary cotinine in non-smokers exposed to outdoor SHS.

Twenty-eight subjects were assigned to outdoor patios of a restaurant and a bar and an open-air location with no smokers (control) on three weekend days in a replicated Latin square design in which subjects visited each site once. Saliva and urine samples were collected before, post-3 h site visits, and next morning, and analyzed for cotinine and total NNAL, respectively. Mixed-effects models were fit and changes in salivary cotinine and urinary NNAL were contrasted between the restaurant and bar locations and the control. Urinary NNAL to salivary cotinine ratios were also computed.

Significant increases in salivary cotinine were measured immediately following visits to the restaurant and bar sites compared to the control ($F = 76.72$, $p < 0.001$). Post minus pre-exposure salivary cotinine at the bar, restaurant, and control were 0.115 (0.105, 0.126), 0.030 (0.028, 0.031), and -0.004 ng/mL, respectively (presented as differences in geometric means and 95% confidence interval), levels that were not statistically different from next-day minus pre-exposure levels by location-type. While significant changes were not observed in post-exposure minus pre-exposure creatinine-corrected urinary NNAL, next-day minus pre-exposure concentrations were significantly higher following visits to the bar and restaurant sites compared to the control ($F = 6.16$, $p = 0.005$). Next-day minus pre-exposure changes at the bar, restaurant and control were 1.858 (0.897, 3.758), 0.615 (0.210, 1.761), and -0.007 pg/mg creatinine, respectively. Urinary NNAL to salivary cotinine ratios ranged from 6.9 to 19.2 (pg/mg creatinine to ng/mL) and lower ratios were observed immediately following 3-hr site visits. Significant but moderate Spearman rank correlation coefficients between salivary cotinine and urinary NNAL were observed, ranging from 0.33 to 0.60.

The results indicate that both salivary cotinine and urinary NNAL increased significantly in a dose-response manner as a result of exposure to SHS outside of a restaurant and a bar in Athens, GA. Measurements of constituents of SHS in air such as nicotine or $PM_{2.5}$ as well as meteorological variables at the locations were infeasible and are limitations of this study. However, the replicated Latin square design employed in this study in which each subject served as his or her reference makes this study particularly strong. Detection of urinary NNAL in non-smokers has historically strengthened epidemiologic studies associating SHS exposure among non-smokers to health risks, risks which are typically small. Thus, the findings of this study are important. Although the concentrations reported for urinary total NNAL are relatively low, the

reported concentrations of NNAL clearly indicate that non-smokers exposed to brief periods of SHS outside establishments, especially bars, may be exposed to concentrations of carcinogenic tobacco-specific nitrosamines such as NNAL and its parent compound NNK.

URINARY CLARA CELL PROTEIN (CC16)

Cigarette smoke transiently increases the permeability of human airways (Olivera et al. 2007), changes that are rapidly reversible and one of the earliest signs of air pollution-induced lung injury (Broeckaert et al. 2000; Mason et al. 1983). Serum concentrations of the 16-kDa Clara cell specific protein (CC16, CC10 or CCSP) have been proposed as a new sensitive marker to detect an increased permeability of the epithelial barrier. Urinary CC16 has also been used to assess the impact of air pollution on lung epithelial permeability (Timonen et al. 2004). Utility of CC16 in any medium to investigate the effect of outdoor SHS exposure on lung epithelial permeability has not been previously reported. Urinary CC16 is considered much less invasive and complicated and can be self-administered compared to serum CC16. Its use in epidemiology studies seems promising.

Therefore, the objective of this study was to assess the utility of urinary Clara cell protein (CC16) as a biomarker of increased lung epithelial permeability in non-smokers exposed to outdoor secondhand smoke (SHS). Twenty-eight subjects were assigned to outdoor patios of a restaurant and a bar where outdoor smoking is allowed and an open-air control site with no smokers on three weekend days in a replicated Latin square design; subjects visited each site once. Urine samples were collected before, post-3 h visits, and next morning, and analyzed for CC16. Males discarded the first 100 mL of urine as suggested in a previous study to eliminate or successfully diminish post-renal CC16 contamination (Andersson, Lundberg, and Barregard

2007). Number of lit cigarettes was counted per sampling occasion. Mixed-effects models were fit with log(post)-log(pre) or log(nextday)-log(pre) creatinine-corrected CC16 concentrations as the response and location-type as the predictor. Models were fit to all subjects and then stratified by gender. A second set of models was fit with cigarette count as the predictor, also fit to all subjects and stratified by gender.

Urinary CC16 was higher in males (n = 9) compared to females (n = 18), up to an average of 10 times per sampling occasion, signifying that post-renal CC16 levels were still sufficiently high in urine samples to confound CC16 originating from the lungs. When all subjects or males only were considered, differences in post-/pre-exposure or next day/pre-exposure across location-type were non-significant (p-value s> 0.50) but p-values were smaller when females only were considered ($p < 0.19$). There was a trend of increasing post-exposure to pre-exposure ratios of urinary CC16 with increasing SHS exposure among females: control, 0.53 (0.30,0.93); restaurant, 0.57 (0.35,1.14); and bar, 0.94 (0.56,1.56) (geometric mean and 95% confidence interval). Cigarette count had a significant effect on post-exposure to pre-exposure urinary CC16 among females (estimate = 0.002, $p = 0.048$).

The results indicate a tendency towards increasing urinary CC16 with increasing SHS exposure outside the restaurant and bar sites among female subjects. A significant positive effect of cigarette count on creatinine-adjusted urinary CC16 concentrations was observed, suggesting that outdoor SHS exposure increases lung epithelial permeability. The effect of outdoor SHS exposure on increasing lung epithelial permeability assessed by changes in urinary CC16 may be masked somewhat by physiological factors that may reduce elimination of CC16 in urine. Further, possible effect of prostatic CC16 on male urine samples variability may limit the use of urinary CC16 as a biomarker of outdoor SHS and other air pollution-induced lung epithelial

changes. Still, this study shows that urinary CC16 remains a promising biomarker of increased lung epithelial permeability, especially among women.

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CHAPTER 7

PUBLIC HEALTH IMPLICATIONS

The results of the studies in Chapters 3 to 5 have presented the most current data on non-smokers' exposure to secondhand smoke (SHS) in outdoor locations. The data presented shows that non-smokers are systemically exposed to constituents of tobacco smoke, including those that are known carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Although the results of these studies should be placed in proper context and not overstated, these results show that exposure to SHS, specifically outside public locations where indoor smoking has been banned, remains a public health hazard.

Public health efforts such as restrictions on smoking have been hugely effective in reducing non-smokers' exposure to SHS. At any moment, approximately 79.4% of the U.S. population is covered by a 100% smoke-free provision in workplaces, and/or restaurants, and/or bars, by either a state, commonwealth, or local law (ANRF 2011). A study consisting of a series of National Health and Nutrition Examination Surveys (NHANES) investigating the trends in serum cotinine levels among non-smokers in the U.S. population over a 14 year period from 1988 through 2002 reported an approximate 70% decline in serum cotinine levels over that period (Pirkle et al. 2006). However, previous studies (Hall et al. 2009) and the studies presented in this dissertation show that while restrictions on indoor smoking have been effective, outdoor sources of SHS in public places remain a significant source of exposure to the general public. It has to be noted though, that the exposure levels reported in this dissertation are relatively low compared to SHS in indoor areas such as casinos, restaurants or bars where indoor smoking is

allowed. Urinary NNAL measured in non-smoking workers of the hospitality industry in several studies (Jensen et al. 2010; Parsons et al. 1998; Stark et al. 2007) were two to five times higher than what was measured in subjects following visits to the bar site in the study reported in Chapter 4. One study measured urinary NNAL in non-smokers before and 24 h after a 4 h visit to a casino (Anderson et al. 2003). Total urinary NNAL reported in that study was two times higher than the reported creatinine-corrected urinary NNAL measured in subjects following visits to the bar site in Chapter 4. The reported NNAL levels in this dissertation are at least two orders of magnitude lower than what has been measured in active smokers (Byrd, and Ogden 2003; Stepanov, and Hecht 2005). In spite of these low exposure levels, the findings of the study in Chapter 4 are significant. SHS leads to 3,000 lung cancer deaths (USDHHS 2006) and greater than 30,000 coronary heart disease deaths per year in the United States (Adhikari et al. 2008).

Further, although the findings of the transitional epidemiology study presented in Chapter 5 did not show a significant difference in Clara cell protein (CC16) when subjects visited outdoor locations at the bar and restaurant compared to the control, a significant cigarette-count effect on urinary CC16 in females was observed. This indicates that outdoor SHS is associated to a transient increase in lung epithelial permeability. In addition, numerous other studies have shown that brief exposures to SHS leads to harmful effects such as endothelial dysfunction (Bonetti et al. 2010), heart attacks (IOM 2009), and various markers of inflammation (Flouris et al. 2009).

The U.S. Surgeon General report in 2006 concluded that there is no safe level of exposure to tobacco smoke (USDHHS 2006). Keeping this in mind, the studies presented in this dissertation provide evidence of significant systemic exposure to SHS and its toxic constituents in outdoor settings as well as evidence of SHS-induced lung epithelial permeability changes.

Therefore, it seems that implementation of indoor smoking bans alone and placing no restrictions on outdoor smoking in public places does not go far enough to fully protect the public from the deleterious effects of SHS. Restrictions on smoking outside public places should be part of tobacco control policies. This will eliminate a significant source of SHS exposure to children and adults who otherwise would not be exposed to SHS at their homes and school or workplaces where indoor smoking bans are enforced.

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APPENDICES

APPENDIX A: STUDY CONSENT FORM

Consent Form #1: Outdoors second hand smoke in downtown Athens, Georgia

I, _____ agree to take part in the research study titled" **Outdoors second hand smoke in downtown Athens, Georgia**" conducted by Dr. Luke Naeher from the Department of Environmental Health Science at the University of Georgia, USA [706-542-4104]. I understand that I do not have to take part if I do not want to. I can refuse to participate and can stop taking part without giving any reason, and without penalty or loss of benefits to which I am otherwise entitled. I can ask to have all of the information about me returned to me, removed from the research records, or destroyed.

The reason for this study is to obtain information on my exposure to second hand smoke.

If I volunteer to take part in this study, I will be asked to do the following things:
1) Answer questions about my personal and work activities that might affect my exposure to second hand smoke and other chemicals in the environment (2 minutes per day).
2) Wear personal air sampling equipment for 6-12 hours each day of my participation in the study (6 to 12 hours each day for 1-2 study days)
3) Provide a urine sample (approximately 3 tablespoons per sample) and a saliva sample the night prior to, during (1-3 samples), and after the study period. Part of my urine samples will be sent to the Centers for Disease Control and Prevention (CDC), National Center for Environmental Health where the amounts of some chemicals related to second hand smoke will be measured in the urine samples.
4) If I am willing, a small amount of my urine and saliva will be stored for analysis of measures of exposure or effect in the future.

I will receive a Target gift card valued at $25 for each day of participation at the end of the study as compensation for being in this study.

In order to process the payment for my participation, the researchers need to collect my name, mailing address, and social security number on a separate payment form. This completed form will be sent to the College of Public Health's business office and then to the UGA Business Office. The researchers have been informed that these offices will keep my information private, but may have to release my name and the amount of compensation paid to me to the IRS, if ever asked. The researchers connected with this study have gone to great lengths to protect my survey information and will keep this confidential in locked files. However, the researchers are not responsible once my name, social security number, and mailing address leave their center for processing of my payment. If I do not want to provide my social security number, I can still participate in the study.

There are no discomforts or risks anticipated.

I understand these questions and air and urine tests are not for diagnostic purposes and are not going to be used to screen for drugs. If I have questions about my test results I should see a physician. The benefits for my workplace and community are that the second hand smoke data may help leaders in occupational and public health agencies reduce elevated occupational and environmental exposures to me and others in my workplace and community.

Any information that is obtained in connection with this study and that can be identified with me will remain confidential and will be disclosed only with my permission or as required by law. I will be assigned an identifying number and this number will be used on all air monitoring and questionnaires I fill out.

The investigator will answer any further questions about the research, now or during the course of the project (706-542-2454).

I give my permission for the researchers to release my urine and saliva analysis information to my health care provider.
Circle one: YES / NO. Initial _____.

I understand that I am agreeing by my signature on this form to take part in this research project and understand that I will receive a signed copy of this consent form for my records.

_____ _____

Signature of Investigator Date Signature of Participant
Date

Additional questions or problems regarding your rights as a research participant should be addressed to: The Chairperson, Institutional Review Board, University of Georgia, 612 Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411, USA; Telephone (706) 542-3199; E-Mail Address IRB@uga.edu

Consent form #2: Consent to bank urine and saliva This form requests you to allow us to store a portion of your urine and saliva sample in the University of Georgia, Department of Environmental Health Science Laboratory in Athens, Georgia, USA or at the CDC so that it could be used for possible future studies. No study which identifies you will be performed in the future without your written permission. If you agree to have the urine and saliva stored, you can ask that the stored urine and saliva be destroyed at any time by contacting Dr. Luke Naeher at 706-542-2454. Refusal to agree to this testing would in no way prevent you from being in the program.

Please Check One:

( ) 1. **I agree** to allow a portion of my urine and saliva sample from this program to be stored for possible future testing as explained above.

OR

( ) 2. **I do not agree** to allow a portion of my urine and saliva sample from this program to be stored for future testing. These samples should not be used for anything but this program.

Print Name: _____

Signature: _____

Witness: _____

Date: _____

If you have questions, concerns or complaints, please contact one of the investigators:

Luke P. Naeher, PhD
706-542-2454

I understand that I am agreeing by my signature on this form to take part in this research project and understand that I will receive a signed copy of this consent form for my records.

_____ _____

Signature of Investigator Date Signature of Participant
Date

Additional questions or problems regarding your rights as a research participant should be addressed to: The Chairperson, Institutional Review Board, University of Georgia, 612 Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411, USA; Telephone (706) 542-3199; E-Mail Address IRB@uga.edu

APPENDIX B: BASELINE QUESTIONNAIRE

Secondhand Smoke Exposure Questionnaire, Spring 2010

Name: _____ Subject I.D.: _____ Date: _____

_____

1. Age: _____
2. Sex: Male / Female
3. Race: White White, non-Hispanic African-American Hispanic
   Asian-Pacific Islander Native American Other: _____
4. Do you currently smoke cigarettes? Yes / No
   If yes, how many cigarettes do you smoke a day? _____
5. Does anyone smoke cigarettes within your home on a regular basis? Yes / No
   If yes, how many cigarettes does that person smoke a day? _____
   If yes, how long are you exposed to smoke in your home? _____
6. Does anyone at your workplace smoke? Yes / No
   If yes, how many cigarettes does that person smoke a day? _____
   If yes, how long are you exposed to the smoke each day? _____
7. Do you currently use smokeless tobacco? Yes / No
   If yes, how often do you use smokeless tobacco? _____
8. Do you currently were nicotine patches? Yes / No
   If yes, how often do you wear the patch? _____
9. Do you currently chew nicotine gum? Yes / No
   If yes, how often do you chew nicotine gum? _____
10. Do you smoke any tobacco products, other than cigarettes? Yes / No
    If yes, what do you smoke and how often do you smoke? _____

APPENDIX C: DAILY QUESTIONNAIRE

Secondhand Smoke Exposure Daily Questionnaire, Spring 2010

Subject I.D.: _____

Date: _____

Time of questionnaire: _____

Time of start of exposure: _____

Time of end of exposure: _____

Salivette I.D. pre-exposure: _____

Salivette I.D. post-exposure: _____

Urine Sample I.D. pre-exposure: _____

Urine Sample I.D. immediate post-exposure: _____

Urine Sample I.D. day after exposure _____

---

1. Have you smoked in the last 48 hours? Yes / No
   If yes, how many cigarettes did you smoke? _____
2. Have you been exposed to secondhand smoke in the last 48 hours? Yes / No
   If yes, how long were you exposed to secondhand smoke? _____
3. Have you used smokeless tobacco in the last 48 hours? Yes / No
   If yes, how long did you use smokeless tobacco? _____
4. Are you currently wearing a nicotine patch? Yes / No
5. Have you worn a nicotine patch in the last 48 hours? Yes / No
   If yes, how long did you wear the patch? _____
6. Have you chewed nicotine gum in the last 48 hours? Yes / No
   If yes, how many pieces of gum have you chewed? _____