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Utility of urinary Clara cell protein (CC16) to demonstrate increased lung epithelial permeability in non-smokers exposed to outdoor secondhand smoke

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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 visited 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. Urine samples were collected at baseline, immediately post-exposure, and next-morning, and analyzed for CC16. Changes in CC16 across location-types or with cigarette count were analyzed using mixed-effect models, stratified by gender.

Results—Urinary CC16 was higher in males (n=9) compared to females (n=18) at all measurement occasions ($p<0.002$), possibly reflecting prostatic contamination. Urinary CC16 from pre-exposure to post-exposure was higher following visits to restaurant and bar sites compared to the control among females but this increase did not reach statistical significance. Post-exposure to pre-exposure urinary CC16 ratios among females increased with cigarette count ($p=0.048$). Exposure-related increases in urinary CC16 were not seen among males.

Conclusion—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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Keywords

Secondhand smoke; CC16; Epithelial permeability; Biomarkers; Smoking bans; Tobacco control

INTRODUCTION

Secondhand smoke (SHS) is a combination of smoke emitted from a burning tobacco product and the smoke exhaled by the smoker (USDHHS, 1986). Scientific evidence continues to show that SHS exposure is causally associated with lung cancer in never- or non-smokers (Vineis, et al., 2007). Secondhand smoke has also been shown to increase the risk of cardiovascular disease by ~30% (Barnoya and Glantz, 2005) as well as increase the risk of respiratory diseases (Flouris, et al., 2009).

The 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. Smoke-free air laws have been very effective in reducing exposures to constituents of SHS (Bondy, et al., 2009) as well as decreasing SHS-related diseases (Herman and Walsh, 2010). The state of Georgia passed a state-wide smoking ban in 2005 which covers 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. A few published studies have measured SHS outside establishments using environmental markers of SHS (Kaufman, et al., 2010; Klepeis, et al., 2007; St.Helen, et al., 2011). We also previously reported the first biomonitoring of non-smokers exposed to outdoor SHS using salivary cotinine (Hall, et al., 2009). Further, health endpoints following acute outdoor SHS exposure have not been previously reported. Nonetheless, there is a growing body of evidence linking low-level tobacco smoke exposure to health effects assessed through the use of biological markers or biomarkers (Flouris, et al., 2009; Heiss, et al., 2008), supporting the conclusion of several leading institutions that there is no “risk-free” or “safe” level of tobacco smoke (USDHHS, 2006; USEPA, 1992; WHO, 1999). Biomarkers are useful tools that can serve as early indicators of adverse effects before onset of symptoms following exposures to environmental pollutants. 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). 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 reversible (Mason, et al., 1983). Serum concentration of the 16-kDa Clara cell specific protein (CC16) 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., 2000). 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., 2000). 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

(Hantson, et al., 2008). Increased epithelial permeability may result in higher rates of passive diffusion and a transient increase in the concentration of CC16 protein in blood and subsequently in urine following CC16 elimination through glomerular filtration.

Serum and/or urinary CC16 have been used to evaluate the impact of several air pollutants such as ozone and photochemical smog in humans (Arjomandi, et al., 2008; Broeckaert, et al., 2000) as well as mainstream tobacco smoke in rats (Van Miert, et al., 2005). The utility of CC16 in any biological fluids as a biomarker of increased lung 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. This project was carried out as one component of a larger study investigating outdoor SHS exposure (St.Helen, et al., 2012). In order to assess the utility of urinary CC16 as a biomarker of SHS-induced 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 1. Previous data showed 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 visit 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 students. Potential participants were administered questionnaires to determine their eligibility. Questions included current and past smoking status and current SHS exposure at home, work or elsewhere. Eligible participants were healthy males and females aged 21 to 40 years who did not use tobacco or nicotine in any form. Females who were pregnant or could be pregnant, were excluded. Enrollment was directed to a target size of 24 participants. Respondents who met the eligibility requirements attended personal information sessions in which the study and protocol were discussed and concerns or questions were addressed. We assigned subjects to study sites in this crossover study based on a replicated Latin square. A Latin square is a design with two blocking factors or sources of variability (participants and study week) and one treatment factor (type of outdoor location). In this study, each Latin square comprised of three participants which we replicated to enroll at least 24 participants. 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 enrolled in the study and they gave written informed consent before participating. The Institutional Review Boards at UGA and the Centers for Disease Control and Prevention (CDC) approved the study.

Site Visits

Participants arrived at the EHS building about 1 hr before site visits. 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 as much as possible, which ranged from about 0.5 m to 5 m at any given time. Participants at the restaurant sites obtained a table in the central part of the seating areas. Participants ate dinner while they were at the restaurant and control sites or prior to visits when assigned to the bar site. One pre-assigned subject and a lab technician at each location took the cumulative smoker and pedestrian or patron counts for every 10 min of 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 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. We collected post-exposure saliva and urine samples within 30 minutes of participants leaving the study sites and we administered another questionnaire to assess participants' exposures to SHS for the 48-h period prior to site visits. Participants were then given materials for next-day sample collection.

Biological Sample Collection and Analysis

Participants provided urine samples, immediately pre- and post-site visits and at 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 post-adolescent 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, et al., 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 at –80 oC in 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 at the CDC.

CC16 was determined in urine by a commercially available ELISA kit (IBL-America, Minneapolis, MN) and analyzed in duplicate, according to the manufacturer's protocol. The limit of detection (LOD) of this assay was 20 pg/mL. The variability in readings was 6.5%. 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-normal 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. Analysis of the changes in urinary CC16 immediately following 3-h site visits (post-exposure) and next day relative to preexposure 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, Model 1.1 was assumed. In this study design, participants were assigned only one of two weekend days (Friday or Saturday), therefore, day was nested in week ($w_{(j)l}$).

$$y_{ijk}=\mu_i+w_{(j)l}+s_k+e_{ijk} \quad \text{Model 1.1}$$

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 for 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 the regression models 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 by replacing location-type with cigarette-count as a continuous variable. 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), seven were black (three females), three were Asian (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 highest value (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) of post-100 mL urine portions were not provided (n = 7 pre, n = 3 post, and n = 3 next-day). CC16 was analyzed in the 75–100 mL urine portions from these subjects and 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 2 and Figure 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 within subjects ($F = 0.45$, $p = 0.639$). Geometric means and 95% CIs of the ratios between post- and pre-exposure and next-day and pre-exposure are also presented in Table 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 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 (post vs. pre-exposure, $F=0.21$, $p=0.811$; next-day vs. pre-exposure, $F=0.05$, $p=0.956$).

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 3. In models where all subjects or males only 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 > 0.48). 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 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 ($F = 4.30$, $p = 0.048$). The cigarette count effect was negative and non-significant when males were considered ($F = 0.24$, $p = 0.635$). 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 analyses were consistent with those reported above.

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 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. Such panel studies are critical in bridging the gap between laboratory experimentation and population-based epidemiology (Hulka, 1991). When all subjects and/or males only were considered, we observed no significant differences in changes of post-exposure to pre-exposure and next-day to pre-exposure creatinine-adjusted urinary CC16 across locations. However, post-exposure to pre-exposure urinary CC16 ratios among female subjects increased with increasing outdoor SHS exposure across locations but this increase did not reach statistical significance (Figure 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 a positive association between outdoor SHS exposure and increased lung epithelial permeability. This relationship was not observed when we analyzed changes in next-day compared to pre-exposure creatinine-adjusted urinary CC16, most likely due to changes in respiratory epithelial permeability being reversible once exposure to SHS is terminated (Mason, et al., 1983).

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. There is currently no information on the utility of serum or urinary CC16 in assessing the health impact of real-life outdoor SHS levels. Although the use of urinary CC16 was considered more feasible than serum levels in this study, 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 or dilution (Andersson, et al., 2007).

We observed significantly higher creatinine-adjusted urinary CC16 in males compared to females, up to 10 times higher per sampling occasion (Table 2, Figure 1). Because serum CC16 concentrations from males and females have not been shown to be significantly different at baseline (Arjomandi, et al., 2008) 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 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 simultaneous decline in urinary CC16 excretion 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 (Plot A of Figure 1 demonstrates the magnitude of the declining urinary CC16 over 3 hr between pre-exposure and post-exposure times in participants at the control). A diurnal variation in urinary CC16 excretion 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 urinary CC16 levels

measured in morning samples. However, pre-exposure urinary CC16 concentrations measured in samples collected just before 6:00 pm (when subjects visited the control and restaurant site) did not differ significantly from pre-exposure urinary CC16 collected just before 11:00 pm when these same subjects visited the bar site. This suggests that the decline in creatinine-adjusted CC16 over the 3-hr sampling period from pre- to post-exposure among female participants may be attributable to reasons other than a diurnal variation in CC16 excretion during these study times. On the other hand, protein elimination in urine has been shown to decrease during periods of relaxation or physical inactivity (Poortmans, et al., 1989) and after meals and that may be likely explanations for the declining background urinary CC16 among female subjects. 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-minus pre-exposure and next-day minus pre-exposure creatinine-adjusted CC16 and post-exposure minus pre-exposure creatinine-adjusted urinary NNAL (rs = 0.25 and 0.26, respectively). Correlations between changes in urinary CC16 and changes in salivary cotinine were non-significant. This lends support to the hypothesis that cotinine does not provide an accurate measure of short-term exposure to the toxic constituents of tobacco smoke compared to NNAL (Benowitz, et al., 2010). Cotinine is not known to have toxic effects on the human body while NNAL and its parent compound, NNK, are potent lung carcinogens (Hukkanen, et al., 2005). The significant correlations between SHS biomarkers and urinary CC16, although relatively small, is indicative of a relationship between known toxic SHS constituents and health effects and need further study.

The current study has several strengths but also some limitations. Among the strengths, we used a crossover design in which subjects served as their own control. This design was chosen to minimize the effect of the variability commonly observed in biomarker data both within and between individuals. Further, this study provides novel data on the use of a noninvasive biomarker 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 measurement of SHS exposure through ambient monitoring of PM2.5, CO or nicotine which was infeasible at the bar and restaurant settings. Instead, we used cigarette count to assess SHS exposure at each site, which showed a linear increase with changes in urinary CC16 excretion. We also measured validated tobacco-specific biomarkers, cotinine and NNAL, in participants, which indicated significant outdoor SHS exposure at the restaurant and bar sites. Further, we did not collect variables such as temperature and wind-speed that would more accurately characterize exposure conditions at the study sites and we did not control for the possible effect of ozone on urinary CC16 excretion. Ozone exposure has been shown to increase lung permeability (Arjomandi, et al., 2008). 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.

While we did not find overwhelming evidence of increased urinary CC16 excretion following outdoor SHS exposure, we do not think that this indicates that outdoor SHS does not induce health effects and therefore, is not a public health hazard (Flouris, et al., 2009). On the contrary, several studies have demonstrated convincingly that low-level SHS exposure is associated with health effects but they often employ costly and more invasive techniques. One study reported significant arterial endothelial dysfunction in nonsmokers

after 30 minutes of exposure to passive smoke in a room with smokers by measuring changes in coronary flow velocity reserve (Otsuka, et al., 2001). In other studies, nonsmokers exposed to real-world levels of SHS for 30 – 60 minutes showed significant vascular injury, diminished function of their endothelial progenitor cells (Heiss, et al., 2008), disturbance of endothelial function (Bonetti, et al., 2010), and significant decrements of lung function and increases in inflammatory cytokines (Flouris, et al., 2009). Although our study focused on SHS in outdoor environments, we, among others, have shown that SHS in outdoor seating areas and near entrances of bars and restaurants can reach sustained peak concentrations comparable to the SHS levels reported in the studies described above (Cameron, et al., 2010; Kaufman, et al., 2010; St.Helen, et al., 2011) and leads to significant systemic exposure to toxic tobacco smoke constituents (St.Helen, et al., 2012). Finally, low-level outdoor SHS exposure has been shown to increase the risk of respiratory symptoms and bronchodilator use in asthmatic adults (Eisner, et al., 2001). We therefore encourage further research into developing and validating non-invasive and easy to use biomarkers for population-based studies of low level SHS exposures.

CONCLUSION

Our results support a positive relationship between urinary CC16 excretion and outdoor SHS exposure among female subjects. We observed a significant positive effect of cigarette count on pre-exposure to post-exposure creatinine-adjusted urinary CC16 concentration among females, suggesting that outdoor SHS exposure increases lung epithelial permeability. We also saw a significant positive association between urinary CC16 and urinary NNAL, a breakdown product of a toxic constituent of SHS. 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 influence renal clearance of CC16. 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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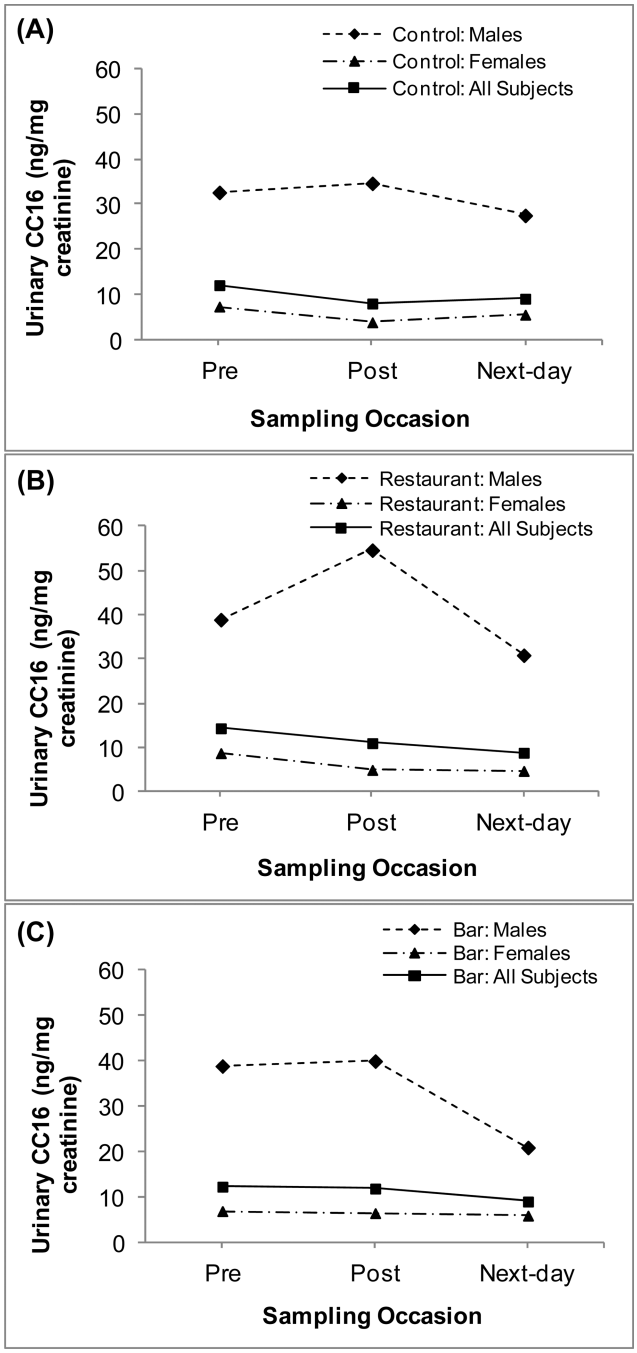

FIGURE 1.

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 2. (A) Control, no exposure to second hand smoke; (B) subjects exposed outside of Restaurant; (C) subjects exposed outside of Bar.

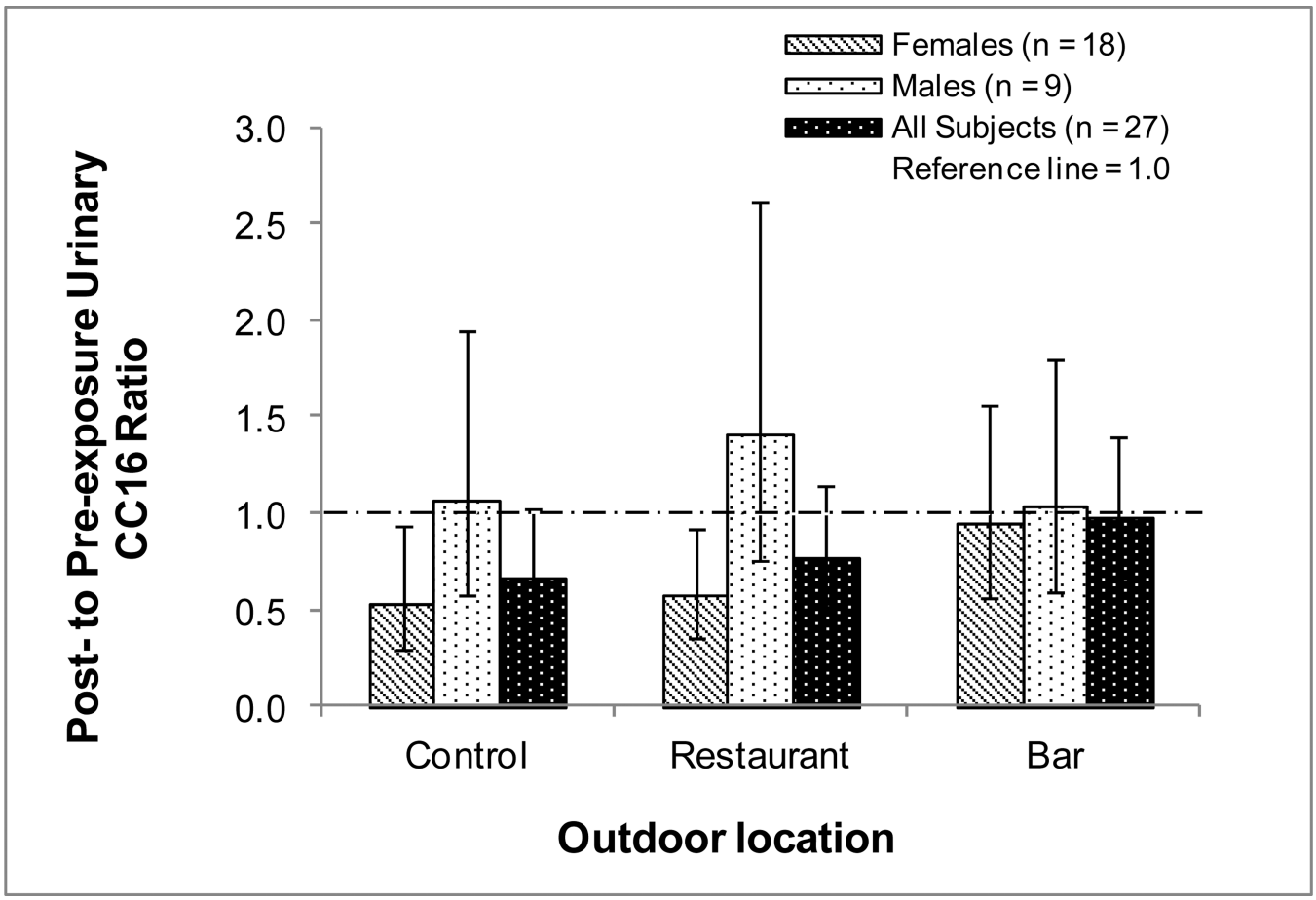

FIGURE 2.

Ratio of post-exposure to pre-exposure creatinine-adjusted urinary Clara cell protein (CC16) in subjects following a 3-h visit to outdoor locations. The reference line is at 1.0. Values > 1.0 indicate that post-exposure CC16 levels were higher than pre-exposure CC16 levels. Values represent geometric means and 95% confidence intervals.

TABLE 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 (0.110, 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 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 3

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

Main effect	Response variable	Data fitted	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)	1.77	0.191
		Females (n = 15)	4.30	**0.048**
		Males (n = 9)	0.24	0.635
	log(next day)-log(pre)	All subjects (n = 24)	0.23	0.633
		Females (n = 15)	2.65	0.116
		Males (n = 9)	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. For the location effect the degrees of freedom (d.f) for the model with all subjects was (2, 43), females only (2, 25), and males only (2, 11); for the cigarette effect the d.f for the model with all subjects was (1, 44), females only (1, 26), and males only (1, 12).

TABLE 4

Spearman rank correlation coefficients (r_s) between changes in Clara cell protein (CC16) and biomarkers of tobacco smoke, salivary cotinine and urinary NNAL (r_s, 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				
r_s	0.02	−0.06	**0.25**	0.07
p-value	0.886	0.618	0.026	0.524
n	75	76	81	80
Next day – Pre				
r_s	−0.07	−0.10	**0.26**	0.01
p-value	0.574	0.399	0.018	0.916
n	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