

# The Exposure-Dependent Effects of Aged Secondhand Smoke on Endothelial Function

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- Objectives** The aim of this study was to investigate whether exposure to a range of relatively low concentrations of aged secondhand smoke (SHS), similar to those encountered commonly in the community, would impair endothelial function in a concentration-dependent manner.
- Background** Exposure to SHS impairs endothelial function in humans. The concentration-dependent relationship for aged SHS effects on endothelial function after an exposure of short duration is unknown.
- Methods** Thirty-three healthy nonsmokers were exposed to 1 of 2 low levels of aged SHS or to conditioned filtered air for 30 min. The primary end point was change in maximal percent brachial artery flow-mediated dilation after exposure.
- Results** In a linear regression model for each increase in SHS exposure by 100  $\mu\text{g}/\text{m}^3$  respirable suspended particles, the absolute maximal percent brachial artery flow-mediated dilation was reduced by 0.67%. We did not find evidence of a threshold for the effect of SHS on flow-mediated dilation.
- Conclusions** Short-term exposure to real-world levels of aged SHS for 30 min resulted in a concentration-dependent decrease in endothelial function as measured by flow-mediated dilation. (J Am Coll Cardiol 2012;59:1908-13) © 2012 by the American College of Cardiology Foundation

Secondhand smoke (SHS) causes cardiovascular disease (1), and bans on public smoking have led to rapid reductions in myocardial infarctions (2,3). The Institute of Medicine found “an increased risk . . . even at the lowest levels of exposure” (4). One key mechanism of cardiovascular disease is endothelial dysfunction (5). Experimental data indicate substantial effects on endothelial function in response to chronic SHS exposures (6–8) and in response to a single short-term exposure at levels encountered in hospitality venues (9). Smoke typically lingers and ages in public sites;

aged smoke is more toxic to the respiratory epithelium, per unit mass, than fresh SHS (10). We tested the hypothesis that aged SHS impairs endothelial function across a range of relatively low exposures encountered in the community.

## Methods

We exposed healthy (no diabetes, hypertension, chronic respiratory disease, kidney disease, coronary artery disease, prior myocardial infarction, or heart failure) nonsmokers ( $n = 33$ ) to aged SHS for 30 min. Subjects were lifelong nonsmokers 18 to 40 years old with no SHS exposure within 30 days by history and confirmed by a salivary cotinine level  $<10$  ng/ml (11). Our experimental SHS exposures were evaluated by urinary cotinine before and 4 h after each study, with a limit of quantification  $<0.05$  ng/ml (12).

We assigned subjects to 1 of 3 exposure levels of aged SHS generated the day the subjects were available: 1) smoke-free, conditioned filtered air; 2) 100  $\mu\text{g}/\text{m}^3$  respirable suspended particles (RSPs; typical of the homes of smokers or restaurants); or 3) 400  $\mu\text{g}/\text{m}^3$  RSPs (typical of smoky bars and casinos) (1). Vascular studies were performed at baseline and immediately after SHS exposure. Urinary high-sensitivity cotinine levels were assessed at 0 and 4 h and blood asymmetric dimethyl-L-arginine (ADMA) and

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nitrotyrosine levels at baseline and at 0, 2, and 4 h after SHS exposure. The study protocol was approved by the University of California, San Francisco, Committee on Human Research; all subjects gave written informed consent.

**SHS exposure.** Marlboro Red hard-pack cigarettes were smoked using a TE10-z smoking machine (Teague Enterprises, Woodland, California). The smoke was diluted with filtered air at 45% to 55% relative humidity and 20°C to 22.5°C, aged for 60 min in a stainless steel chamber, and then routed to the hood (Hood H-410-10/07037; 3M, St. Paul, Minnesota) for 30-min human exposure. Control exposures used the same hood and purified air, free of smoke. RSP concentration was monitored continuously immediately upstream of the subject using a photometer (Dusttrak 8350, TSI Corporation, Shoreview, Minnesota). The range of individual exposures appears in Figure 1.

Endothelium-dependent dilation of the brachial artery was measured by high-resolution ultrasound (MicroMaxx, Sonosite, Bothell, Washington) before and immediately after SHS exposure (13). Baseline diameter and blood flow velocity of the brachial artery were quantified after 15 min of supine rest in a dark, quiet room after an overnight fast. A blood pressure cuff was placed on the forearm and inflated to  $\geq 200$  mm Hg for 5 min. After cuff deflation, the maximal increase in brachial artery end-diastolic diameter was measured at 40, 60, 80, and 100 s using a digital analysis system (Medical Imaging Applications, Iowa City, Iowa) and averaged over at least 3 consecutive cardiac cycles.

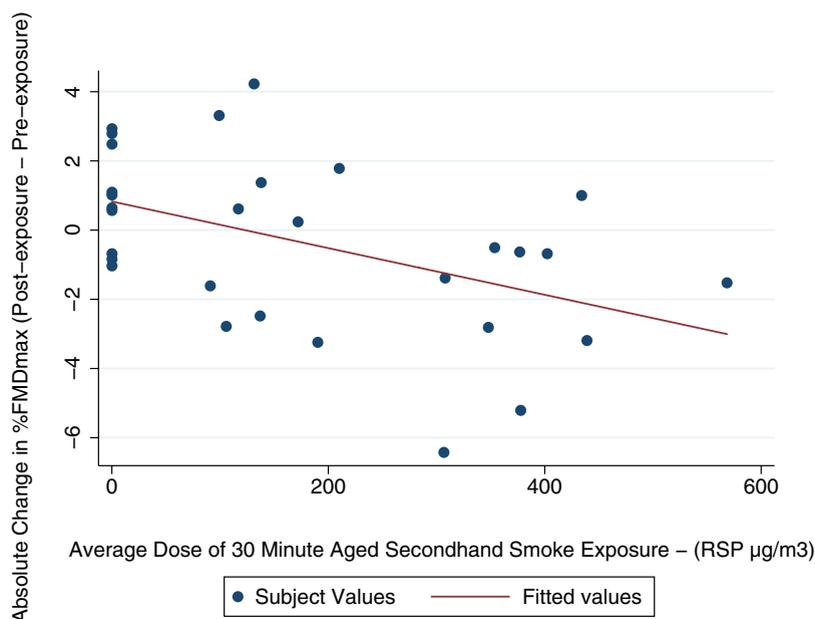
Before data analysis, the image quality of 3 participants was deemed insufficient by a blinded investigator, and their flow-mediated dilation (FMD) data were excluded from further analysis, leaving 30 participants with complete FMD data.

Maximal percent brachial artery FMD (%FMD<sub>max</sub>) was calculated as:  $[(\text{diameter}_{\text{max}} - \text{diameter}_{\text{baseline}}) / (\text{diameter}_{\text{baseline}})] \times 100\%$ . To account for any differences from subject to subject in baseline %FMD<sub>max</sub>, we also examined relative change in %FMD<sub>max</sub>. The absolute and relative changes yielded similar results. The images were measured by a single investigator (P.F.F.) blinded to the subject's exposure level. Plasma ADMA and symmetric dimethyl-L-arginine (SDMA) levels (14) and plasma free nitrotyrosine levels (15) were measured by high-performance liquid chromatography (Ox-ononon Inc., Emeryville, California).

**Statistical analysis.** Subjects' baseline characteristics are expressed as mean  $\pm$  SD, and comparisons between exposure groups were made using 1-way analysis of variance for continuous variables and chi-square tests for categorical variables.

**Abbreviations and Acronyms**

- ADMA** = asymmetric dimethyl-L-arginine
- FMD** = flow-mediated dilation
- MED** = minimal effective dose
- %FMD<sub>max</sub>** = maximal percent brachial artery flow-mediated dilation
- %FMD<sub>0</sub>** = baseline percent brachial artery flow-mediated dilation
- RSPs** = respirable suspended particle
- SDMA** = symmetric dimethyl-L-arginine
- SHS** = secondhand smoke



**Figure 1. Absolute Change in %FMD<sub>max</sub> (Post-Exposure Minus Pre-Exposure) Versus Dose of SHS With Linear Regression Line**

The correlation coefficient (*r*) is  $-0.47$ . **Solid circles** = subject values, and the **line** = fitted values by linear regression model. FMD = flow-mediated dilation; %FMD<sub>max</sub> = maximal percent brachial artery flow-mediated dilation; RSP = respirable suspended particle; SHS = secondhand smoke.

We tested for a linear relationship between absolute and relative change in %FMD<sub>max</sub> and SHS exposure using linear regression (Fig. 1). We also tested for evidence of a threshold or minimal effective dose (MED) of SHS by fitting a piecewise linear model (16) using nonlinear regression, in which FMD response was constant at some baseline level up to the MED, then increased linearly after that with nonlinear regression to estimate the baseline level (%FMD<sub>0</sub>), MED, and slope above the MED (*b*):

$$\%FMD_{\max} = \begin{cases} \%FMD_0 & \text{if } RSP_s < MED \\ \%FMD_0 + b(RSP_s - MED) & \text{if } RSP_s \geq MED \end{cases}$$

This analysis did not produce evidence of a threshold effect, so we used simple linear regression to quantify the relationships between absolute and relative %FMD<sub>max</sub> change and RSPs.

To assess the influence of accepted risk factors of impaired vascular function on FMD, we tested univariate linear regressions of absolute change in %FMD<sub>max</sub> against age, sex, body mass index, total cholesterol, baseline cotinine values, baseline arterial diameter, and baseline %FMD<sub>max</sub>.

To see if they were related to SHS exposure, we also correlated changes in the biomarkers ADMA, SDMA, nitrotyrosine, and high-sensitivity urinary cotinine (the value at 4 h after exposure minus the value at time zero before exposure) with SHS concentration using Spearman rank-order correlations.

## Results

Our subjects had few cardiovascular risk factors (Table 1), with no significant differences among exposure groups. Baseline mean brachial artery diameter was  $3.79 \pm 0.74$  mm. Baseline mean %FMD<sub>max</sub> was  $5.2 \pm 2.3\%$ , similar

to previous adult study populations free of cardiovascular disease (17). The linear regression showed that %FMD<sub>max</sub> decreased by 0.67% (95% confidence interval: 0.18 to 1.17,  $p < 0.01$ ) for each 100  $\mu\text{g}/\text{m}^3$  increase in SHS (RSPs) (Fig. 1). Risk factors for impaired vascular function (age, sex, body mass index, total cholesterol, baseline cotinine values, and baseline arterial diameter) were not significantly related to absolute changes in %FMD<sub>max</sub> ( $p > 0.30$ ) in this healthy population. Baseline %FMD<sub>max</sub> was correlated with absolute change in %FMD<sub>max</sub> ( $p < 0.01$ ). The analysis examining relative change in %FMD<sub>max</sub> similarly yielded a significant correlation between SHS exposure and relative change in %FMD<sub>max</sub> ( $p < 0.02$ ) (Fig. 2).

**Cotinine, ADMA, SDMA, and nitrotyrosine levels.** The median urinary cotinine level at baseline before exposure was 0.16 ng/ml (interquartile range: 0.07 to 0.85 ng/ml), consistent with nonsmokers without recent SHS exposure (18). As expected, there was a significant correlation between SHS exposure and change in urinary cotinine levels 4 h after exposure ( $p < 0.02$ ).

The mean nitrotyrosine levels for all participants were  $15.7 \pm 7.3$  nmol/l at baseline and  $24.6 \pm 18.5$  nmol/l 4 h after exposure. Nitrotyrosine levels increased after exposure in all 3 groups, suggesting a circadian increase in nitrotyrosine in fasting subjects. There was no correlation between the change in nitrotyrosine levels and SHS exposure level ( $p > 0.99$ ) (Table 2).

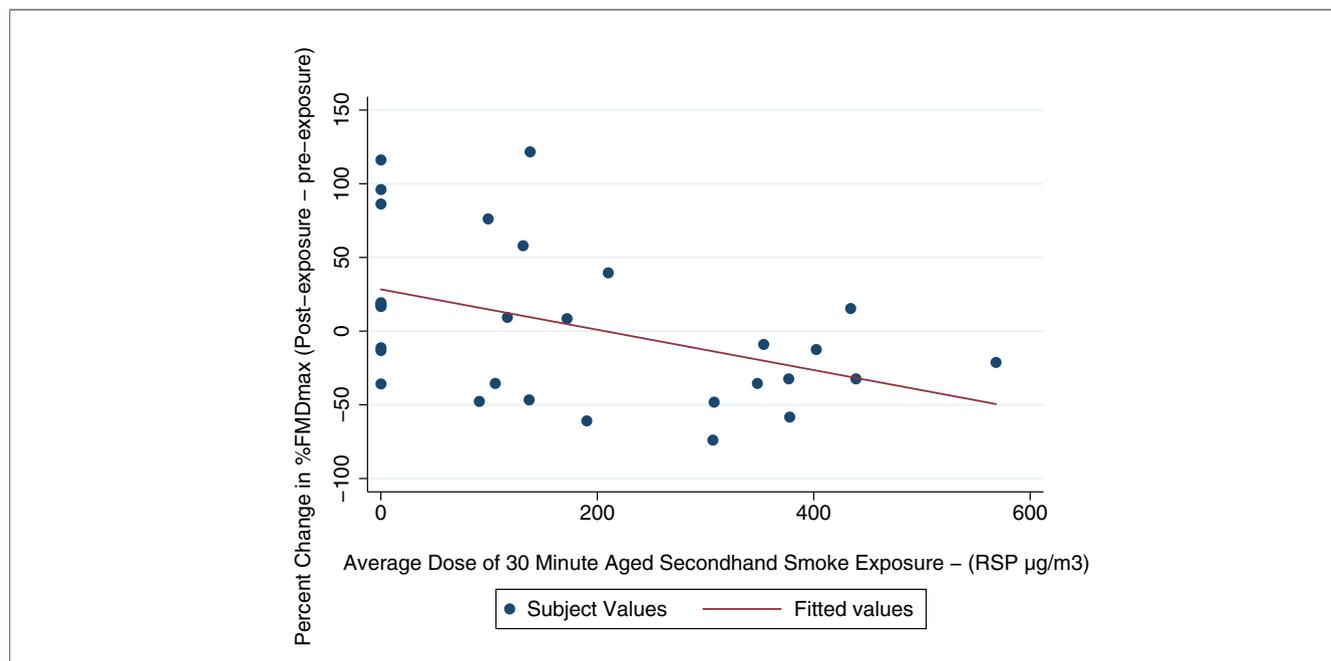
The mean baseline ADMA and SDMA levels for all study participants were  $0.44 \pm 0.09$   $\mu\text{mol}/\text{l}$  and  $0.48 \pm 0.10$   $\mu\text{mol}/\text{l}$ , respectively, and levels obtained 4 h after exposure were  $0.44 \pm 0.09$   $\mu\text{mol}/\text{l}$  and  $0.48 \pm 0.10$   $\mu\text{mol}/\text{l}$ , respectively. There was no correlation between the change in ADMA from baseline to 4 h after exposure and the concentration of RSP ( $p = 0.65$ ), nor for SDMA ( $p = 0.48$ ) (Table 3).

**Table 1. Baseline Characteristics**

Characteristics	Control (n = 11)	Lowest Dose (n = 11)	Low Dose (n = 11)	p Value*
Dose ( $\mu\text{g}/\text{m}^3$ RSPs)	0	142 ± 39	392 ± 77	
Men/women	5/6	6/5	6/5	<0.24
Age (yrs)	24 ± 2	25 ± 4	29 ± 7	<0.08
BMI (kg/m <sup>2</sup> )	26.2 ± 4.8	23 ± 2.3	23.7 ± 3.2	<0.12
HbA <sub>1c</sub>	5.3 ± 0.4	5.3 ± 0.4	5.3 ± 0.4	<0.89
Total cholesterol	158 ± 26	157 ± 21	152 ± 33	<0.85
High-density lipoprotein (mg/dl)	57 ± 13	58 ± 14	50 ± 11	<0.40
Low-density lipoprotein (mg/dl)	85 ± 19	90 ± 21	88 ± 28	<0.92
Triglycerides	71 ± 30	73 ± 45	67 ± 25	<0.90
Baseline artery diameter	3.79 ± 0.9	3.73 ± 0.62	3.84 ± 0.75	<0.95
Baseline SBP (mm Hg)	110 ± 15	106 ± 10	107 ± 11	<0.69
Baseline DBP (mm Hg)	72 ± 8	67 ± 6	70 ± 10	<0.40

Values are mean ± SD. \*For analysis of variance or chi-square test (for gender). p Values illustrate that the allocation of subjects to different exposure groups did not lead to significant differences in subject characteristics between exposure groups.

BMI = body mass index; DBP = diastolic blood pressure; HbA<sub>1c</sub> = glycosylated hemoglobin; RSP = respirable suspended particle; SBP = systolic blood pressure.



**Figure 2** Relative Change in %FMD<sub>max</sub> (Post-Exposure Minus Pre-Exposure) in Percent Versus Dose of SHS With Linear Regression Line

The correlation coefficient (*r*) is  $-0.44$ . **Solid circles** = subject values, and the **line** = fitted values by linear regression model. Abbreviations as in Figure 1.

### Discussion

Our results demonstrate a concentration-dependent decrease in endothelium-dependent dilation of the brachial artery after a single 30-min exposure to aged SHS, extending even to the low range of SHS concentrations: for each increase in SHS exposure by  $100 \mu\text{g}/\text{m}^3$ , the absolute %FMD<sub>max</sub> was reduced by 0.67%.

We have extended to lower exposure concentrations the findings of Heiss et al. (9), who demonstrated that 30-min exposure to fresh SHS concentrations of  $367 \pm 43 \mu\text{g}/\text{m}^3$  resulted in an average absolute %FMD<sub>max</sub> value reduction of 3%, comparable with the reduction in %FMD<sub>max</sub> of 2.48% at  $367 \mu\text{g}/\text{m}^3$  (95% confidence interval: 0.66% to 4.29%) we found.

Our findings are consistent with the epidemiological findings of the INTERHEART study (19), analyses of the American Cancer Society cohort (20), and meta-analyses conducted by the U.S. Surgeon General's Office on smoking and health (21). These studies showed a dose-response relationship between exposure to SHS and the risk for

cardiovascular disease, with higher levels or longer exposures leading to greater levels of risk. Epidemiological studies of ambient particulate pollution have shown that decreases as small as  $10 \mu\text{g}/\text{m}^3$  annual average concentration of fine particulate matter were associated with increased life expectancy (22). This result is consistent with our observation of no threshold for the effects of SHS on endothelial function. Our research strengthens the evidence that SHS is detrimental to cardiovascular health even at very short exposures and low particulate concentrations.

We hypothesized that 1 biologic pathway for the endothelium-dependent dysfunction detected would be increased degradation of endothelium-derived nitric oxide, detected by elevated plasma nitrotyrosine levels, and by increased accumulation of the competitive inhibitor of nitric oxide synthesis ADMA. Our data did not support this hypothesis. There was no statistical difference between baseline nitrotyrosine, ADMA, and SDMA values and values 4 h after exposure. Published data correlating tobacco smoke exposure and ADMA levels have yielded conflicting

**Table 2** Effect of Exposure to Secondhand Smoke on Systemic Nitrotyrosine Levels

Nitrotyrosine Level (nmol/l)	Control (n = 11)	Lowest Dose (n = 11)	Low Dose (n = 11)	p Value
Baseline	14 (13-19)	12.5 (10-15)	12 (12-20)	<0.54
Immediately after exposure	15 (13-17)	12.5 (11-14)	13 (12-23)	<0.47
2 h after exposure	17.5 (13-22)	17 (12-48)	14 (12-21)	<0.63
4 h after exposure	21 (13-35)	18.5 (12-27)	17.5 (12-22)	<0.80

Values are median (interquartile range). Nitrotyrosine values were not normally distributed, so they were analyzed using Kruskal-Wallis 1-way analysis of variance on ranks.

**Table 3** Effect of Exposure to Secondhand Smoke on Systemic Levels of ADMA and SDMA

Variable	Control (n = 11)	Lowest Dose (n = 11)	Low Dose (n = 11)	p Value
<b>ADMA (<math>\mu\text{mol/l}</math>)</b>				
Baseline	0.44 $\pm$ 0.10	0.45 $\pm$ 0.12	0.42 $\pm$ 0.09	<0.88
Immediately after exposure	0.44 $\pm$ 0.09	0.45 $\pm$ 0.11	0.44 $\pm$ 0.08	<0.97
2 h after exposure	0.43 $\pm$ 0.08	0.45 $\pm$ 0.10	0.43 $\pm$ 0.10	<0.82
4 h after exposure	0.44 $\pm$ 0.07	0.44 $\pm$ 0.11	0.44 $\pm$ 0.09	<0.99
<b>SDMA (<math>\mu\text{mol/l}</math>)</b>				
Baseline	0.46 $\pm$ 0.09	0.48 $\pm$ 0.12	0.47 $\pm$ 0.09	<0.93
Immediately after exposure	0.74 $\pm$ 0.09	0.49 $\pm$ 0.12	0.49 $\pm$ 0.09	<0.91
2 h after exposure	0.47 $\pm$ 0.10	0.40 $\pm$ 0.12	0.49 $\pm$ 0.09	<0.73
4 h after exposure	0.47 $\pm$ 0.09	0.49 $\pm$ 0.13	0.48 $\pm$ 0.09	<0.88

Values are mean  $\pm$  SD and were analyzed using 1-way analysis of variance.

ADMA = asymmetric dimethyl-L-arginine; SDMA = symmetric dimethyl-L-arginine.

results, complicated by varied study populations and exposures (23–25).

**Study limitations.** Because we exposed subjects briefly to low concentrations of SHS, it is possible that increases in intracellular nitrotyrosine and ADMA in endothelial cells were not large enough to spill over into systemic circulation and be detected in plasma. Our sample size had power to detect ADMA changes  $>0.22 \mu\text{mol/l}$ . In daily life, non-smokers may be exposed to SHS for much longer, and the time course of change in ADMA and nitrotyrosine is not known. Our subjects were at rest. During exercise, exposure to SHS is higher because of increased minute ventilation. Our subjects were healthy and may have been less susceptible to decrements in endothelial function than patients with vascular disease who are at greatest risk for SHS-induced acute cardiovascular events.

## Conclusions

Short-term exposure to low levels of SHS for 30 min results in a concentration-dependent decrease in endothelial function, a key mechanism for all stages of atherosclerosis, making policies to limit SHS exposure at low concentrations important.

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**Key Words:** endothelium ■ flow-mediated dilation ■ secondhand smoke.