Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Association of serum aryl hydrocarbon receptor activity and RBC omega-3 polyunsaturated fatty acids with flow-mediated dilation in healthy, young Hispanic cigarette smokers

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HIGHLIGHTS

- Flow-mediated dilation (FMD) was significantly impaired in young, healthy Hispanic cigarette smokers.
- Serum aryl hydrocarbon receptor (AHR) activity and RBC% of α -linolenic acid (ALA%) were significantly correlated with FMD in smokers in simple regression, but not in never smokers.
- Serum AHR activity, ALA%, and systolic blood pressure were significantly correlated with FMD in smokers in multivariable regression.
- Serum AHR activity and ALA% could serve as biomarkers of impaired FMD in health smokers and could be used to screen the potential cardiovascular effects of other tobacco products.

ARTICLE INFO

Article history:

Received 23 September 2014

Received in revised form 24 November 2014

Accepted 2 December 2014

Available online xxx

Keywords:

Aryl hydrocarbon receptor
Omega-3 polyunsaturated fatty acids
Cigarette smoke
Flow-mediated dilation
Endothelial dysfunction

ABSTRACT

Impaired flow-mediated dilation (FMD) occurs prior to clinical disease in young cigarette smokers. We investigated two potential biomarkers of FMD: serum aryl hydrocarbon receptor (AHR) activity and RBC omega-3 polyunsaturated fatty acids in healthy young Hispanic cigarette smokers. We recruited never ($n=16$) and current ($n=16$) Hispanic smokers (32 ± 7 years old), excluding individuals with clinical cardiovascular disease. We measured FMD with duplex ultrasound, RBC fatty acids and serum AHR activity using a luciferase reporter assay. FMD was significantly impaired in smokers ($5.8 \pm 4\%$) versus never smokers ($12.3 \pm 7.4\%$, $p=0.001$). Serum AHR activity was significantly increased in smokers (1467 ± 358 relative light units (RLU)) versus never smokers (689 ± 251 RLU, $p<0.001$), and correlated positively with FMD only in smokers ($r=0.691$, $p<0.004$). RBC percentage of α -linolenic acid (ALA%) was significantly increased in smokers ($0.14 \pm 0.03\%$) versus never smokers ($0.11 \pm 0.03\%$, $p=0.018$), and correlated inversely with FMD only in smokers ($r=-0.538$, $p=0.03$). The combination of serum AHR activity, ALA%, and systolic blood pressure significantly correlated with FMD in a multivariable regression model ($r=0.802$, $p<0.008$). These results suggest that serum AHR activity and RBC ALA% could serve as biomarkers of FMD in healthy, young Hispanic cigarette smokers.

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Abbreviations: AHR, aryl hydrocarbon receptor; ALA, α -linolenic acid; CALUX, chemically activated luciferase expression; CYP, cytochrome P450; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; FMD, flow-mediated dilation; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; PAH, polycyclic aromatic hydrocarbons; PUFA, polyunsaturated fatty acid; TG, triglycerides.

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1. Introduction

Cigarette smoking is a major independent risk factor for atherosclerosis and cardiovascular diseases, including myocardial infarction, stroke, aortic aneurysm, and peripheral artery disease (Ockene and Miller, 1997; Selvin and Erlinger, 2004). Although the pathophysiology of cigarette smoke-induced cardiovascular disease is not fully understood, changes in the structure and function

<http://dx.doi.org/10.1016/j.toxlet.2014.12.002>

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of blood vessels are major contributing factors (Rahman and Laher, 2007). One early event in cigarette smoke-induced vascular injury is endothelial dysfunction, which results from the loss of endothelial-derived vasodilators. Endothelial dysfunction is a pathological precursor to many vascular diseases (Munzel et al., 2008) and has been shown to occur prior to symptoms of overt clinical disease in young cigarette smokers (Neunteufl et al., 2000; Ozaki et al., 2010).

Flow-mediated dilation (FMD) is frequently used to measure endothelial dysfunction. Studies have shown that impaired FMD is an independent predictor of future cardiovascular events in individuals without clinical cardiovascular disease or in individuals with low Framingham risk scores (Rossi et al., 2008; Shechter et al., 2009). However, FMD cannot be employed in a cost-effective manner on a wide scale basis, because equipment is costly and image acquisition is technically difficult. Thus, there is a need for a simple, reproducible and less expensive biomarker of endothelial dysfunction that could be used to identify those individuals at greatest risk.

Cigarette smoke-induced endothelial dysfunction is mediated, in part, by oxidative stress. Cigarette smoke is a source of free radicals, including superoxide anion and peroxynitrite (Pryor and Stone, 1993). In addition, cigarette smoke contains high levels of polycyclic aromatic hydrocarbons (PAHs) (Ding et al., 2005), leading to activation of the aryl hydrocarbon receptor (AHR) (Denison and Nagy, 2003; Gebremichael et al., 1996; Kasai et al., 2006; Kitamura and Kasai, 2007) and to induction of various cytochrome CYPs, including CYP1A1 and 1B1 in the liver and extra hepatic tissues (Granberg et al., 2003; Kiyohara and Hirohata, 1997). These CYPs can generate reactive oxygen species directly by the inefficient coupling of NADPH consumption to substrate oxidation (Kopf and Walker, 2010; Morel et al., 1999; Schlezinger et al., 2006; Zangar et al., 2004) and indirectly by the metabolic activation of PAHs to highly reactive diol-epoxides and dihydrodiols (Bolton et al., 2000; Shimada, 2006). Furthermore, Hispanics commonly carry the CYP1A1*2A polymorphism (Swinney et al., 2011), which results in higher levels of CYP1A1 activity, and this increased CYP1A1 activity may put them at higher risk of cigarette smoke-induced oxidative stress. Thus, the degree to which the AHR is activated and the presence of the CYP1A1*2A polymorphism could be potential predictors of endothelial dysfunction in cigarette smokers.

In addition, studies have shown that cigarette smokers have lower levels of omega-3 polyunsaturated fatty acids (PUFAs), specifically eicosapentanoic acid (EPA, 20:5n-3) and docosahexanoic acid (DHA, 22:6n-3) (Ionescu et al., 2013; Simon et al., 1996). These fatty acids are metabolized by CYPs to potent vasodilators that contribute to endothelial-mediated dilation (Wang et al., 2011). Thus, the levels of omega-3 PUFAs also could be potential biomarkers of endothelial dysfunction in cigarette smokers.

The purpose of this study was to determine the degree to which serum AHR activity and RBC omega-3 PUFAs were associated with FMD in healthy, young Hispanic current and never cigarette smokers.

2. Methods

2.1. Study subjects

We recruited age- and sex-matched, healthy never ($n = 16$) and current smokers ($n = 16$) of self-described Hispanic ethnicity. Age was restricted to between 19–50 years and current smokers were defined as having smoked >0.5 pk/d for at least the past year. Patients excluded from the study included those who were pregnant, those who had the atherosclerosis risk factors of hypertension or diabetes, and those who had a history of ischemic

heart disease, stroke, or heart failure. All subjects were questioned regarding their consumption of fish and their use of dietary supplements, including fish oil. This study was approved by the University of New Mexico Institutional Review Board (HRRC: 12-168) and written informed consent was obtained from all participants.

2.2. Study design

Subjects were asked to fast and refrain from exercise, smoking, or drinking caffeinated beverages for 10 h prior to their appointment for assessment of flow-mediated dilation (FMD). FMD was conducted between 7–10 am, in the same room, at a set temperature of 70–72 °F and was performed by the same Registered Vascular Technologist. Subjects were placed in a supine position and ECG leads were placed. Blood pressure was measured in both arms; subjects rested quietly for 15 min with the room darkened, then brachial artery diameter was imaged using a 15–7 linear array transducer in the arm with the highest systolic blood pressure. The ECG was monitored continuously and directly connected to the scanner to allow for post-exam analysis during the same phase of the cardiac cycle. Reactive hyperemia was induced by inflating a blood pressure cuff on the upper forearm to 50 mm Hg above the subject's systolic blood pressure. The cuff was inflated for 5 min and continuous scanning of the brachial artery was performed from 30 s prior to cuff release until 2 min after cuff release. Longitudinal view diameter measurements were recorded and the peak percent change in brachial artery diameter from baseline to the point of greatest diameter during the hyperemic phase was used as a measure of FMD. Measurements were conducted by a vascular surgeon that was blinded to the patient group.

Following assessment of FMD, blood samples were taken to measure cotinine, HbA1c, lipids (VAP test, AtheroTech, Birmingham, AL), AHR activation potential in serum, RBC omega-3 and omega-6 PUFAs (OmegaQuant, Souix Falls, SD), and cytochrome P4501A1 (CYP1A1) and CYP1B1 mRNA and presence of CYP1A1*2A polymorphism. To assess serum AHR activation potential, we used a recombinant cell-based bioassay, termed CALUX (chemically activated luciferase expression), to detect the ability of serum to induce a luciferase reporter construct requiring AHR activation (He et al., 2008; Ziccardi et al., 2000). Human hepatoma cells (HepG2) stably transfected with an AHR-luciferase reporter were treated with benzo(a)pyrene (10^{-8} M) as a positive control or 25% serum from never or current smokers for 24 h, and luciferase expression measured (Promega, Madison, WI). To assess CYP1A1 and 1B1 mRNA expression, RNA was isolated from whole blood (PAXgene RNA kit, Qiagen) and analyzed by qPCR and normalized to RNA polymerase 2 as described previously (Kopf and Walker, 2010). To determine the CYP1A1*2A genotype, DNA was isolated from whole blood (PAXgene kit, Qiagen) and analyzed by a PCR/restriction digest method as described (Jarvis et al., 2010). The wild type genotype was identified by a single 340 bp band; the heterozygous genotype was identified by the presence of a 340 bp band (wild type allele) and two bands of 200 and 140 bp resulting from MspI digestion (variant allele); and the homozygous variant identified by presence of only the 200 and 140 bp bands.

2.3. Statistical analysis

We expected that serum AHR activity and n-3 PUFAs would correlate with FMD in all subjects and thus our objective was to have adequate power to detect a relationship in multivariate regression analyses. We estimated needing a sample size of 36 to achieve 80% power to detect a correlation ($r = 0.35$) in multivariable regression model with three predictors and $\alpha = 0.05$. Data are

expressed as mean $\pm$ standard deviation (SD) and were compared between never and current smokers using a paired *t*-test when the data were normally distributed and a Wilcoxon Signed Rank test when the data were not normally distributed. In addition, we tested categorical variables with a chi square test. Based on our hypothesis that serum AHR activity and n-3 PUFAs would be predictive of FMD, we conducted correlation analysis of FMD versus serum AHR activity, CYP1B1 mRNA, ALA%, DHA%, or EPA%, using the Pearson correlation test. We also conducted correlation analysis of FMD versus the individual factors used to calculate the Framingham risk score, including age, total cholesterol, high density lipoprotein, and systolic blood pressure, using the Pearson correlation test. Since we found that correlation of FMD with serum AHR activity and ALA% was statistically significant for each variable alone, we conducted multivariate regression analysis for FMD versus both serum AHR activity and ALA% (Model #1). Finally, we added individual factors used to calculate the Framingham risk score to the multivariate regression Model #1 to assess the degree to which they improved prediction of FMD. $P < 0.05$ was considered statistically significant.

3. Results

The cigarette smokers recruited for this study were in good cardiovascular health, failing to exhibit any significant changes in classic cardiovascular disease risk factors, including blood pressure, triglycerides (TG), and low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol (Table 1). In addition, total cholesterol was significantly lower in smokers than never smokers. The Framingham risk score, an estimate of the 10-year risk of developing coronary heart disease, was significantly elevated in smokers solely as a result of their smoking. However, the score of 6.8% represented a low risk of developing coronary heart disease in the next ten years.

Cigarette smokers exhibited significantly impaired FMD, compared to never smokers (Fig. 1, $p = 0.001$), while baseline diameter of the brachial artery prior to reactive hyperemia did not differ between groups (never smokers, 3.78 ± 0.12 ; smokers, 3.98 ± 0.12 ; $p > 0.2$).

We also assessed AHR-dependent luciferase expression from serum and CYP1A1 and 1B1 mRNA expression from WBCs, representing indices of exposure to PAHs. Cigarette smokers exhibited significantly higher levels of AHR-dependent luciferase expression when serum was applied to the CALUX assay ($p < 0.001$, Fig. 2A). Smokers also exhibited significantly higher levels of CYP1B1 mRNA in WBCs ($p < 0.017$, Fig. 2B). CYP1A1 mRNA was detectable from only 1 of 17 smokers and none of the never smokers (data not shown). Smokers tended to have a higher

Fig. 1. Change in brachial artery diameter as a percent of baseline resulting from flow-mediated dilation (FMD). Cigarette smokers exhibited significantly impaired FMD. Data are expressed as mean $\pm$ SD of never smokers and current smokers ($n = 16/\text{group}$).

frequency of carrying one CYP1A1*2A variant allele (never smokers, 20%; current smokers, 60%), but this was not significantly different, and no individuals were identified with two CYP1A1*2A variant alleles. Further, the presence of the CYP1A1*2A polymorphism did not correlate with serum AHR activation potential in the CALUX assay.

Cigarette smokers also exhibited significant differences in the levels of two omega-3 PUFAs; α -linolenic acid (ALA, 18:3n-3) and DHA, expressed as a percentage of total RBC fatty acids. ALA% was significantly increased in smokers ($p < 0.018$), while DHA% was significantly decreased ($p < 0.018$, Fig. 3A and B). There was no significant difference in the percentage of EPA in RBCs (Fig. 3C). We also failed to find any differences in the percentages of omega-6 PUFAs, including linoleic acid (18:2n-6) and arachidonic acid (20:4n-6), and failed to find any differences in fish meals consumed per week or fish oil supplement usage.

In correlation analysis, we failed to find any association between FMD and RBC fatty acids, CYP1B1 mRNA expression or serum AHR-activated luciferase expression when all subjects were included. However, we did find significant associations when considering smokers alone. We found that AHR-dependent luciferase expression correlated positively and significantly with FMD in smokers ($r = 0.691$, $p < 0.004$), but showed no relationship to FMD in never smokers ($r = -0.184$, $p = 0.530$; Fig. 4A and B). In addition, we found that ALA% in RBCs correlated inversely and significantly with FMD in smokers ($r = -0.538$, $p = 0.03$), but showed no relationship to FMD in never smokers ($r = 0.09$, $p = 0.75$; Fig. 5A and B). There was no association between FMD and other omega-3 PUFAs, including EPA and DHA, nor was there a correlation between ALA and AHR-dependent luciferase expression. Finally, none of the individual factors used to calculate the Framingham risk score showed a significant correlation with FMD.

Multivariate regression analysis was used to develop a model to predict FMD. There was no significant association found when all factors used to calculate the Framingham risk score were included in the model (data not shown). However, a significant association was found when using AHR-dependent luciferase expression and ALA% as independent variables (Table 2). Furthermore, the addition of systolic blood pressure (model #2) was the only Framingham risk score variable that increased both *R* and adjusted *R*² values.

Table 1
Characteristics of study subjects.

Characteristic	Never smoker	Current smoker	P-value
Male/female, <i>n</i>	8/8	8/8	1.000
Age (yr)	30.6 $\pm$ 6.6	32.1 $\pm$ 6.8	0.066
Cotinine (mg/dL)	1.1 $\pm$ 0.4	100 $\pm$ 62	<0.001
HbA1c (%)	5.3 $\pm$ 0.2	5.3 $\pm$ 0.2	0.520
Systolic BP (mmHg)	122 $\pm$ 11	125 $\pm$ 16	0.555
Diastolic BP (mmHg)	78 $\pm$ 10	83 $\pm$ 12	0.303
BMI (kg/m ²)	31 $\pm$ 11	27 $\pm$ 4	0.250
Total cholesterol (mg/dL)	198 $\pm$ 28	173 $\pm$ 31	0.014
LDL (mg/dL)	117 $\pm$ 31	100 $\pm$ 31	0.088
HDL (mg/dL)	58 $\pm$ 23	50 $\pm$ 12	0.194
TG (mg/dL)	120 $\pm$ 44	136 $\pm$ 93	0.575
Framingham risk score (%) ^a	0.6 $\pm$ 4.1	6.5 $\pm$ 3.9	<0.001

^a Estimate of the 10-year risk of developing coronary heart disease taking into account age, sex, total cholesterol, HDL cholesterol, blood pressure, treatment for hypertension, and smoking. A value <10% is considered low risk.

Fig. 2. Serum AHR activation potential and WBC CYP1B1 mRNA expression, representing indices of PAH exposure. Current smokers exhibited significantly higher (A) AHR-dependent luciferase expression resulting from serum applied to CALUX assay and (B) CYP1B1 mRNA expression. Data are expressed as mean ± SD of never smokers and current smokers ($n = 16/\text{group}$). rPOL2, RNA polymerase 2.

4. Discussion

In this study we found that FMD was significantly impaired in healthy, young Hispanic male and female cigarette smokers, compared to never smokers, confirming numerous previous reports of impaired FMD in healthy young smokers of other ethnicities (Celermajer et al., 1993; Neunteufl et al., 2000; Ozaki et al., 2010; Siasos et al., 2013). However, our data showed for the first time that serum AHR-dependent luciferase expression and RBC ALA% were independent predictors of FMD in healthy, young Hispanic cigarette smokers. Furthermore, the best prediction of FMD in smokers was observed when serum AHR activity, ALA%, and systolic blood pressure were used as independent variables in a multivariable regression model.

Cigarette smoking is the primary route of exposure to PAHs, which are formed from the incomplete combustion of organic material (Ding et al., 2005; Suwan-ampai et al., 2009). Thus, it is not surprising that current smokers have significantly higher serum AHR activity than never smokers. The serum AHR activity measured from never smokers likely results from the “background” exposure to AHR active chemicals from outdoor air pollution and dietary sources. Interestingly, while serum AHR activity was not associated with FMD in never smokers, it was positively associated with FMD in current smokers, suggesting that smokers with the worst FMD responses had lower serum AHR activity. This finding was unexpected, but would make sense if PAH metabolism is considered. PAHs bind the AHR with high affinity, resulting in its nuclear translocation and activation of gene expression via binding to dioxin response elements (core

sequence: 5′-GCGTG-3′) in the promoters of gene transcriptionally regulated by the AHR, including CYP1A1 and CYP1B1. These P450s subsequently metabolize PAHs to highly reactive products, leading to the formation of reactive oxygen species and vascular injury (Bolton et al., 2000; Ramos and Moorthy, 2005; Shimada, 2006). Thus, it has been shown that PAH toxicity occurs secondary to metabolism and the levels of unmetabolized parent PAHs are inversely related to P450 activity (Uno et al., 2006; Uppstad et al., 2010). Consequently, the AHR-dependent luciferase expression in a recombinant cell-based bioassay would represent as a surrogate index of unmetabolized PAHs from the serum of smokers (Han et al., 2004; He et al., 2008; Ziccardi et al., 2000). Thus, we speculate that smokers with the worst FMD responses have higher rates of PAH metabolism and lower levels of parent PAHs in the serum.

Cigarette smoking also has been shown to significantly reduce long chain omega-3 PUFAs, EPA and DHA, and DHA specifically has been shown to correlate with FMD in healthy young cigarette smokers (Leeson et al., 2002). Further, omega-3 PUFA supplements containing EPA and DHA have been shown to significantly improve endothelial function in healthy young cigarette smokers (Din et al., 2014; Siasos et al., 2013). We observed a significant decrease in RBC DHA% in current smokers consistent with previous studies; however, we did not find an association between DHA% and FMD. The reason for this is not clear. While the levels of DHA can be influenced by dietary intake and use of fish oil supplements, we did not find that dietary intake or supplement use differed in our cohort of smokers. It is possible that our small sample size precluded detecting a relationship between these two variables.

Fig. 3. Omega-3 PUFAs expressed as a percentage of total fatty acids in RBCs. Cigarette smokers exhibited significantly (A) higher RBC percentage of ALA and (B) lower RBC percentage of DHA. There was no difference in RBC percentage of EPA between groups. Data are expressed as mean ± SD of never smokers and current smokers ($n = 16/\text{group}$).

Fig. 4. Relationship between AHR-dependent luciferase expression and FMD. There was no correlation between AHR-dependent luciferase expression and FMD in never smokers (A, $r = -0.184$, $p = 0.530$). In contrast, there was a significant positive correlation between AHR-dependent luciferase expression and FMD in current smokers (B, $r = 0.691$, $p < 0.004$). Dotted lines represent 95% confidence interval around the regression line.

In addition to the decrease in RBC DHA%, we found a significant increase in RBC ALA% in smokers. Furthermore, we observed that RBC ALA% was significantly and inversely associated with FMD. ALA is a medium chain omega-3 PUFA that can be converted to longer chain omega-3 PUFAs, EPA and DHA, via desaturase and elongase enzymes, although this conversion is inefficient in humans. One previous study has reported that ALA% is increased in smokers (Simon et al., 1996) and a number of studies have reported that cigarette smoke inhibits the activities of the desaturase enzymes, slowing the conversion of ALA to EPA and DHA (Ghezzi et al., 2007; Marangoni et al., 2004). Our data show that individuals with the worst FMD responses have a higher percentage of RBC ALA. This would be consistent with an inhibition of ALA conversion to longer chain omega-3 PUFAs and subsequently lower levels of DHA. The relationship between ALA and cardiovascular disease risk is mixed, however. High dietary linolenic acid intake (combination of both ALA and γ -linolenic acid, 18:3n-6) has been reported to have cardiovascular protective effects, including being associated with a lower prevalence of coronary artery disease, lower prevalence of carotid plaques, and lower plasma TG (Djousse et al., 2001, 2003a,b). In contrast, higher RBC ALA% was associated with a significantly higher risk of sudden myocardial infarction (Lemaitre et al., 2009). The reasons for this disparity are not known. It is possible that the higher incorporation of ALA into cell membranes may reflect poor conversion into longer chain omega-3 PUFAs, while higher dietary intake may reflect better bioavailability of omega-3 PUFAs in general.

Fig. 5. Relationship between RBC percentage of ALA and FMD. There was no correlation between ALA% and FMD in never smokers (A, $r = 0.09$, $p = 0.75$). In contrast, there was a significantly inverse correlation between ALA% and FMD in current smokers (B, $r = -0.538$, $p = 0.03$). Dotted lines represent 95% confidence interval around the regression line.

4.1. Study limitations

The cross-sectional design of this study prevents us from establishing a cause-and-effect relationship between these potential blood-based biomarkers and impaired FMD. In addition, we only measured FMD, which represents a combination of the release of vasodilators from the endothelium and the response of the smooth muscle to these vasodilators. Thus, we did not establish that impaired FMD in our cohort of cigarette smokers resulted from endothelial dysfunction. Nonetheless, many previous studies in healthy young cigarette smokers have established that impaired FMD results from endothelial dysfunction and is independent of smooth muscle responses. Additionally, we used serum AHR-dependent luciferase expression as an index of unmetabolized PAHs in the systemic circulation, but we did not directly measure PAHs, PAH metabolites or P450-dependent

Table 2
Multiple regression models for FMD in current cigarette smokers.

Model	B coefficient $\pm$ SE	P-value	R, adjusted R ² values
Model #1		0.013	R = 0.718, adj R ² = 0.434
Intercept	4.1 $\pm$ 6.0	0.681	
Serum AHR activity	0.006 $\pm$ 0.002	0.036	
ALA%	-4621 $\pm$ 2573	0.098	
Model #2		0.008	R = 0.802, adj R ² = 0.545
Intercept	-5.7 $\pm$ 7.3	0.451	
Serum AHR activity	0.006 $\pm$ 0.002	0.021	
ALA%	-6455 $\pm$ 2486	0.025	
Systolic BP	0.10 $\pm$ 0.05	0.073	

activity. Lastly, our data are limited to a small group of healthy, young Hispanic cigarette smokers without clinical cardiovascular disease. It remains unknown whether these potential biomarkers of FMD are correlated with FMD in individuals with other cardiovascular disease risk factors, such as hypertension and hyperlipidemia.

5. Conclusions

FMD is an independent predictor of future cardiovascular events in individuals without clinical cardiovascular disease. Our results suggest that the ability of serum to activate the AHR and the percentage of ALA in RBC have the potential to serve as independent biomarkers of impaired FMD in healthy cigarette smokers. These biomarkers could be used by regulatory agencies to screen the potential cardiovascular disease risk posed by use of other tobacco products or possibly by occupational or environmental exposure to PAHs. Additional studies are warranted to confirm these associations in larger population samples and in multiple ethnic groups as well as to determine if these represent potential biomarkers of cardiovascular risk in individuals with clinical cardiovascular disease.

Conflict of interest

No conflict of interests declared.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

Acknowledgments

This research was supported by the National Center for Research Resources and the National Center for Advancing Translational Sciences of the National Institutes of Health through Grant Number 8UL1TR000041. The authors would like to thank the nursing and administrative staff of the University of New Mexico Health Sciences Center, Clinical and Translational Science Center, and William Goldstein of the University of New Mexico Hospital Vascular Lab for their expert assistance.

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