Columbia University Human Subjects Study Description Data Sheet

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Principal Investigator:	Sanja Jelic (527)

Study Description

A. Study Purpose and Rationale

A growing body of data indicates that vascular endothelial dysfunction plays an important role in the cardiovascular complications associated with exposure to cigarette smoke. Endothelial dysfunction may explain in part the increased risk of cardiovascular diseases associated with exposure to cigarette smoke. We have developed a new minimally invasive technique of endothelial harvesting from a superficial forearm vein that allows safe collection of endothelial cells (ECs) in human subjects. The well demonstrated capacity to study freshly harvested ECs will allow a better understanding of the pathobiology of cardiovascular complications associated with exposure to cigarette smoke. We hypothesize that increased endothelial oxidative stress, inflammation, and impaired endothelial repair capacity contribute to vascular endothelial dysfunction and thereby to alterations in vasomotor tone and reactivity in habitual smokers (HS) and healthy nonsmokers acutely exposed to secondhand cigarette smoke (SHS). To address this hypothesis we are proposing the following 2 specific aims:

Aim 1. To assess the level of endothelial oxidative stress, inflammation, and NO availability in habitual smokers (HS) and healthy nonsmokers acutely exposed to secondhand cigarette smoke (SHS). We propose to compare levels of oxidative stress, inflammation, and NO availability in both venous and arterial endothelium of HS and acute SHS. The hypothesis: Isoprostane plasma levels, nitrotyrosine (N) formation, and cyclooxygenase-2 (COX-2) expression are increased, and NO synthesis/release and flow-mediated vasodilation (FMD) are reduced in HS when compared to healthy nonsmokers, and acute exposure to SHS causes similar alterations in healthy nonsmokers.

Aim 2. To assess vascular remodeling by quantifying the degree of apoptosis and endothelial repair capacity in HS and healthy nonsmokers acutely exposed to SHS. We propose to quantify the degree of apoptosis and endothelial repair capacity in HS and acute SHS. The hypothesis: endothelial cell-derived CD31+/annexinV+ apoptotic microparticles and percentage of apoptotic nuclei per total nuclei in harvested ECs are increased and circulating EPCs quantity and/or activity is decreased in HS when compared to healthy nonsmokers, and acute exposure to SHS causes similar alterations in of healthy nonsmokers.

Secondhand smoke (SHS) increases the risk of heart disease by approximately 30%, accounting for at least 35 000 deaths annually in the United States [11,20]. Protection of nonsmokers through smoke-free environments leads to a decrease in cardiovascular mortality [22]. The dose of smoke delivered to active smokers is 100 times or more that delivered to a passive smoker; however, the relative risk of coronary heart disease for smokers is 1.78, compared with 1.31 for passive smokers [18]. The precise molecular links between the exposure to cigarette smoke and the increased risk for cardiovascular diseases are not clearly understood.

Endothelial activation and dysfunction associated with exposure to cigarette smoke may trigger the process of accelerated atherogenesis. Thirty minutes of SHS exposure impairs endothelium-dependent vasodilation in coronary arteries of nonsmokers almost to the same extent as seen in habitual smokers [23]. Cigarette smoke extract increases generation of reactive oxygen species (ROS), especially superoxide anions [27]. Superoxide rapidly scavenges nitric oxide (NO), generating peroxynitrate, a toxic metabolite that nitrosylates proteins on tyrosine residues forming nitrotyrosine [14]. NO is essential for endothelium dependent vasodilation and reduced NO bioavailability is the basis for endothelial dysfunction [13]. NO suppresses atherosclerosis by reducing endothelial cell activation, smooth muscle proliferation, leukocyte activation and leukocyte-endothelial interactions, and platelet aggregation and adhesion [2]. Decreased basal nitric oxide release in hypercholesterolemia increases neutrophil adherence to rabbit coronary artery endothelium [19, 25]. Light (<1 pack per week) and heavy (1 pack per week) smokers have similarly decreased levels of endothelial NO, suggesting that cigarette smoke has an effect at low exposure that saturates at high exposures [3]. SHS reduces endothelium-dependent vasodilatation of the brachial artery in healthy nonsmokers in a dose-dependent manner [5]. Smoking a single cigarette rapidly reduces combined concentrations of nitrate and nitrite and concentrations of antioxidants in plasma [31]. Reduced NO bioavailability associated with cigarette smoke exposure may result in a proatherogenic state. Adequate endogenous NO production may be an important protective mechanism against smoking-induced endothelial apoptosis. Endothelin NOS (eNOS) pre-activation by L-arginine reduced cigarette smoke induced apoptosis, and eNOS inhibition has been shown to be accentuated in cultured human aortic endothelial cells [28]. Cigarette smoke-induced increased endothelial apoptosis would require adequate endothelial repair capacity to maintain adequate endothelial homeostasis. Endothelial progenitor cells (EPCs) are bone-marrow-derived cells that enter the systemic circulation to replace defective or injured mature endothelial cells. EPCs also contribute to neovascularization and limit the progression of atherosclerosis. Patients with reduced EPC levels or dysfunctional EPCs are at increased risk for coronary artery disease. Smoking is inversely correlated with the number and functional activity of circulating EPCs [12,30]. Cotinine, a metabolite of nicotine, suppresses the growth of EPCs at concentrations equivalent to its serum levels in smokers, and smoking cessation rapidly increases levels of circulating EPCs in chronic smokers [16,29]. The possible impact of SHS on the mobilization and the circulating levels of EPCs is an important issue that needs to be addressed.

Endothelium mediates several other physiological and pathological processes besides NO-mediated control of the vasomotor tone. Oxidative stress, such as observed in habitual smokers and SHS, activates cultured endothelial cells by inducing nuclear translocation of the transcription factor NF-kappaB [4, 17]. In turn, NF-kappaB promotes the expression of several proinflammatory genes, including cyclooxygenase2 (COX-2) [1, 10]. These nonvasomotor functions of the vascular endothelium are not routinely characterized in subjects exposed to cigarette smoke, primarily because of limited access to the vascular endothelium.

Our preliminary data show that a novel technique of minimally invasive vascular endothelial cells harvesting can be safely performed in human subjects. Measurements of protein and gene expression indicating level of oxidative stress can be reliably performed in 1,000-3,000 endothelial cells collected from superficial forearm vein [21, 15].

We propose to directly assess the level of endothelial oxidative stress, inflammation, and NO availability in habitual smokers and healthy nonsmokers acutely exposed to SHS (specific aim 1), and to assess vascular remodeling by quantifying the degree of apoptosis and endothelial repair capacity in habitual smokers and healthy nonsmokers acutely exposed to SHS (specific aim 2).

B. Study Design and Statistical Analysis

Overview: The major goal of the present proposal is to assess the level of oxidative stress, inflammation, NO availability, the degree of apoptosis, and endothelial repair capacity in habitual smokers and healthy nonsmokers acutely exposed to SHS. To address this previously unexplored question we propose the following experiments: (Please refer to Figure 1, attached document)

Study participants: Thirty habitual smokers and 30 age and gender-matched healthy nonsmokers will be prospectively recruited for this study. We anticipate 5% drop-out rate in each group which leaves 28 patients available for analysis in

each group. All attempts will be made to recruit disparate ethnic minorities and both genders into this study. All enrolled subjects will give written informed consent in accordance with the regulations of the Columbia University institutional ethical review board. All enrolled subjects will undergo a complete history and physical examination by the physician investigators. Blood samples will be collected for lipid levels measurement from all study participants, and sent to off-site laboratory (QUEST Diagnostics, NJ). Study participants medical insurance will not be charged for this test. Demographic data including age, gender, BMI, and history of smoking will be collected using standardized questioner.

Inclusion criteria for the habitual smokers group: active self-reported cigarette smoking of at least 10 cigarettes per day for at least 10 years.

Exclusion criteria for the habitual smokers group: a history of stroke, pharmacologically treated depression, chronic obstructive or restrictive lung disease, overweight or obesity (BMI>25), diabetes mellitus, abnormal plasma lipid levels, cardiovascular disease including hypertension, and subjects receiving any medications or nutritional supplements such as antioxidants.

Inclusion criteria for the healthy nonsmokers group: never smoked, no exposure to SHS at home or workplace.

Exclusion criteria for the healthy nonsmokers group: same as for the habitual smokers group.

Rationale: We aim to assess the direct effect of cigarette smoke on endothelial function in humans. Habitual smokers and healthy nonsmokers should be free of any condition that could potentially affect endothelial function.

Identification of subjects: Patients will be recruited by standard advertising within Columbia University.

Method of data collection: Two healthy nonsmokers will sit with 1 habitual smoker who will smoke 3 cigarettes (Marlborough Light), each cigarette containing 1.0 mg of nicotine, consecutively within 30 minutes in a 5 x 7 x 5 feet car (owned by the PI) parked just outside the Columbia Presbyterian Medical Center, or at PH10 in the laboratory of Dr. Herbert Kleber located just outside of the Columbia Presbyterian Medical Center. This habitual smoker will not be enrolled into the study and will serve as a "smoking generator".

ECs harvesting, blood sample collection, and FMD will be performed in the morning in fasting subjects to exclude circadian variation in measured parameters. All experimental procedures will be repeated 2 hours and 24 hours after a 30-minute exposure to SHS in all healthy nonsmokers. Experimental procedures will not be repeated in habitual smokers. All sample collection will be performed in the Cardiovascular Sleep and Ventilatory Disorders Laboratory at Columbia University. FMD and EndoPAT® measurements will be performed in the Echocardiography and Vascular Ultrasound Laboratory at Columbia University. Each venous endothelial biopsy will be taken from a different antecubital vein on the same arm. FMD and EndoPAT® will be performed on the opposite arm. The order in which each subject will undergo FMD, venous endothelial harvesting, and EndoPAT® measurements after exposure at 2 hrs and 24 hours will be randomized. [EndoPAT® FDA approval and directions are attached as separate documents.]

Confirmation of cigarette smoke exposure will be assessed by measuring urine cotinine. Urine samples will be collected from HS and healthy nonsmokers before SHS exposure, and 2 hours and 24 hours after a 30-minute exposure to SHS in all healthy nonsmokers. Urine cotinine levels will be measured by gas chromatography-mass spectrometry (GC-MS) as an assessment of smoking exposure. Samples will be sent to Clinical Pharmacology Laboratory, UCSF, San Francisco, CA [5, 26].

Study Design and Procedures

A 1. Specific Aim 1. To assess the level of endothelial oxidative stress, inflammation, and NO availability in habitual smokers (HS) and healthy nonsmokers acutely exposed to secondhand cigarette smoke (SHS).
A 2. Experiments

A 2. 1. Assessment of the endothelial oxidative stress and NO synthesis
Endothelial cells harvesting: A 20-gauge angiocath will be inserted into a superficial forearm vein. Under sterile conditions, 5 J-shaped vascular guide wires (Arrow) will be sequentially advanced into the vein a few centimeters. Tips of the wires will be removed and washed in ECs dissociation buffer kept at 4°C. One half of the harvested sample will be used for immunohistochemistry and the other half for real-time PCR analysis. Potential complications (e.g., pain, phlebitis, infection, thrombosis) will be assessed by history and physical examination at a 1-wk clinical follow-up.

Immunohistochemistry for protein expression. ECs will be recovered by centrifugation and fixed with 3.7% formaldehyde in PBS for 10 min, washed twice with PBS, transferred to poly-L-lysine coated slides (Sigma Chemical), and air dried at 37°C. The slides will be stored at -80°C until analysis. ECs will be permeabilized in PBS/0.5% Triton X-100. Non-specific sites will be blocked with PBS-5% donkey serum. ECs will be incubated with monoclonal antibodies against nitrotyrosine (Upstate Biotechnology), COX-2 (Cayman Chemicals), and eNOS (Transduction Laboratories) followed by Cy3-conjugated donkey anti-mouse antibodies (Jackson ImmunoResearch Laboratories). Appropriate negative control slides will be generated using preimmune IgG. Polyclonal anti-von Willebrand factor antibodies (DAKO) will then be used, followed by FITC-conjugated secondary antibodies. Nuclei will be stained with diaminophenylindole (DAPI) (Molecular Probes). Between experiments variability will be standardized using reference slides of HUVECs obtained from the same culture dish. Slides from patients will be stained concurrently with one slide of HUVECs. ECs will be observed with a fluorescent microscope using identical conditions. Nuclear and von Willebrand factor staining will identify ECs. Slides will be systematically read left to right and top to bottom. Twenty-five consecutive ECs will be analyzed from each slide. The number of positive (bright) intracellular pixels will be quantified using commercially available software, and normalized to reference HUVEC slides to calculate pixel ratios (arbitrary units) [32].

Real-time PCR analysis for mRNA. Harvested endothelial sample will be exposed to magnetic beads (Dynal Biotech) coated with a mouse monoclonal antibody specific for ECs (P1H12) (Chemicon International). ECs will attach to the beads by binding to the antibody and non-endothelial cells will be removed by three washes with saline as previously described [6,8]. Endothelial mRNA will be linearly amplified using the RiboAmp HS RNA Amplification kit (Arcturus). Agilent Bioanalyzer will be used to demonstrate purity and integrity of amplified RNA. The amplified RNA will then be then subjected to real-time PCR analysis of eNOS and COX-2 transcripts using the ABI Prism 7900 HT sequence detection system (Perkin Elmer). Beta-actin will be used as an endogenous control.

Isoprostane plasma levels. Five ml of venous blood will be collected in the vacutainer containing EDTA 1 mg/ml after the insertion of the angiocath. 8-Isoprostane will be determined in plasma by ELISA (Cayman Chemical, Ann Arbor, MI, USA) using a purification step performed according to the manufacturer's instructions.

A 2. 2. Assessment of the NO availability
Flow-mediated dilation (FMD). Vascular response in the brachial artery will be assessed by FMD according to the guidelines of the International Brachial Artery Reactivity Task Force [6]. Brachial artery diameter will be measured in the opposite arm in which the endothelial harvesting was performed, using a 15 MHz linear array ultrasound transducer connected to a Hewlett-Packard Sonos 5500 ultrasound machine. Brachial artery diameter will be measured at rest and during peak hyperemia for 2 minutes after a 5-minute occlusion of arterial flow. The percent change from baseline diameter to maximum diameter after hyperemia will be calculated as an index of FMD. Equipment and technical support will be provided by Dr. Shunichi Homma (Director of the Echocardiography and Vascular Ultrasound Laboratory, at Columbia Presbyterian Medical Center).

EndoPAT®, Itamar Medical Inc. EndoPAT®, is a noninvasive measurement of endothelial function by determining peripheral arterial tone. It uses pulse wave analysis that allows the accurate recording of peripheral pressure waveforms and pulse wave velocity from the index finger, to provide information about arterial stiffness. It is non user dependent which has been FDA cleared. Each subject will be asked to place one index finger on the EndoPAT® probe for 2-3 minutes for measurement of peripheral arterial tone [25]. [EndoPAT® FDA approval and directions are attached as separate documents.]

A 3. Outcome measurements
Intracellular pixel ratios of eNOS, COX-2 and nitrotyrosine, relative copy number of mRNA for eNOS and COX-2 to betaactin, isoprostane plasma levels, and percent change in brachial artery FMD will be compared between HS and healthy nonsmokers before SHS exposure. All healthy nonsmokers will have repeat analysis of both protein and gene expression, and FMD measurements after a 30-minute exposure to SHS, and their baseline and post-SHS exposure measurements will be compared using Student's t-test.

A4. Anticipated results and experimental concerns
Endothelial oxidative stress as quantified by nitrotyrosine formation and COX-2 expression is increased, and NO bioavailability measured by eNOS expression and FMD is decreased in HS compared with healthy nonsmokers before SHS exposure. Arterial NO availability measured by FMD is reduced in HS compared with healthy nonsmokers before SHS exposure. After a 30-minute exposure to SHS, healthy nonsmokers develop endothelial alterations similar to those observed in HS. If the hypothesis of the present proposal proves not to be correct, other mechanisms will be investigated to explain the detrimental effect of cigarette smoke on vascular endothelium in humans. In this respect we will use spared amplified mRNA from this project to study the effect of cigarette smoke on the expression of other relevant genes. ECs will be harvested from venous endothelium. Venous sampling allows serial measurements with minimal hazard and discomfort to patients. We recognize potential difference in arterial and venous ECs activation after cigarette smoke exposure and acknowledge that characterization of arterial ECs could be important. However, cannulation of the radial or brachial artery could not be justified in the absence of a clinical indication due to the risks of thrombosis and chronic reduction in lumen diameter. We strongly believe that analysis of protein and gene expression in freshly isolated human venous ECs provides useful mechanistic insights to the pathophysiology of endothelial dysfunction. 'Low-pressure' veins are chronically exposed to the same circulating levels of pro-inflammatory factors as the arterial compartment.

B 1. Specific Aim 2. To assess vascular remodeling by quantifying the degree of apoptosis and endothelial repair capacity in HS and healthy nonsmokers acutely exposed to SHS.
B 2. Experiments
Blood sample collection. Thirty-five mL of venous blood will be withdrawn for a forearm superficial vein via the angiocath inserted for the endothelial harvesting [Angiocath insertion directions attached as separate document]. Twenty-five mL of blood will be used for quantification of EPCs. Plasma will be separated by centrifugation of the remaining 10 mL of blood and used for quantification of EAMs.

B 2.1. Assesment of endothelial apoptosis
Immunohistochemistry for ECs apoptosis. Venous ECs will be collected, fixed, and permeabilized as described above. Apoptotic ECs will be analyzed by TdT-mediated dUTP nick end labeling (TUNEL) assay and fluorescence microscopy [7]. Nuclear and von Willebrand factor staining will identify ECs as described above. The number of TUNEL-positive ECs will be scored in 10 randomly chosen high-power fields.

Flow cytometry for circulating EAMs. Plasma derived from 10-mL of blood will be centrifuged at 2000 rpm for 6 minutes to generate platelet-poor plasma. Fifty µL of plasma will be incubated with 4 µL of PE-conjugated monoclonal antibody against CD31 (Becton Dickinson) followed by incubation with fluorescein isothiocyanateconjugated annexin V (Sigma). IgG 2a-fluorescein isothiocyanate (Pharmingen) will serve as a negative control. Data will be gated using sizing of microparticles, and 20 000 events will be collected in the gated region for each sample. FACSCalibur flow cytometer (BD Biosciences) and CellQuest Software will be used for data acquisition. Population of CD31+/annexin V+ microparticles smaller than 1.5 µm will be expressed as the number of EAMs per µL of platelet poor plasma.

B 2.2. Assesment of endothelial repair capacity
Flow cytometry for circulating EPCs. Mononuclear cells will be isolated by density-gradient centrifugation with Ficoll (Sigma) from 25 mL of peripheral blood and counted using a Coulter Counter (Beckman Coulter). One million mononuclear cells will be aliquoted and incubated with 15 µL mouse serum (Sigma-Aldrich) at room temperature to block nonspecific binding of antibodies. Mononuclear cells will be incubated for 30 minutes in the dark with monoclonal antibodies against human KDR (PE-conjugated), and for 10 minutes with CD34 (FITC-labeled), and

CD133 (APC-labeled). Isotype-identical antibodies will serve as controls. Data will be gated on the mononuclear population during data acquisition, and 20 000 events will be collected in the gated region for each sample. FACSCalibur flow cytometer and CellQuest Software will be used for data acquisition [12]. The percent of KDR+/CD34+/CD133+ cells will be expressed as the percent of the total mononuclear cells.

Colony-forming assay of EPCs. In order to determine functional capacity of circulating EPCs, we will measure the number of colony-forming units of EPCs. The remaining mononuclear cells which are not used in the above flow cytometry analysis will be incubated in EPCs growing media (GIBCO BRL Life Technologies). Cells will be resuspended in media, plated at a density of 5x106 per well on dishes coated with human fibronectin (BIOCOAT, Becton Dickinson), and incubated at 37°C in humidified 5% CO2. After 48 hours, nonadherent cells suspended in the growth media will be replated onto fibronectin-coated 24-well plates at a density of 106 per well. Media will be changed every 3 days, and EPCs colony-forming units, defined as a central core of rounded cells surrounded by elongating and spindle-shaped cells, will be counted after 7 days in culture. Cell clusters alone without emerging spindle cells will not be counted as positive. Colonies will be counted in a minimum of 4 wells of a 24-well plate and averaged [24].

B 3. Outcome measurements
The quantity of EPCs colony-forming units, KDR+/CD34+/CD133+ cells and CD31+/annexin V+ microparticles, and percentage of apoptotic nuclei per total nuclei in harvested ECs will be compared between HS and healthy nonsmokers before SHS exposure. All healthy nonsmokers will have repeat measurements after a 30-minute exposure to SHS, and their baseline and post-SHS exposure measurements will be compared using Student's t-test.

B4. Anticipated results and experimental concerns
Circulating EAMs and percentage of apoptotic nuclei per total nuclei in harvested ECs are increased, and circulating EPCs quantity and activity is decreased in HS compared with healthy nonsmokers before SHS exposure. After a 30-minute exposure to SHS, healthy nonsmokers develop alterations in endothelial homeostasis similar to those observed in HS. We recognize that the degree of apoptosis in arterial ECs would be more relevant to determine the mechanisms responsible for cardiovascular complications after cigarette smoke exposure. However, similar concerns as described above apply. If no difference in the degree of apoptosis and levels of circulating EPCs levels is observed in HS compared with healthy nonsmokers before SHS exposure or after a 30-minute exposure to SHS, such negative findings may be explained by the need for a longer cigarette smoke exposure time, which would be explored in a future study.

Statistical Analysis
A. Descriptive Statistics. The outcome measurements are: intracellular pixel ratios of eNOS, COX-2 and nitrotyrosine; relative copy number of mRNA for eNOS and COX-2 to betaactin; isoprostane plasma levels; percent change in brachial artery FMD; the quantity of EPCs colony-forming units, KDR+/CD34+/CD133+ cells, and CD31+/annexin V + microparticles; percentage of apoptotic nuclei per total nuclei in harvested ECs. Continuous variables will be summarized by the mean, median, standard deviation, and range. Categorical variables will be summarized by frequencies with 95% confidence intervals.

B. Bivariate Comparison of HS to healthy nonsmokers before SHS exposure. We will compare each dependent variable (all outcome measurements) between HS and healthy nonsmokers before SHS exposure. If the outcome variables are normally distributed, we will use unpaired Student's t-tests to compare the groups. If the data are not normally distributed, they will be transformed to meet these assumptions. If this does not achieve normality, we will use Wilcoxon rank sum tests. Pearson's correlation coefficients and Spearman rank correlation coefficients will be used as appropriate.

C. Bivariate Analysis of healthy nonsmokers before and after SHS exposure. Each healthy nonsmoker will be his or her own control before and after SHS exposure. We will use paired Student's t tests to assess whether each dependent variable changes from baseline to follow-up after SHS exposure. If these data are not normally distributed, we will use sign rank tests.

D. Multivariate Predictors of Endothelial Function in healthy nonsmokers before and after SHS exposure. We will

construct linear regression models with the post-SHS exposure outcome measurements as dependent variables and the following as independent variables: age, gender, and BMI. Models will contain up to three variables to avoid overfitting.

E. Sample Size. We have used Bonferroni's method to account for the multiple hypotheses we are testing, setting alpha= 0.007 for each hypothesis, and therefore making alpha= 0.05 for the entire study. We have set ß= 0.10 (or 90% power).
HS to healthy nonsmokers before SHS exposure. Based on our experience, we anticipate 1-2 eligible new subjects each week during the period of recruitment (60 subjects). We anticipate a drop-out rate of 5%, leaving 56 to participate. Our preliminary studies have shown a greater than 1 standard deviation (SD) difference in protein expression and FMD between other patient population (patients with obstructive sleep apnea) and healthy subjects. Therefore, we will have more than adequate power to detect these differences (Table 1, please see attached document). For example, the mean ± SD eNOS expression in OSA is 0.74±0.24 while in healthy subjects is 0.40±0.24. We will have > 90% power to detect this difference. The mean±SD FMD is 4.4±3.1% in HS and 3.1±2.7% in healthy subjects after chronic SHS from previously published data [5]. Even if we have a higher drop-out rate than anticipated (10%, for example, leaving only 52 patients for inclusion), we will still have > 90% power to detect this clinically important difference.

Healthy nonsmokers before and after SHS exposure. We presume that 30 healthy nonsmokers will consent for the study, and 28 of them will complete the baseline and follow-up assessments (assuming 5% drop-out rate). The mean ±SD change in FMD after SHS exposure is approximately 5.1±3.1% [5]. Assuming that drop-out rate subjects will not change in terms of outcome measures from baseline to follow-up, we will have 90% power to detect a mean change of approximately 0.7% between baseline and follow-up assessment of FMD (corresponding to a 0.5 SD difference). Assuming that patients who drop out (and therefore are unavailable for follow-up) have a substantial decrease (worsening) of their FMD values, giving a mean SD change of 0.6 %, we will still have > 80% power to detect this difference. We are therefore adequately powered to detect clinically significant differences despite very conservative assumptions.

Study Drugs: Not applicable.

Medical Devices: The devices used in the study include a 20 gauge angiocath which is commercially available, sphygmomanometer, and 0.018 in. diameter J shaped wire (Arrow, Reading, PA). EndoPAT® which is commercially available (Itamar Medical Inc., 160 Speen Street, Suite 201,Framingham, MA 01701, USA)

Study Participants:
See above

Recruitment: Participants will be recruited from flier as per included sample.

Confidentiality of Study Data: All collected data for this study will be confidential and confidentiality will be ensured with an assigned unique code for each venous endothelial cell sample, such that each sample cannot be associated with the participants by anyone other than the investigator. No collected sample will have any other direct identifier (i.e. participant name). All data collected will be stored in a password protected database (Microsoft Access/Excel) with paper records and data storage disk locked in a facility only accessible by the investigator.

Potential Conflict of Interest: None of the investigators or the University have a proprietary interest in a device or the procedure used in this study, nor will any investigation member benefit financially in any other way from the results of this study.

Location of the Study: All sample collection will be performed in the Cardiovascular Sleep and Ventilatory Disorders Laboratory at Columbia University located in PH8 Center. FMD and EndoPAT® measurements will be performed in the Echocardiography and Vascular Ultrasound Laboratory at Columbia University located in PH3. Quantitative

immunofluorescence and gene expression analysis of the harvested endothelial cells, will be performed in Dr. Phillip Factor's laboratory P&S 8 by the physician investigator.

Potential Risks: Potential risks incurred during the study include inflammation, swelling, redness, pain and thrombosis at the local area of the venous instrumentation. All participants included in the study will be assessed for potential complications at 1 week clinical follow up after vascular endothelial biopsy and appropriate treatment will be administered if necessary. All participants will be educated about possible risks and given a telephone number to contact in case any complications from the instrumentation should arise. Overall we have performed more than 100 blood and venous endothelial cell collections and no complications were observed at clinical follow-up appointments, even when the procedure was repeated twice in the same patient at a 4-day interval in a previous study by our lab. There are no known associated risks from vascular ultrasound of the brachial artery.

Potential Benefits: Potential direct benefits from the participant include health screening and blood pressure evaluation before venous instrumentation. Potential direct benefits to society include the practice of a novel technique that will augment research and understanding of vascular endothelial cell function in humans who actively smoke cigarettes or are exposed to cigarette smoke. This will in turn, will more importantly, augment knowledge of on the mechanisms of cardiac and vascular risk factors of smoking which may lead to development of novel diagnostic and therapeutic pathways to improve earlier detection, prevention, treatment and further research on smoking related vascular and cardiac disease.

Alternatives: Not applicable.

Compensation to Participant: Participants will be paid $40 cash after vascular endothelial cell collections to cover the costs of transportation and/or parking.

Cost to Participants: Participants will incur no additional costs as a result of participation in the study

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