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Secondhand smoke exposure induces acutely airway acidification and oxidative stress

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pH

Summary
Previous studies have shown that secondhand smoke induces lung function impairment and increases proinflammatory cytokines. The aim of the present study was to evaluate the acute effects of secondhand smoke on airway acidification and airway oxidative stress in never-smokers.

In a randomized controlled cross-over trial, 18 young healthy never-smokers were assessed at baseline and 0, 30, 60, 120, 180 and 240 min after one-hour secondhand smoke exposure at bar/restaurant levels. Exhaled NO and CO measurements, exhaled breath condensate collection (for pH, H_2O_2 and NO_2^-/NO_3^- measurements) and spirometry were performed at all time-points.

Secondhand smoke exposure induced increases in serum cotinine and exhaled CO that persisted until 240 min. Exhaled breath condensate pH decreased immediately after exposure ($p < 0.001$) and returned to baseline by 180 min, whereas H_2O_2 increased at 120 min and remained increased at 240 min ($p = 0.001$). No changes in exhaled NO and NO_2/NO_3 were observed, while decreases in

Abbreviations: COPD, chronic obstructive pulmonary disease; EBC, exhaled breath condensate; eCO, exhaled carbon monoxide; $FEF_{25-75\%}$, mid-expiratory flow; FeNO, fraction of exhaled nitric oxide; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; H_2O_2, hydrogen peroxide; MANOVA, multivariate analysis of variance; NO_2^-, nitrate; NO_3^-, nitrite; PEF, peak expiratory flow; SD, standard deviation; SHS, secondhand smoke.

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FEV_1 ($p < 0.001$) and FEV_1/FVC ($p < 0.001$) were observed after exposure and returned to baseline by 180 min.

A 1-h exposure to secondhand smoke induced airway acidification and increased airway oxidative stress, accompanied by significant impairment of lung function. Despite the reversal in EBC pH and lung function, airway oxidative stress remained increased 4 h after the exposure.

Clinical trial registration number (EudraCT): 2009-013545-28.

Introduction

Secondhand smoke (SHS) or passive smoking is a risk factor for several diseases. Previous clinical studies have shown that SHS may represent a risk factor for lung cancer[1] and chronic obstructive pulmonary disease (COPD),[2] and plays a significant role in the development of ischemic heart disease.[3] The Third National Health and Nutrition Examination Survey in the US has also shown that SHS is associated with deterioration in lung function in adult females, especially those with asthma.[4] It is estimated that, before the introduction of smoke-free policies, one employee in the EU hospitality industry died every day from SHS exposure, whereas exposure to passive smoking at home causes the deaths of 16,600 nonsmokers each year, or one death every 32 min[5] Despite large anti-smoking campaigns, SHS still represents a major health problem in many countries.

Several experimental trials have evaluated the acute effects of active and passive smoking in subjects with and without airway disorders. In a recent study we showed that smoking two cigarettes can induce an acute oxidative stress burst in the airways of asthmatic smokers.[6] Moreover, a single session of water pipe smoking leads to decreased lung function, as expressed by forced mid-expiratory flow (FEF_{25-75}), and reduction in the levels of the fraction of exhaled nitric oxide (FeNO).[7] Our group has further shown that a one-hour SHS exposure at bar/restaurant levels induces significant decrements on lung function and marked increases in inflammatory cytokines.[2,8]

Exhaled breath biomarkers have been extensively evaluated in current smokers, with and without airway disease. FeNO levels are lower in healthy smokers compared to healthy non-smokers,[9] and smokers with allergic rhinitis have lower values compared to non-smokers with allergic rhinitis.[10] Exhaled breath condensate (EBC) is a totally non-invasive method for the evaluation of airways inflammation and EBC pH levels, a robust and reproducible biomarker of airway acidification,[11] are lower in current smokers compared to controls.[12] Moreover, we have shown that acute smoking induces acute airways acidification in asthmatic current smokers.[6] Additionally, EBC hydrogen peroxide (H_2O_2) levels increased 30 min after smoke exposure,[13] whereas plasma nitrate (NO_2^-) and nitrite (NO_3^-) levels decreased for a short time after smoking one cigarette.[14]

Despite a robust body of evidence on the effects of active smoking on airway inflammation, only a few studies have investigated the effects of SHS on exhaled biomarkers. A single study has provided experimental evidence of short-term reduction of exhaled NO after a 1-h exposure to SHS in a separately ventilated chamber.[15] A cross-sectional study has shown that asthmatic allergic children with significant exposure to SHS present lower FeNO levels compared to those without SHS exposure,[16] and a population-based cross-sectional study in adults suggested that SHS exposure is associated with lower FeNO levels in adults.[17] A small cross-sectional study has also evaluated oxidative stress biomarkers in the EBC and has provided some evidence for an effect of SHS the balance between oxidative stress and antioxidant capacity of the lungs.[18] However, the experimental data on the effects of SHS on exhaled biomarkers are still limited.

The aim of the present randomized controlled cross-over trial was to evaluate the effects of a one-hour exposure to SHS at bar/restaurant levels on airway acidification (as expressed by EBC pH), airway oxidative and nitrosative stress (as expressed by H_2O_2 and NO_2^-/NO_3^- levels, respectively), exhaled carbon monoxide (eCO) and lung function.

Materials and methods

Study design

In a randomized controlled cross-over trial (EudraCT: 2009-013545-28), 18 young healthy never smokers (9 males, 9 females, 32.7 $\pm$ 6.0 years) with normal spirometry were evaluated during an exposure and a control visit, separated by 7 days. Subjects were non-atopic with a normal spirometry, had not suffered a respiratory tract infection for at least 8 weeks, and arrived in the lab following a 10-h fast. In the exposure visit subjects were submitted to a 1-h SHS exposure, whereas in the control visit they were exposed to normal room air in a well-ventilated room. All exposures started at 09:00 a.m. At baseline as well as at 0, 30, 60, 120, 180 and 240 min following each exposure subjects were submitted sequentially to FeNO measurement, eCO measurement, EBC collection and simple spirometry, and blood samples were drawn, in that order. The experimental protocol conformed to the standards of the Declaration of Helsinki, was approved by the University of Thessaly ethical review board (Approval No 164/2009), and subjects provided written informed consent.

Second-hand smoke exposure

During the exposure visit, subjects were instructed to remain seated at rest (i.e., reading a book or magazine) for 1-h inside a 6 $\times$ 5 $\times$ 4 m environmentally controlled chamber (air temperature: 24 °C; air velocity: 0.05 m s^{-1};

humidity: 45%). The second-hand smoke (SHS) exposure was adjusted at a carbon monoxide (CO) concentration of 23 ± 1 ppm to meet levels previously reported for bar/restaurant environments.[2,19] Gradients of gas concentrations and particle density were checked by continuous measurement of different areas inside the chamber by a CO90 (Martindale Electric Ltd., Watford, UK) CO—CO_2 analyzer. The desired CO concentration of the gas mixture was achieved by combustion of cigarettes from various popular brands (i.e., equal number of Camel, Davidoff Classic, Gauloises Filter, Original Red Lucky Strike, Marlboro Reds, Prince Classic and Silk Cut Purple King Size cigarettes). The cigarettes were lit and placed on ashtrays to burn (just prior to reaching the filter) at different areas in the chamber. When the desired CO concentration was reached, the subject would enter the chamber.

Study measurements

Spirometry

Spirometry was performed with a dry spirometer (KoKo Legend; Ferraris, UK), and forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, mid-expiratory flow (FEF_{25-75}) and peak expiratory flow (PEF) were recorded as absolute values and percentages of predicted values, according to the American Thoracic Society guidelines.[20]

Measurements of exhaled biomarkers

Exhaled CO was measured with a commercial analyzer (PICO Smokerlyser; Bedfont, Rochester, UK). FeNO was measured with a commercial NO analyzer (NIOX MINO; Aerocrine, Solna, Sweden) at 50 mL/s exhalation flow rate.[21]

Exhaled breath condensate measurements

Exhaled breath condensate (EBC) was collected using a commercially available condenser (EcoScreen; Viasys, Hoechberg, Germany) according to the ATS/ERS recommendations.[22] Subjects rinsed their mouths with distilled water and were asked to perform tidal breathing for 20 min through a mouthpiece wearing a nose clip, while sitting comfortably on a chair. Approximately 2 mL of condensate were collected and were separated in two aliquots. The first aliquot was used for the measurement of EBC pH, immediately after EBC collection as previously described.[22] Stable pH was achieved after deaeration with an inert gas (argon, 350 ml/min for 10 min) and was measured using a commercially available pH meter (Model 3510, Jenway, Essex, UK).

The second aliquot of the EBC was immediately frozen in −80 °C and was used for the rest of the measurements (i.e. H_2O_2, and NO_2^-/NO_3^-). EBC H_2O_2 levels were measured colorimetrically by means of horseradish peroxidase-catalyzed oxidation of tetramethylbenzidine, according to the method previously described by Gallati and Pracht.[23] Briefly, 100 μL 3,3′,5,5′-tetramethylbenzidine and 10 μL horseradish peroxidase (Sigma Chemicals, St Louis, MO, USA) were reacted with 100 μL Serum or EBC for 20 min at room temperature. Subsequently, the mixture was acidified to pH 1 with 10 μL sulfuric acid. The reaction product was measured spectrophotometrically at 450 nm using a microplate reader (Bio-TeK, Instruments INC, Highland Park, USA). A separate standard curve for H_2O_2 was constructed for each assay and all samples were analyzed in triplicate and in one run to circumvent interassay variation. Assays of H_2O_2 were performed on undiluted and non-concentrated EBC samples. The lower limit of detection (LOD), defined as the lowest concentration of the standard curve was 0.1 μM for H_2O_2.

Nitrite/nitrate (NO_2^-/NO_3^-) measurements were performed as previously described.[24] Briefly, NO_3^- was measured as NO_2^- after enzymatic conversion by NO_3^- reductase, and the total NO_2^-/NO_3^- (converted NO_3^- plus NO_2^-/NO_3^-) was measured by using the Griess reaction, as previously described.[25] For the enzymatic conversion of NO_3^- to NO_2^-, aliquots of EBC were incubated for 30 min at 37 °C with nitrate reductase (10 mU) and nicotinamide adenine dinucleotide phosphohydrogenase (100 μM). Griess reagent (100 ml; 5% v/v H_3PO_4 containing 1% w/v sulphanilic acid and 0.1% w/v N-1-napthylethylenediamine) was then added and samples incubated for a further 15 min (37 °C). The absorbance at 540 nm was measured using a microplate reader (Bio-TeK, Instruments INC, Highland Park, USA). The concentration of NO_2^-/NO_3^- sum in each sample was determined by comparing the measured absorbance to a standard curve with known sodium nitrite ($NaNO_2$) concentrations. Each EBC sample was analyzed in triplicate. The lowest detection limit of the method was 0.2 μM.

Serum cotinine measurements

Blood samples were immediately centrifuged and aliquots were stored in −80 °C. Serum cotinine was measured via electron ionization mass spectrometric confirmatory analysis, as previously described.[2]

Statistical analysis

Demographic data are presented as mean values with standard deviation (SD). Normality of data was evaluated with Kolmogorov Smirnov's test. Sample size calculations were conducted based on FEV_1 values before and after [4.9(0.4) vs. 4.5(0.3) in men and 3.7(0.4) vs. 3.2(0.3) in women] a similar 1-h SHS exposure from a previous experiment by our group.[2] The resulting minimum required sample size was 11 for 2-sided type 1 and type 2 errors of 5%. Exposure and control visits were performed using a random allocation algorithm (SPSS 14.0.1, SPSS Inc., Chicago, Illinois). Analysis of data was performed using a factorial multivariate analysis of variance (MANOVA) incorporating three factors [visit (exposure, control), time (0, 30, 60, 120, 180 and 240 min) and gender (male, female)]. In the event of a significant F-ratio for a time point and visit or interactive effect, post-hoc analyses using Bonferroni corrections were employed to identify significant differences over time within each group or between groups. Correlations were performed using Spearman's rank correlation coefficient (r_s). p values of <0.05 were considered statistically significant. Statistical analysis was performed with SPSS 14.0.1 (SPSS Inc., Chicago, IL), and graphs were created with Graph Pad Prism 5 (GraphPad Software Inc., La Jolla, CA).

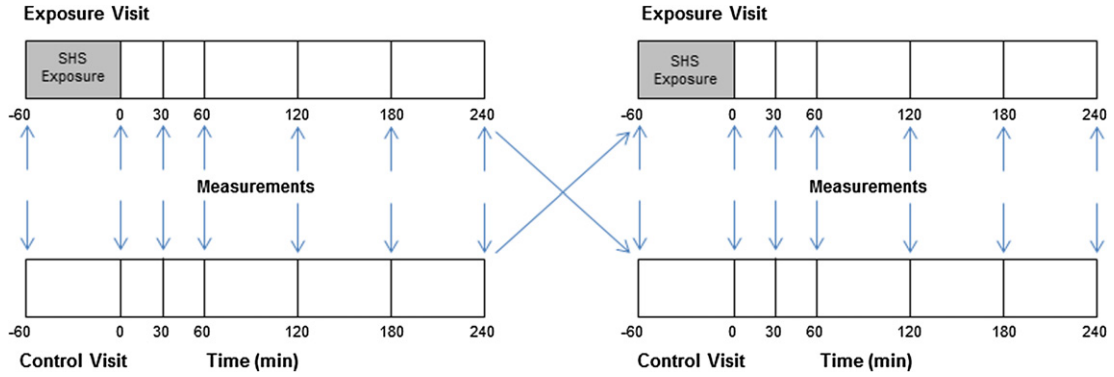

Figure 1 Study design and times of exposure to secondhand smoke (SHS) and measurements.

Results

The design of the experiment is provided in Fig. 1. Spirometric changes throughout the trial are presented in Table 1. Significant reductions in the values of FEV_1 ($F = 38.416$, $p < 0.001$) and FEF_{25-75} ($F = 10.633$, $p < 0.001$) were observed immediately after the exposure and until 120 min, whereas the FEV_1/FVC ratio ($F = 14.765$, $p < 0.001$) was significantly reduced immediately after the exposure and until 60 min, returning to baseline values thereafter. No significant changes were observed in either FVC ($F = 0.270$, $p = 0.946$) or PEF ($F = 1.274$, $p = 0.300$).

Changes in the levels of serum and exhaled biomarkers throughout the trial are presented in Table 2. Significant increases were observed in the levels of serum cotinine ($F = 1937.475$, $p < 0.001$) and exhaled CO ($F = 25.759$, $p < 0.001$; Fig. 2A); these increases were observed immediately after the exposure and remained increased until the end of the trial (240 min). A reduction in EBC pH ($F = 5.755$, $p = 0.001$; Fig. 2B) was found immediately after the exposure and remained significant until 120 min after the exposure. EBC H_2O_2 levels were significantly increased 120 min after the exposure, compared to baseline, and remained significantly increased until 240 min ($F = 2.645$, $p = 0.036$; Fig. 2C). No changes were observed in FeNO levels ($F = 0.434$, $p = 0.850$) or EBC NO_2/NO_3 levels ($F = 0.359$, $p = 0.899$) throughout the trial. No differences were observed between in either exhaled biomarkers or spirometry measurements between genders.

EBC pH levels throughout the trial presented a modest negative correlation with exhaled CO levels ($r_s = -0.444$, $p < 0.001$) and a weak correlation with EBC NO_2^-/NO_3^- levels ($r_s = -0.142$, $p = 0.024$), as well as a modest positive correlation with the FEV_1/FVC ratio ($r_s = 0.352$, $p < 0.001$) and FEF_{25-75} ($r_s = 0.180$, $p = 0.004$). Additionally, exhaled CO levels and presented a significant negative correlation with the FEV_1/FVC ratio ($r_s = -0.525$, $p < 0.001$), whereas EBC H_2O_2 levels presented a weak negative correlation with FEF_{25-75} ($r_s = -0.184$, $p = 0.003$). No other significant correlations between exhaled biomarkers and/or spirometric values were observed. Additionally, there were no significant correlations between EBC biomarkers and serum cotinine levels.

Discussion

The current randomized controlled cross-over trial revealed that a 1-h exposure of young healthy never

Table 1 Changes in spirometric values at each time point during the secondhand smoke exposure and control visits.

Variable	Visit	Time points (min)						
		−60	0	30	60	120	180	240
FEV_1 (L)	Exposure	4.11 (0.89)	3.73 (0.89)[a,b]	3.79 (0.91)[a,b]	3.89 (0.91)[a,b]	3.99 (0.91)[a,b]	4.10 (0.89)	4.10 (0.90)
	Control	4.11 (0.89)	4.11 (0.87)	4.10 (0.88)	4.10 (0.90)	4.11 (0.91)	4.11 (0.91)	4.11 (0.90)
FVC (L)	Exposure	5.05 (1.20)	5.03 (1.21)	5.01 (1.20)	5.01 (1.25)	4.98 (1.27)	5.00 (1.27)	5.02 (1.27)
	Control	5.03 (1.18)	5.03 (1.20)	5.02 (1.20)	5.01 (1.23)	4.99 (1.22)	5.01 (1.24)	5.02 (1.24)
FEV_1/FVC	Exposure	0.82 (0.04)	0.74 (0.04)[a,b]	0.76 (0.03)[a,b]	0.78 (0.04)[a,b]	0.81 (0.04)	0.83 (0.05)	0.83 (0.04)
	Control	0.82 (0.04)	0.82 (0.05)	0.82 (0.04)	0.82 (0.04)	0.83 (0.04)	0.83 (0.05)	0.82 (0.04)
PEF (L/min)	Exposure	9.89 (2.39)	9.70 (2.18)	9.43 (1.76)	9.25 (1.18)	9.54 (2.04)	9.43 (2.06)	9.54 (1.98)
	Control	9.54 (2.35)	9.52 (2.22)	9.48 (2.10)	9.34 (1.90)	9.43 (2.10)	9.65 (2.03)	9.52 (1.90)
FEF_{25-75} (L)	Exposure	3.98 (0.78)	3.58 (0.73)[a,b]	3.57 (0.74)[a,b]	3.66 (0.71)[a,b]	3.81 (0.72)[a,b]	3.91 (0.73)	4.04 (0.77)
	Control	4.03 (0.76)	4.03 (0.76)	4.08 (0.79)	4.12 (0.80)	4.09 (0.74)	4.14 (0.76)	4.10 (0.73)

FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; FEF_{25-75}: mid-expiratory flow; PEF: peak expiratory flow.
Data are presented as mean (SD).
[a] Statistical difference compared to −60.
[b] Statistical difference between visits.

Table 2 Serum cotinine and exhaled biomarkers at each time point during the secondhand smoke exposure (E) and control (C) visits.

Variable	Visit	Time points (min)						
		−60	0	30	60	120	180	240
Serum cotinine (ng/mL)	Exposure	1.18 (1.88)	14.73 (6.13)[a,b]	23.88 (2.84)[a,b]	22.76 (2.02)[a,b]	22.40 (3.19)[a,b]	22.33 (6.14)[a,b]	21.52 (6.33)[a,b]
	Control	0.76 (0.22)	0.77 (0.23)	0.74 (0.18)	0.70 (0.12)	0.63 (0.14)	0.68 (0.17)	0.59 (0.10)
Exhaled CO (ppm)	Exposure	2.8 (1.0)	7.3 (1.7)[a,b]	7.5 (1.6)[a,b]	6.8 (1.4)[a,b]	5.8 (1.4)[a,b]	5.3 (1.3)[a,b]	4.0 (1.0)[a,b]
	Control	2.6 (0.6)	2.6 (0.8)	2.6 (0.9)	2.4 (1.1)	2.5 (1.0)	2.3 (1.0)	2.3 (0.8)
FeNO (ppb)	Exposure	11.9 (4.5)	11.3 (4.3)	10.8 (4.2)	10.9 (4.0)	11.3 (4.7)	10.9 (4.2)	11.0 (4.0)
	Control	13.1 (4.9)	12.6 (4.8)	12.1 (4.5)	12.5 (4.4)	12.2 (4.6)	12.6 (4.1)	11.9 (4.1)
EBC pH	Exposure	7.69 (0.14)	7.23 (0.27)[a,b]	7.05 (0.29)[a,b]	7.16 (0.35)[a,b]	7.32 (0.26)[a,b]	7.40 (0.36)	7.61 (0.17)
	Control	7.67 (0.18)	7.57 (0.18)	7.54 (0.22)	7.63 (0.16)	7.61 (0.25)	7.57 (0.27)	7.67 (0.20)
EBC H_2O_2 (μM)	Exposure	0.10 (0.08)	0.15 (0.19)	0.17 (0.18)	0.21 (0.19)	0.27 (0.21)[a,b]	0.31 (0.19)[a,b]	0.28 (0.24)[a,b]
	Control	0.12 (0.10)	0.12 (0.11)	0.14 (0.13)	0.14 (0.11)	0.15 (0.12)	0.13 (0.12)	0.14 (0.11)
EBC NO_2/NO_3 (μM)	Exposure	5.08 (3.89)	5.44 (4.21)	5.15 (3.55)	4.47 (3.45)	4.74 (3.09)	4.48 (3.75)	4.85 (3.65)
	Control	5.34 (4.10)	5.23 (3.62)	4.70 (3.02)	3.98 (2.57)	4.75 (3.35)	5.63 (4.17)	5.82 (3.89)

CO: carbon monoxide; FeNO: fraction of exhaled nitric oxide; EBC: exhaled breath condensate; NO_2/NO_3: nitrites/nitrates.
Data are presented as mean (SD).
[a] Statistical difference compared to −60.
[b] Statistical difference between visits.

smokers to SHS induced acutely airways acidification, as expressed by EBC pH that subsided in 180 min. We also observed a delayed increase in airway oxidative stress, as expressed by EBC H_2O_2 that was evident for at least 4 h after the exposure. These changes were accompanied by increases in serum cotinine and exhaled CO levels that persisted for at least 4 h, as well as by transient decreases in FEV_1, FEV_1/FVC ratio and FEF_{25-75} that returned to baseline levels 2 h after the exposure. We were additionally able to find significant correlations between EBC pH, exhaled CO and H_2O_2 levels with spirometric parameters expressing airway obstruction. This finding provides indications of possible mechanisms regarding the development of obstructive airways disease in healthy subjects exposed to SHS. To our knowledge, this is the first controlled trial that evaluates the acute effects of SHS on airway inflammation biomarkers in young healthy never smokers.

Our study further contributes to the vast evidence regarding the deleterious effects of SHS in the respiratory system. For instance, SHS is an established risk factor for the development of COPD.[26] Lifetime exposure to SHS at home and/or at work has been found to be associated with respiratory symptoms especially dyspnea, shortness of breath at rest and wheeze.[27] The randomized cross-over design of our study provides evidence that even a single 1-h exposure may lead to a transient, yet statistically and possibly clinically significant, airway obstruction in spirometry. These results support previous findings by our group in a similar setting of SHS exposure,[2] providing additional information about the airway inflammatory process that may underlie this obstructive disorder. Our present findings are even more important given that the observed increases in serum cotinine and exhaled CO levels suggest a moderate and brief SHS exposure.[2]

Airway acidification, as expressed by EBC pH, is present in patients with COPD and is associated with sputum neutrophilia and oxidative stress.[24] Moreover, patients with severe refractory asthma have lower values of EBC pH compared with to patients with moderate asthma or healthy subjects,[28] whereas acute airway acidification is present in acute asthma.[29] In a cross-sectional study, a subgroup of otherwise healthy smokers presented significant airway acidification (pH <7.2), but EBC pH did not differ between smokers and non-smokers.[30] The origin of EBC pH reduction after smoke exposure is still under debate, with some authors supporting a possible role for salivary contamination,[31] yet there is now evidence that EBC pH expresses acidification of the airway at all levels,[32] therefore expressing a deleterious effect in the whole respiratory tract. Nevertheless, EBC pH represents the most robust and reproducible biomarker in EBC,[11] with well-established normal values.[33] Using the methodology adopted in the present study, Paget-Brown et al. have shown that only 6.4% of normal subjects present pH values <7.4.[33] Our findings indicate that exposure to SHS caused a significant reduction in EBC pH, well below the aforementioned "normal" range, however not reaching the extreme levels of airway acidification reported in acute asthma.[29] This acidification was transient, returning to baseline after 180 min, but the significant correlations of EBC pH with FEV_1/FVC ratio and $FEF_{25-75\%}$ suggest a possible role of airway acidification in the development

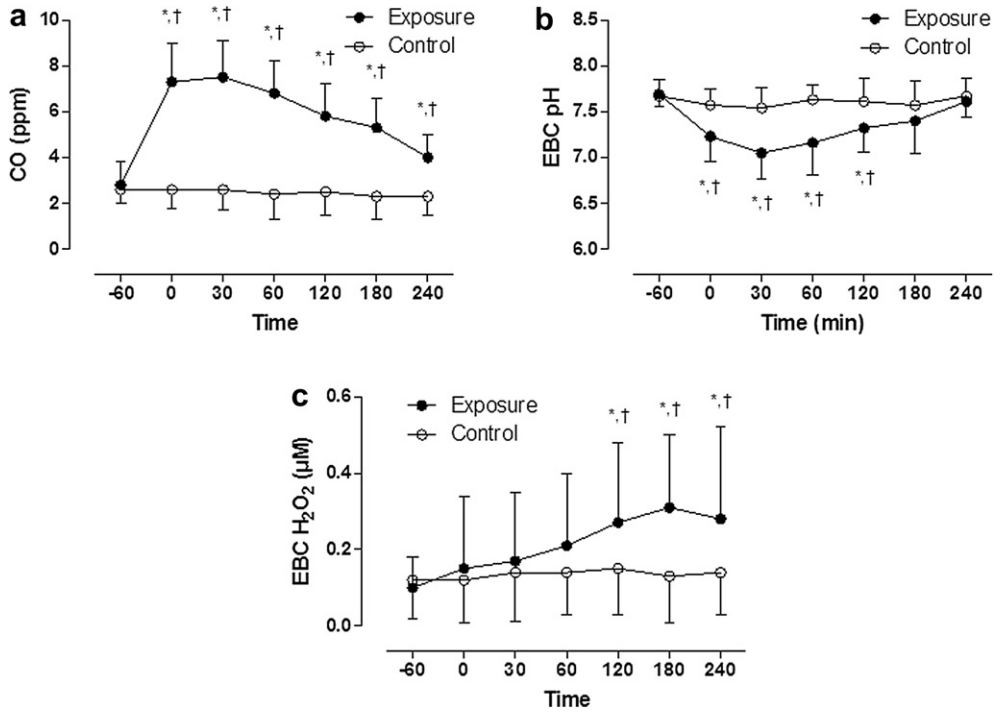

Figure 2 Changes in (A) exhaled CO (eCO) (B) exhaled breath condensate (EBC) pH and (C) exhaled breath condensate (EBC) H_2O_2 levels during the exposure and control visits. Error bars represent standard deviation. *Statistical difference compared to −60 min, †Statistical difference between visits.

of airway obstruction in our model of SHS exposure in healthy young adults.

The second important finding of the present study is the increase in exhaled H_2O_2, a biomarker of airway oxidative stress. Despite measurement problems with exhaled breath condensate that still need to be resolved, H_2O_2 is elevated in patients with COPD[34] and is a reproducible biomarker of oxidative stress.[35] The reproducible results in the control visit of our experiment further support a role of this biomarker in the evaluation of airway oxidative stress. The increase of H_2O_2 in our experiment presented a different pattern compared to airflow impairment and airway acidification, with its increase starting 2 h after exposure and persisting at least until 4 h. This may be of importance, since airway oxidative stress is one of the major pathogenic mechanisms in COPD development.[26] Previous studies have shown that active smoking acutely increases exhaled biomarkers of oxidative stress, in both healthy subjects and asthmatic smokers.[6] To our knowledge, this is the first study that shows an increase in airway oxidative stress after a brief moderate SHS exposure. This observation is important, especially in relation to previous data from our group showing that the same model of SHS led to increases of systemic inflammatory cytokines that persisted until 3 h after exposure.[2] Taken together these findings suggest that the deleterious effects of SHS may persist long after the exposure.

Exhaled NO represents the most widely evaluated exhaled biomarker today, being elevated in atopic asthma and rhinitis,[21] whereas smoking represents a significant confounding factor in its evaluation, with asthmatic smokers presenting lower values of FeNO compared to non-smokers.[36] Total NO_2^-/NO_3^- are end-products of NO metabolism and biomarkers of nitrosative stress that have been found to be elevated in asthma.[37] The effects of acute smoking on exhaled NO levels are contradictory, with some studies reporting reduction,[38] no effect,[6] or even increase[39] of its levels in asthmatic smokers. In the present study, exposure to SHS had no effect on FeNO or NO_2^-/NO_3^- levels during the 4 h follow-up. Previous cross-sectional studies have provided evidence that FeNO is reduced in allergic asthmatic children[16] and asthmatic and non-asthmatic adults[17] with regular exposure to SHS, but these studies have different design and our model of 1-h exposure in bar-restaurant levels may differ significantly from the exposure of their subjects. In a crossover study with a similar experimental design to our study, Yates et al. have shown that FeNO levels decrease rapidly in normal subjects exposed to SHS when compared with sham exposure. Differences in the study populations (higher age and inclusion of former smokers in the study by Yates) and in the measurement of FeNO (at 250 ml/min) may account in part for the discrepancy in our results. Furthermore, FeNO suppression in smokers is achieved through decreased NO formation by reduced expression of inducible NO synthase,[40] mechanisms that may not be activated acutely by the 1-h exposure to SHS in the young healthy never-smokers of our population.

A weak correlation between EBC pH and NO_2^-/NO_3^- was additionally observed in our population. We believe that this may be associated with a trend for a reduction of NO_2^-/NO_3^- in the exposure group (see Table 2, Columns 60—240 min) that, however, did not approach statistical significance. This may reflect a trend for reduction in NO

production in the presence of low pH that has been reported in previous studies.[24,29] Another possible explanation for that may be that the 1-h exposure to SHS in our model may not be adequate for the suppression of FeNO and NO-related products in the exhaled air of our study participants, and this needs to be investigated in further studies with longer exposure.

In conclusion, the present randomized controlled crossover trial has revealed that a 1-h exposure to SHS induced airway acidification and increased airway oxidative stress, accompanied by significant impairment of lung function in young healthy never-smokers. Despite the reversal in EBC pH and lung function, airway oxidative stress remained increased 4 h after the exposure, representing a possible mechanism for the persistence of the deleterious effects of secondhand smoke after the end of the exposure.

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Conflict of interest statement

None of the authors has any conflict of interest related to the present manuscript.

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