BMJ Open

Assessing the Impact of Tobacco-Induced Volatile Organic Compounds on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to Tobacco Study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-019850
Article Type:	Protocol
Date Submitted by the Author:	05-Oct-2017
Complete List of Authors:	Keith, Rachel; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Fetterman, Jessica; Boston Medical Center, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center Shah, Jasmit; University of Louisville; American Heart Association- Tobacco Regulation and Addiction Center O'Toole, Timothy; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Nystoriak, Jessica; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Holbrook, Monika; Boston Medical Center, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center Lorkiewicz, Pawel; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Bhatnagar, Aruni; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center DeFilippis, Andrew; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Hamburg, Naomi ; Boston University, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center
Keywords:	smoking, tobacco, electronic cigarette, cardiovascular risk, vascular injury, cigarettes

Assessing the Impact of Tobacco-Induced Volatile Organic Compounds on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to Tobacco Study

Rachel J. Keith, Jessica L. Fetterman, Daniel W. Riggs, Tim O'Toole, Jessica Nystoriak, Monica Holbrook, Pawel Lorkiewicz, Aruni Bhatnagar, Andrew DeFilippis*, Naomi M. Hamburg*

Rachel J. Keith-Division of Cardiovascular Medicine, University of Louisville School of Medicine 580 S. Preston St. Louisville KY, 40202 rachel.keith@louisville.edu 502-852-4211

Jessica L. Fetterman- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston University School of Medicine Evans Building, Boston, MA USA

Jasmit Shah- University of Louisville School of Medicine Louisville, KY USA

Timothy O-Toole- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Jessica L. Nystoriak- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Monika Holbrook- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston University School of Medicine Boston, MA USA

Pawel Lorkiewicz- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Aruni Bhatnagar- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Andrew P. DeFilippis- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA (co-senior author)

Naomi M. Hamburg- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston University School of Medicine Boston, MA USA (co-senior author)

Word Count: 2581

ABSTRACT

Introduction: Tobacco use leads to increased mortality, the majority of which is attributed to cardiovascular disease. Despite this knowledge, the early cardiovascular impact of tobacco product use is not well understood. Tobacco use increases exposure to harmful and potentially harmful constituents including volatile organic compounds (VOCs) such as acrolein and crotonaldehyde, which may contribute to cardiovascular risk. The link between exposure patterns, risk profiles and demographic distribution of tobacco product users, particularly users of new and emerging products, are not well known. Therefore, we designed the Cardiovascular Injury due to Tobacco Use (CITU) study to assess population characteristics, demographic features, exposure patterns and cardiovascular risk in relation to tobacco.

Methods and analysis: This is a cross-section observational study conducted in Boston MA and Louisville KY from 2014 through 2018. Healthy participants 21 to 45 years of age who use tobacco products, including ENDS, or who never used tobacco are being recruited. The study aims to recruit an evenly split cohort of African Americans and Caucasians that is sex balanced for evaluation of self-reported tobacco

exposure, VOC exposure and tobacco-induced injury profiling. Detailed information about participant's demographics, health status and lifestyle is also collected.

Ethics and dissemination: The study protocol was approved institutional review boards at both participating universities. All study protocols will protect participant confidentiality. Results from the study will be disseminated via peer-reviewed journals and presented at scientific conferences.

Strengths and limitations

- Young age to allow for evaluation of early stage disease (e.g. inflammation, endothelial function) as opposed to end stage clinical consequence (e.g. myocardial infarction)
- Diverse tobacco product use allows for assessment of a wide range of tobacco-induced VOC exposure
- All study visits are in English introducing selection bias
- Data will inform regulatory agencies on the cardiovascular health effects of multiple tobacco products and the contribution of HPHCs

Keywords: Tobacco, smoking, electronic cigarette, vascular injury, cardiovascular risk, cigarettes.

INTRODUCTION

Tobacco product use and smoking are the leading causes of preventable deaths throughout the world. Of those deaths, one-third are attributed to cardiovascular disease

(CVD) [1]. The cardiovascular (CV) effects of tobacco exposure can include atherogenesis, vascular injury, thrombosis, arrhythmias and inflammation [2] and may be attributable to the many different harmful and potentially harmful constituents (HPHCs) present in tobacco products.

The HPHCs found in tobacco products include volatile organic compounds (VOCs) of which reactive aldehydes, such as acrolein and crotonaldehyde, are likely the most significant contributors to CV toxicity [3]. High levels of aldehydes are present in cigarette smoke [4 5] as well as smokeless tobacco (ST) [6]. Risk assessments, using the prevalence of each individual chemical weighed by its potency, suggest that the non-cancer risk of smoking is dominated by acrolein, which contributes 40-100 times more to risk than any other chemical present in cigarette smoke [3].

Although HPHCs, including VOC reactive aldehydes, have been suspected to be major contributors to the toxicity of cigarette smoke for over 4 decades, their contribution to CV injury and early CVD risk has not been rigorously evaluated. Experimental studies in animal models suggest that because of low aldehyde-metabolizing capacity, CV tissues are highly sensitive to aldehydes and exposure to low levels of aldehydes can induce CV injury and accelerate CVD [7-19]. The WHO Study Group on Tobacco Product Regulation (TobReg) has marked acrolein, a VOC, along with 8 other cigarette constituents for monitoring and regulation [20] and the U.S. Environmental Protection Agency lists Acrolein as one of most hazardous air pollutants[21]. Nevertheless, the contribution of tobacco induced VOCs, including acrolein or other aldehydes, toward CV toxicity in humans has not been fully assessed. Greater understanding of how aldehydes affect cardiovascular health and disease will provide

new avenues for evaluating the toxicity of cigarette smoke and for assessing the injurious potential of new and emerging tobacco products, such as ENDS, which may also contain VOCs including acrolein [22-24].

The latency period between tobacco exposure and the development of major clinical adverse health effects is long, therefore biomarkers that provide information over a shorter period allow for the identification of harm decades before clinical outcome data is available. Thus, the Cardiovascular Injury due To Tobacco Use (CITU) study evaluates the association of the urinary metabolites of 18 parent VOCs from tobacco exposure with a comprehensive set of CV biomarkers representative of early disease and predictive of future CV events.[25]

METHODS AND DESIGN

Overall design

The CITU study is an investigator-initiated cross-sectional observational study of around 500 healthy participants 21 to 45 years of age who are never or current tobacco product users in two urban areas at Boston University (BU) and University of Louisville (UofL) (Boston, MA and Louisville, KY) designed to evaluate CV toxicity due to tobacco product use, with correlations to VOCs found in the tobacco products (Figure 1).

Figure 1. Cardiovascular Injury due to Tobacco Use

CITU is designed to assess how tobacco related VOC exposure contributes to cardiovascular risk factors. Our exposure measurements include a panel of 23 urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular phenotyping

includes measures of injury, risk, vascular biomarkers and early vascular dysfunction. Tobacco use included use of traditional cigarettes, smokeless tobacco, waterpipe tobacco (hookah), electronic nicotine devices (ENDS), little cigars, cigarillos, pipes, cigars or any other form of tobacco that is available. Enrollment began in July 2014 and is ongoing.

Participant Eligibility Criteria

The goal of the study is to examine the impact of tobacco products on healthy young adults who could be classified as a current tobacco product users (Defined in table 1), or never-users (does not have lifetime use of any tobacco product); therefore we excluded participants if they had: 1) diagnosis of diabetes (HbA1c >7.0 or treatment for diabetes), hypertension (systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg), hypothyroidism or hyperthyroidism, inflammatory conditions such as lupus or inflammatory bowel disease, HIV/AIDS, hepatitis, liver disease, anemia, cancer of any type or another medical condition that might compromise the successful completion of the study; 2) recipients of organ transplant or renal replacement therapy; 3) individuals that are taking the following medications: immunosuppressant agents estrogen, testosterone, anti TNF agents, certain biologics, Procrit, statins, beta-blockers or other cardiovascular medicine; 4) individuals using nutraceuticals or anabolic steroids beyond the recommended daily allowance; 5) body weight less than 100 pounds; 6) pregnant women; 7) prisoners and other vulnerable populations; and 8) active illness or infection. Participants are rescheduled or considered screen-failures and excluded from the study if symptomatic of an acute illness, i.e. viral upper respiratory infection, on study date.

Table 1. Tobacco product use classifications

Classification	Qualification
Never	Does not meet lifetime limits for any tobacco use (see below)
Smoker	>100 lifetime cigarettes and current use for the past year
Smokeless Tobacco User	>20 lifetime dips or chews and current use for the past year
Cigar/Cigarillo User	>20 lifetime cigars or cigarillos and current use for the past year
Pipe User	>20 lifetime pipefuls and current use for the past year
ENDS User	>20 lifetime vape sessions and current use for the past year
Hookah User	>20 lifetime hookah sessions and current use for the past year

Study participants are screened prior to enrollment for current and past tobacco product use. Participants are characterized and assigned a use group based on self-reported patterns collected during the study visits.

Overall Study Procedure

Study participants fast for 8 h from food and 6 h from tobacco prior to the visit. All study visits occur before 11AM to limit effects due to circadian changes. All vascular function studies are completed after 10 min of supine positioning. All vascular studies are sent to the BU central lab for analysis. BU biologic samples have minimal processing and are shipped overnight to the UofL central laboratory at the completion of each study visit. Samples obtained at UofL are processed to a similar stage, then held overnight prior to analysis for standardization of time to measurement for all samples.

Study visits include a structured interview on demographics, socioeconomics, lifestyle, health, family history of heart disease, allergies, and tobacco use. All surveys are collected and kept in Research Electronic Data Capture (REDCap), a secure web application for building and managing online surveys and databases.

Exposure Variables

Tobacco Product Use & Particulate Matter Exposure

Comprehensive tobacco product exposure is assessed using a modified version of the National Health Interview survey on tobacco use [26]. The survey is modified to include detailed information on electronic nicotine devices (ENDs) and other new or emerging tobacco products. Residential addresses are collected for assessment of ambient airborne particulate matter ($PM_{2.5}$) exposure and future correction of overall exposure. $PM_{2.5}$ data from the day of the study visit, and 3 and 5 days prior to the study is collected from publicly available data associated with EPA monitoring stations. Other exposure variables, including occupation, are collected through interview.

VOC Measurements

Standard clean catch urine specimens are obtained from participants. We have developed a robust Core Lab that utilizes mass spectrometry procedures adopted from the Centers for Disease Control and Prevention (CDC) protocols, to quantify 23 urinary metabolites of tobacco smoking related toxins (aldehydes and other VOCs), including acrolein[27] (**Table 2**). The concentration values of analytes are then normalized to urinary creatinine levels measured using Infinity Creatinine Reagent (Thermo Fisher Scientific, MA) on a COBAS MIRA-plus analyzer (Roche, NJ).

Table 2 Exposure Variables (Please see end of article)

Parent compound	***VOC metabolite***	***Common abbr.***
Acetaldehyde	Acetic acid/Acetate	ACETATE
Acrolein	N-Acetyl-S-(2-carboxyethyl)-L-cysteine	CEMA
	N-Acetyl-S-(3-hydroxypropyl)-L-cysteine	3HPMA
Acrylamide	N-Acetyl-S-(2-carbamoylethyl)-L-cysteine	AAMA

	N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	GAMA
Acrylonitrile	N-Acetyl-S-(2-cyanoethyl)-L-cysteine	CYMA
Acrylonitrile, vinyl chloride, ethylene oxide	N-Acetyl-S-(2-hydroxyethyl)-L-cysteine	HEMA
Anabasine	Anabasine (free)	ANB
Anatabine	Anatabine (free)	ANTB
Benzene	N-Acetyl-S-(phenyl)-L-cysteine	PMA
	trans, trans-Muconic acid	MU
1-Bromopropane	N-Acetyl-S-(n-propyl)-L-cysteine	BPMA
1,3-Butadiene	N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine	DHBMA
	N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine	MHBMA1
	N-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine	MHBMA2
	N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine	MHBMA3
Carbon-disulfide	2-Thioxothiazolidine-4-carboxylic acid	TTCA
Crotonaldehyde	N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine	HPMMA
Cyanide	2-Aminothiazoline-4-carboxylic acid	ATCA
N,N-Dimethylformamide	N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine	AMCC
Ethylbenzene, styrene	Phenylglyoxylic acid	PGA
Formaldehyde	Formate	FORMATE
Nicotine	Nicotine	NIC
	Cotinine	COT
	3-Hydroxycotinine	3HC

Propylene oxide	N-Acetyl-S-(2-hydroxypropyl)-L-cysteine	2HPMA
Styrene	N-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine + N-Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine	PHEMA
	Mandelic acid	MA
Tetrachloroethylene	N-Acetyl-S-(trichlorovinyl)-L-cysteine	TCVMA
Toluene	N-Acetyl-S-(benzyl)-L-cysteine	BMA
Trichloroethylene	N-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine	1,2DCVMA
	N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine	2,2DCVMA
Xylene	N-Acetyl-S-(2,4-dimethylphenyl)-L-cysteine + N-Acetyl-S-(2,5-dimethylphenyl)-L-cysteine + N-Acetyl-S-(3,4-dimethylphenyl)-L-cysteine	DPMA
	2-Methylhippuric acid	2MHA
	3-Methylhippuric acid + 4-Methylhippuric acid	3MHA+ 4MHA

Urine is analyzed for 23 metabolites of 18 parent VOCs and tobacco alkaloids by UPLC-MS/MS. Analytes are listed as parent, metabolite and their common abbreviation.

Circulating Markers of Cardiovascular Injury

To assess tobacco product-induced cardiovascular toxicity, we examine endothelial function, inflammatory mediators, biomarkers, and thrombosis. CV risk is defined through measurements of circulating angiogenic cells, lipid profile, and glucose metabolism [25 28 29]. Plasma (BD367863 and BD366415) and serum (BD367814) samples are obtained from all participants for laboratory testing and long term

biobanking. Whole blood (BD366415) is obtained for flow cytometry on fresh samples at UofL pathology core. BU biologic samples have minimal processing and are shipped overnight to the UofL central laboratory at the completion of each study visit. Samples obtained at UofL are processed to a similar stage, then held overnight prior to analysis to standardize the time to measurement for all samples. The UofL central laboratory, as previously reported, will complete fasting and biomarker measurements (**Table 3**), with the exception of cytomics [13 30]. For cytomic measurements, mononuclear cells are labeled with the peripheral blood phenotyping panel kit (Fluidigm).Samples are shipped at 4 degree C to Core Lab facilities at the University of Rochester for Mass cytometric analysis.

Table 3 Blood analysis

Fasting Measurements
LDL cholesterol, HDL cholesterol, total cholesterol, triglycerides, glucose, uric acid, SAA and fibrinogen
Biomarkers
CAC (1-15)[1], Platelet-monocyte aggregates, MP (1-5)[1], PF4, t-PA, TxA2, Factor VII, IL-6, CRP, D-dimer, PAI-1, s-ICAM-1, s-VCAM, s-thrombomodulin, s-TNFR1, MMP-2, MMP-3, MMP-9, cytomics, endothelin, E-selectin and P-selectin
1: Fifteen different CAP subpopulations and 5 subtypes of microparticles were measured by flow cytometry.

All participants who complete the study visit will have blood samples taken and processed. Flow cytometric analysis is completed on fresh samples. All other analysis will be completed on biobanked samples in batches LDL= low density lipoprotein. HDL= high density lipoprotein. SAA= serum amyloid A. CAC= circulating angiogenic cells. MP= microparticles. PF4= Platelet factor 4. t-PA= tissue plasminogen activator. TxA2=Thromboxane A. IL-6= Interleukin 6. CRP= C-reactive protein. PAI-=- Plasminogen activator. s- ICAM- soluble intercellular adhesion protein inhibitor. s-

VCAM= soluble vascular adhesion protein. TNFR1= Tumor necrosis factor receptor 1. MMP- Matrix metalloproteinase.

Non-Invasive Vascular Function Testing

Smoking, is associated with endothelial damage and vascular dysfunction [31 32]. Endothelial cells are exposed to circulating toxins and measures of endothelial function are reflective of cardiovascular injury [33]. Thus, we examine the non-invasive endothelial vasodilator function using flow-mediated vasodilation [34 35], arterial stiffness with carotid-femoral and carotid-radial pulse wave velocity [36], and peripheral vascular function with ankle brachial index. All vascular imagers where trained at BU. Similar equipment and software is used at both sites. All vascular studies are sent to the BU central lab for analysis.

Anthropometric measures

Anthropometric measures included height, weight, waist and hip circumference and body fat. All anthropometric measures are completed twice and the average recorded. Standing height measurements are completed on a fixed stadiometer. Weight measurements are completed on a digital scale to the nearest tenth of a pound. Waist circumference is measured at the level of the umbilicus to the nearest tenth of a centimeter. Hip circumference is measured at the maximal protrusion of the gluteal muscle to the nearest tenth of a centimeter. Body fat percentage is calculated by the bioelectrical impedance measured with the Omron fat loss monitor (HBF-306C).

DATA ANALYSIS

We expect that from this study we will be able to identify specific biomarkers of cardiovascular injury due to tobacco use and the relationship of these biomarkers to

specific measures of tobacco exposure. For instance, we will identify which biomarkers are affected by tobacco use, and which ones are most sensitive; including their dose-dependence. Additionally we will examine the extent to which biomarkers are associated with exposure to nicotine versus exposure to HPHC of tobacco like aldehydes.

All statistical analysis will be performed using SAS version 9.4 software (SAS Institute, Inc., Cary, North Carolina), and a two-sided p-value of <0.05 will be considered significant for any statistical test. Demographics and other baseline characteristics will be summarized according to product group. The primary outcomes will be analyzed using multiple regression techniques. Appropriate Interaction variables will be tested for in the regression models and subgroup analyses will be conducted according to the following factors: significant interactions, sex, age, race, tobacco product group. Multiple imputation method will be used for missing data where appropriate. Sensitivity analysis using different analytic approaches, such as generalized linear models, as well as considering different covariate adjustments, will be used to build concordant results.

The dose-dependence of the changes in biomarkers will be determined by analyzing the data obtained from individuals that are exposed to different doses of a single product (e.g. smoking 0, <15, 15-20 and >20 cigarettes per day) and by comparing between tobacco products that have different doses of HPHC constituents. In the US the average cigarettes per day is between15-20 [37] and therefore this dose range distribution is reflective of general population exposure. Comparisons of the effects of novel tobacco products and smoking will be informative of the relative toxicity of the two products.

We believe that the methods employed in the current project are exquisitely sensitive and responsive to even low dose insults such as ambient air pollution [13] allowing us to quantify tobacco product-induced changes with high precision. Moreover, levels of acrolein exposure vary between different individuals due to difference in puffing intensity and the time a cigarette is left smoldering. Thus, direct measurements of acrolein metabolites afford better estimates of acrolein exposure than machine yields. We expect to obtain wide variations in acrolein/crotonaldehyde exposure which will enable us to construct a dose-response relationship and identify which injury biomarkers are associated with aldehyde exposure and whether high levels of exposure are associated with high levels of injury, despite similar nicotine delivery.

We consider three major factors for balancing sample selection: age, gender, and race. Given that very few females use e-cigarette, only males will be enrolled in this group. With the balanced design to determine the main effects and interactions in selected scenarios, we justify the sample size. The analysis plan is primarily based on evaluating the effect of tobacco exposure on endothelial function (FMD), and the main biomarkers, EPCs, and platelet-monocyte aggregates (PMA). The sample size is justified in terms of the primary dependent measure, FMD, given the potential importance of this variable as a direct measure of the impact of tobacco exposure. The main comparisons are between non-tobacco users and tobacco users. Due to one control group, we will conservatively adjust our α (significance level) using a Bonferroni correction, and we will set α=0.01. Based on preliminary data for FMD, we have observed mean ± SD in smoker and nonsmoker groups to be 4.0 ±1.6 and 6.8 ±1.0, respectively. We consider at least 25% (mean FMD=3.0 from 4.0) reduction from

smokers to non-smokers is meaningful. Using a two sample, one-sided t test with an α of 0.01 and 80% power (1-β), assuming a common SD of 1.3, we will need 34 evaluable subjects in each group. To examine dose response, smokers will be recruited in 3 groups (<15, 15-20 and >20 CPD). We will recruit 40 participants in each group; total group size = 120 participants. In **Table 4** we provide estimable effect size for different outcome measures.

Table 4 Minimal Detectable Differences in Endpoints at α=0.01 and Power=80%

Variable	Non-smokers	Smokers	n	p	Ref	MDD
Primary Functional Outcome						
FMD	6.8 ± 1%	4.0 ± 1.6%	10	<0.05	[32]	1.0
Primary Biomarkers						
EPC	25 ± 5 cell/ml	10 ± 3 cells/ml	24	0.037	[38]	3.1
PMA	19.7 ± 8.6%	26.6 ± 9%	25	0.02	[39]	7.0
EMP	1.1 ± 0.4	0.5 ± 0.2	32	<0.05	[40]	0.23
Other Biochemical Variables						
PF4	3.9 ± 1.2 IU/ml	5.0 ± 2.6 IU/ml	12	<0.05	[41]	2.0
tPA	3.0 ± 0.6 ng/ml	4.3 ± 2.0 ng/ml	20	<0.05	[42]	1.6
TxA_2	2.2 ± 0.1 pg/ml	3.3 ± 0.02 pg/ml	12	<0.05	[43]	0.016

PMA: Platelet-monocyte aggregates; EMP: Endothelial microparticles (CD62+/CD31+); MDD: minimal detectable difference. Values are mean ± SD

ETHICS AND DISSEMINATION

The CITU study was approved at each institution by their institutional review board (BU #H-32613 and UofL #13.0590) and all participants provide written consent. No study related procedures will be completed until after participant consent.

Participants for the CITU study are being recruited in both Boston, MA and Louisville KY. The two populations show significant differences, therefore recruitment at two sites will ensure a range more reflective of the general population. Although overall racial and ethnic demographics for both cities show a clear majority of Caucasians (70%) and despite smokers typically male, we strive to, and currently are successful in, recruiting a population that was gender balanced and almost evenly split between Caucasian and African Americans. Despite this balanced recruitment, e-cigarette users have been reported as predominantly Caucasian and male[44], and thus far our recruitment mirrors these demographics. We expect very few Hispanic/Latino's to participate, due to data suggesting tobacco use, including ENDS, tends to be lower among Hispanic's/Latino's [44 45]. Thus we have also opted to only recruit English speakers. We have carefully develop our recruitment strategy and exclusion criteria to protect vulnerable populations, which is important since many report a lower socioeconomic status and educational level in smokers in addition to higher rates of reported alcohol and drug use [46 47].

Our study is an observational study where participants have already assumed the risk of using tobacco. Study procedures pose minimal risk. Given the known harms associated with smoking, we will provide information on tobacco treatment when requested by the participant. Participant information is de-identified for analysis and reported in aggregate to protect privacy.

Completion of these studies will enable a greater understanding of the biological responses to use of a variety of tobacco products. Specifically, they will help to identify the constituents of these products; and how a panel of exposure and CV injury biomarkers are associated with these different constituents. This data will be available to the FDA and could help guide new policy measures to reduce or eliminate the harmful components of tobacco smoke and other nicotine products. The study is dedicated to the rapid dissemination of their rigorously characterized and well-controlled research findings to the public in the form of peer-reviewed publications. Subsequent to the initial full-length manuscript publications of the resources generated with funding from this program, the study will make them available to interested and qualified investigators upon written request. The study will provide relevant protocols of published data, upon request (presuming prior publication by the Center members). Participants will be provided a summary of the results as they become available. Finally press releases of relevant findings will inform the general population.

LIST OF ABBREVIATIONS

ABI- Ankle Brachial Index

CAC= circulating angiogenic cells

CRP= C-reactive protein

CVD- Cardiovascular disease

ENDS- Electronic nicotine Device (i.e. e-cigarette)

FACS- Fluorescence-activated cell sorting

FMD- Flow mediated dilation

HDL= high density lipoprotein

IL-6= Interleukin 6

MMP- Matrix metalloproteinase

MP= micoparticles

PAI-=- Plasminogen activator

PF4= Platelet factor 4

PWV- Pulse wave velocity

SAA= serum amyloid A

s-ICAM- soluble intercellular adhesion protein inhibitor

s-VCAM= soluble vascular adhesion protein

TNFR1= Tumor necrosis factor receptor 1

t-PA= tissue plasminogen activator

TxA2=Thromboxane A

VOC- Volatile organic compound

W:H- ratio: Waist to hip ratio

AUTHORS CONTRIBUTIONS

Rachel Keith- Study design, study recruitment, study visits, statistical analysis and manuscript preparation. Jessica Fetterman- study recruitment, study visits, manuscript preparation and editing. Dan Riggs- statistical analysis, manuscript preparation and editing. Tim O'Toole- Biomarker measurements, manuscript preparation and editing. Jessica Nystoriak- study recruitment and study visits. Monica Holbrook- study recruitment and study visits. Pawel Lorkiewicz- VOC measurements and manuscript

preparation. Aruni Bhatnagar- Study design, study funding and manuscript editing. Andrew DeFilippis- Human subject assessment planning, manuscript preparation and editing. Naomi M. Hamburg- Study design, study funding, vascular core, manuscript preparation and editing.

COMPETING INTERESTS

None declared

FUNDING

This work was supported by the National Institutes of Health and the FDA Center for Tobacco Products (CTP) grant number P50HL120163.

Acknowledgements

Study design reported in this publication was supported by the AHA Tobacco Regulation and Addiction Center (A-TRAC) and FDA Center for Tobacco Products (CTP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the Food and Drug Administration.

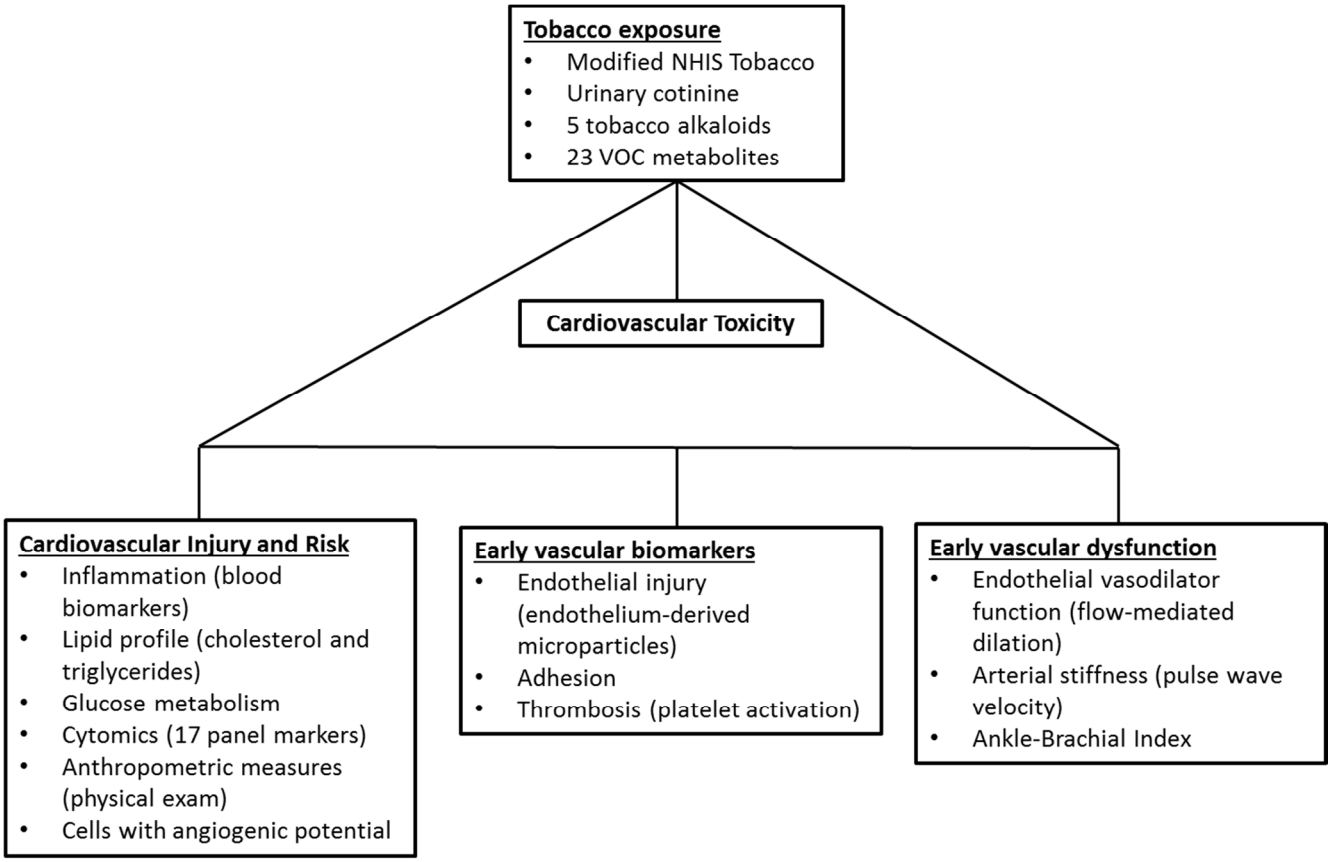
REFERENCES

1. Rostron B. Smoking-Attributable Mortality by Cause in the United States: Revising the CDC's Data and Estimates. Nicotine Tob Res 2013;**15**(1):238-46.
2. Morris PB, Ference BA, Jahangir E, et al. Cardiovascular Effects of Exposure to Cigarette Smoke and Electronic Cigarettes: Clinical Perspectives From the Prevention of Cardiovascular Disease Section Leadership Council and Early Career Councils of the American College of Cardiology. J Am Coll Cardiol 2015;**66**(12):1378-91.
3. Haussmann HJ. Use of hazard indices for a theoretical evaluation of cigarette smoke composition. Chem Res Toxicol 2012;**25**(4):794-810.
4. Ghilarducci DP, Tjeerdema RS. Fate and effects of acrolein. Rev Environ Contam Toxicol 1995;**144**:95-146.
5. Dong JZ, Moldoveanu SC. Gas chromatography-mass spectrometry of carbonyl compounds in cigarette mainstream smoke after derivatization with 2,4-dinitrophenylhydrazine. J Chromatogr A 2004;**1027**(1-2):25-35.
6. Stepanov I, Jensen J, Hatsukami D, et al. New and traditional smokeless tobacco: comparison of toxicant and carcinogen levels. Nicotine Tob Res 2008;**10**(12):1773-82.
7. Awe SO, Adeagbo AS, D'Souza SE, et al. Acrolein induces vasodilatation of rodent mesenteric bed via an EDHF-dependent mechanism. Toxicol Appl Pharmacol 2006;**217**(3):266-76.

8. Conklin DJ, Barski OA, Lesgards JF, et al. Acrolein consumption induces systemic dyslipidemia and lipoprotein modification. Toxicol Appl Pharmacol 2010;**243**(1):1-12.
9. Conklin DJ, Bhatnagar A, Cowley HR, et al. Acrolein generation stimulates hypercontraction in isolated human blood vessels. Toxicol Appl Pharmacol 2006;**217**(3):277-88.
10. Conklin DJ, Haberzettl P, Lesgards JF, et al. Increased sensitivity of glutathione S-transferase P-null mice to cyclophosphamide-induced urinary bladder toxicity. J Pharmacol Exp Ther 2009;**331**(2):456-69.
11. Conklin DJ, Haberzettl P, Prough RA, et al. Glutathione-S-transferase P protects against endothelial dysfunction induced by exposure to tobacco smoke. Am J Physiol Heart Circ Physiol 2009;**296**(5):H1586-97.
12. Ismahil MA, Hamid T, Haberzettl P, et al. Chronic oral exposure to the aldehyde pollutant acrolein induces dilated cardiomyopathy. Am J Physiol Heart Circ Physiol 2011;**301**(5):H2050-60.
13. O'Toole TE, Hellmann J, Wheat L, et al. Episodic exposure to fine particulate air pollution decreases circulating levels of endothelial progenitor cells. Circ Res 2010;**107**(2):200-3.
14. O'Toole TE, Zheng YT, Hellmann J, et al. Acrolein activates matrix metalloproteinases by increasing reactive oxygen species in macrophages. Toxicol Appl Pharmacol 2009;**236**(2):194-201.
15. Sithu SD, Srivastava S, Siddiqui MA, et al. Exposure to acrolein by inhalation causes platelet activation. Toxicol Appl Pharmacol 2010;**248**(2):100-10.
16. Srivastava S, Sithu SD, Vladykovskaya E, et al. Oral exposure to acrolein exacerbates atherosclerosis in apoE-null mice. Atherosclerosis 2011;**215**(2):301-8.
17. Tsakadze NL, Srivastava S, Awe SO, et al. Acrolein-induced vasomotor responses of rat aorta. Am J Physiol Heart Circ Physiol 2003;**285**(2):H727-34.
18. Wang GW, Guo Y, Vondriska TM, et al. Acrolein consumption exacerbates myocardial ischemic injury and blocks nitric oxide-induced PKCepsilon signaling and cardioprotection. J Mol Cell Cardiol 2008;**44**(6):1016-22.
19. Wheat LA, Haberzettl P, Hellmann J, et al. Acrolein inhalation prevents vascular endothelial growth factor-induced mobilization of Flk-1+/Sca-1+ cells in mice. Arterioscler Thromb Vasc Biol 2011;**31**(7):1598-606.
20. WHO Study Group on Tobacco Product Regulation. Report on the scientific basic of tobacco product regulation: fourth report of a WHO study group. World Health Organ Tech Rep Ser 2012(967):1-83, 1 p following 83.
21. De Woskin R, Greenberg M, Pepelko W, et al. Toxicological review of acrolein (cas no. 107-02-08) in support of summary information on the integrated risk information system (Iris). Washington, DC: US Environmental Protection Agency 2003.
22. Kosmider L, Sobczak A, Fik M, et al. Carbonyl compounds in electronic cigarette vapors: effects of nicotine solvent and battery output voltage. Nicotine Tob Res 2014;**16**(10):1319-26.
23. Goniewicz ML, Knysak J, Gawron M, et al. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. Tob Control 2014;**23**(2):133-9.
24. Wang P, Chen W, Liao J, et al. A Device-Independent Evaluation of Carbonyl Emissions from Heated Electronic Cigarette Solvents. PLoS One 2017;**12**(1):e0169811.
25. Hill JM, Zalos G, Halcox JP, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. The New England journal of medicine 2003;**348**(7):593-600.
26. Parsons VL, Moriarity C, Jonas K, et al. Design and estimation for the national health interview survey, 2006-2015. Vital Health Stat 2 2014(165):1-53.
27. Alwis KU, Blount BC, Britt AS, et al. Simultaneous analysis of 28 urinary VOC metabolites using ultra high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI/MSMS). Analytica chimica acta 2012;**750**:152-60.

28. Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001;**89**(1):E1-7.
29. Werner N, Nickenig G. Influence of cardiovascular risk factors on endothelial progenitor cells: limitations for therapy? Arterioscler Thromb Vasc Biol 2006;**26**(2):257-66.
30. DeJarnett N, Yeager R, Conklin DJ, et al. Residential Proximity to Major Roadways Is Associated With Increased Levels of AC133+ Circulating Angiogenic Cells. Arteriosclerosis, thrombosis, and vascular biology 2015;**35**(11):2468-77.
31. Poredos P, Orehek M, Tratnik E. Smoking is associated with dose-related increase of intima-media thickness and endothelial dysfunction. Angiology 1999;**50**(3):201-8.
32. Heiss C, Amabile N, Lee AC, et al. Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production. J Am Coll Cardiol 2008;**51**(18):1760-71.
33. Flammer AJ, Anderson T, Celermajer DS, et al. The assessment of endothelial function: from research into clinical practice. Circulation 2012;**126**(6):753-67.
34. Benjamin EJ, Larson MG, Keyes MJ, et al. Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. Circulation 2004;**109**(5):613-9.
35. Vita JA. Nitric oxide-dependent vasodilation in human subjects. Methods Enzymol 2002;**359**:186-200.
36. Mitchell GF, Guo CY, Benjamin EJ, et al. Cross-sectional correlates of increased aortic stiffness in the community: the Framingham Heart Study. Circulation 2007;**115**(20):2628-36.
37. Benowitz NL, Dains KM, Dempsey D, et al. Racial differences in the relationship between number of cigarettes smoked and nicotine and carcinogen exposure. Nicotine Tob Res 2011;**13**(9):772-83.
38. Kondo T, Hayashi M, Takeshita K, et al. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. Arterioscler Thromb Vasc Biol 2004;**24**(8):1442-7.
39. Harding SA, Sarma J, Josephs DH, et al. Upregulation of the CD40/CD40 ligand dyad and platelet-monocyte aggregation in cigarette smokers. Circulation 2004;**109**(16):1926-9.
40. Gordon C, Gudi K, Krause A, et al. Circulating endothelial microparticles as a measure of early lung destruction in cigarette smokers. Am J Respir Crit Care Med 2011;**184**(2):224-32.
41. Zevin S, Saunders S, Gourlay SG, et al. Cardiovascular effects of carbon monoxide and cigarette smoking. J Am Coll Cardiol 2001;**38**(6):1633-8.
42. Newby DE, McLeod AL, Uren NG, et al. Impaired coronary tissue plasminogen activator release is associated with coronary atherosclerosis and cigarette smoking: direct link between endothelial dysfunction and atherothrombosis. Circulation 2001;**103**(15):1936-41.
43. Schmid P, Karanikas G, Kritz H, et al. Passive smoking and platelet thromboxane. Thromb Res 1996;**81**(4):451-60.
44. Schoenborn CA, Gindi RM. Electronic Cigarette Use Among Adults: United States, 2014. NCHS Data Brief 2015(217):1-8.
45. Maher J, Boysun M, Rohde K, et al. Are Latinos really less likely to be smokers? Lessons from Oregon. Nicotine & Tobacco Research 2005;**7**(2):283-87.
46. Barbeau EM, Krieger N, Soobader M-J. Working Class Matters: Socioeconomic Disadvantage, Race/Ethnicity, Gender, and Smoking in NHIS 2000. Am J Public Health 2004;**94**(2):269-78.
47. Giskes K, Kunst AE, Benach J, et al. Trends in smoking behaviour between 1985 and 2000 in nine European countries by education. Journal of Epidemiology and Community Health 2005;**59**(5):395.

Figure 1.



STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2-3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3-4
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5, 7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5, 7
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6-7
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7-12
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*7-12*
Bias	9	Describe any efforts to address potential sources of bias	7
Study size	10	Explain how the study size was arrived at	14-16
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	12-14
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	12-14
		(*b*) Describe any methods used to examine subgroups and interactions	13
		(*c*) Explain how missing data were addressed	13
		(*d*) If applicable, explain how loss to follow-up was addressed	N/A (study protocol)
		(*e*) Describe any sensitivity analyses	13
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	N/A (study protocol)
		(b) Give reasons for non-participation at each stage	N/A (study protocol)
		(c) Consider use of a flow diagram	N/A (study protocol)
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	N/A (study protocol)
		(b) Indicate number of participants with missing data for each variable of interest	N/A (study protocol)
		(c) Summarise follow-up time (eg, average and total amount)	N/A (study protocol)
Outcome data	15*	Report numbers of outcome events or summary measures over time	N/A (study protocol)
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	N/A (study protocol)
		(*b*) Report category boundaries when continuous variables were categorized	N/A (study protocol)
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A (study protocol)
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N/A (study protocol)
Discussion			
Key results	18	Summarise key results with reference to study objectives	N/A (study protocol)
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	17
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Protocol to Assess the Impact of Tobacco-Induced Volatile Organic Compounds on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to Tobacco Study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-019850.R1
Article Type:	Protocol
Date Submitted by the Author:	02-Jan-2018
Complete List of Authors:	Keith, Rachel; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Fetterman, Jessica; Boston Medical Center, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center Riggs, Daniel; American Heart Association- Tobacco Regulation and Addiction Center; University of Louisville, Medicine O'Toole, Timothy; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Nystoriak, Jessica; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Holbrook, Monika; Boston Medical Center, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center Lorkiewicz, Pawel; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Bhatnagar, Aruni; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center DeFilippis, Andrew; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Hamburg, Naomi ; Boston University, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Public health
Keywords:	smoking, tobacco, electronic cigarette, cardiovascular risk, vascular injury, cigarettes

Protocol to Assess the Impact of Tobacco-Induced Volatile Organic Compounds on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to Tobacco Study

Rachel J. Keith, Jessica L. Fetterman, Daniel W. Riggs, Tim O'Toole, Jessica Nystoriak, Monica Holbrook, Pawel Lorkiewicz, Aruni Bhatnagar, Andrew DeFilippis*, Naomi M. Hamburg*

Rachel J. Keith-Division of Cardiovascular Medicine, University of Louisville School of Medicine 580 S. Preston St. Louisville KY, 40202 rachel.keith@louisville.edu 502-852-4211

Jessica L. Fetterman- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston University School of Medicine Evans Building, Boston, MA USA

Jasmit Shah- University of Louisville School of Medicine Louisville, KY USA

Timothy O-Toole- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Jessica L. Nystoriak- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Monika Holbrook- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston University School of Medicine Boston, MA USA

Pawel Lorkiewicz- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Aruni Bhatnagar- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Andrew P. DeFilippis- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA (co-senior author)

Naomi M. Hamburg- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston University School of Medicine Boston, MA USA (co-senior author)

Word Count: 2581

ABSTRACT

Introduction: Tobacco use leads to increased mortality, the majority of which is attributed to cardiovascular disease. Despite this knowledge, the early cardiovascular impact of tobacco product use is not well understood. Tobacco use increases exposure to harmful and potentially harmful constituents including volatile organic compounds (VOCs) such as acrolein and crotonaldehyde, which may contribute to cardiovascular risk. The link between exposure patterns, risk profiles and demographic distribution of tobacco product users, particularly users of new and emerging products, are not well known. Therefore, we designed the Cardiovascular Injury due to Tobacco Use (CITU) study to assess population characteristics, demographic features, exposure patterns and cardiovascular risk in relation to tobacco.

Methods and analysis: We present the design and methodology of the CITU study a cross-section observational tobacco study conducted in Boston MA and Louisville KY starting in 2014. Healthy participants 21 to 45 years of age who use tobacco products, including ENDS, or who never used tobacco are being recruited. The study aims to recruit an evenly split cohort of African Americans and Caucasians that is sex balanced

for evaluation of self-reported tobacco exposure, VOC exposure and tobacco-induced injury profiling. Detailed information about participant's demographics, health status and lifestyle is also collected.

Ethics and dissemination: The study protocol was approved institutional review boards at both participating universities. All study protocols will protect participant confidentiality. Results from the study will be disseminated via peer-reviewed journals and presented at scientific conferences.

Strengths and limitations

- Young age to allow for evaluation of early stage disease (e.g. inflammation, endothelial function) as opposed to end stage clinical consequence (e.g. myocardial infarction)
- Diverse tobacco product use allows for assessment of a wide range of tobacco-induced VOC exposure
- All study visits are in English introducing selection bias
- Data will inform regulatory agencies on the cardiovascular health effects of multiple tobacco products and the contribution of HPHCs

Keywords: Tobacco, smoking, electronic cigarette, vascular injury, cardiovascular risk, cigarettes.

INTRODUCTION

Tobacco product use and smoking are the leading causes of preventable deaths throughout the world. Of those deaths, one-third are attributed to cardiovascular disease (CVD) [1]. The cardiovascular (CV) effects of tobacco exposure can include atherogenesis, vascular injury, thrombosis, arrhythmias and inflammation [2] and may be attributable to the many different harmful and potentially harmful constituents (HPHCs) present in tobacco products.

The HPHCs found in tobacco products include volatile organic compounds (VOCs) of which reactive aldehydes, such as acrolein and crotonaldehyde, are likely the most significant contributors to CV toxicity [3]. High levels of aldehydes are present in cigarette smoke [4 5] as well as smokeless tobacco (ST) [6]. Risk assessments, using the prevalence of each individual chemical weighed by its potency, suggest that the non-cancer risk of smoking is dominated by acrolein, which contributes 40-100 times more to risk than any other chemical present in cigarette smoke [3].

Although HPHCs, including VOC reactive aldehydes, have been suspected to be major contributors to the toxicity of cigarette smoke for over 4 decades, their contribution to CV injury and early CVD risk has not been rigorously evaluated. Experimental studies in animal models suggest that because of low aldehyde-metabolizing capacity, CV tissues are highly sensitive to aldehydes and exposure to low levels of aldehydes can induce CV injury and accelerate CVD [7-18]. The WHO Study Group on Tobacco Product Regulation (TobReg) has marked acrolein, a VOC, along with 8 other cigarette constituents for monitoring and regulation [19] and the U.S. Environmental Protection Agency lists Acrolein as one of most hazardous air pollutants[20]. Nevertheless, the contribution of tobacco induced VOCs, including acrolein

or other aldehydes, toward CV toxicity in humans has not been fully assessed. Greater understanding of how aldehydes affect cardiovascular health and disease will provide new avenues for evaluating the toxicity of cigarette smoke and for assessing the injurious potential of new and emerging tobacco products, such as ENDS, which may also contain VOCs including acrolein [21-23].

The latency period between tobacco exposure and the development of major clinical adverse health effects is long, therefore biomarkers that provide information over a shorter period allow for the identification of harm decades before clinical outcome data is available. Thus, in this paper we present the design and methodology of the Cardiovascular Injury due To Tobacco Use (CITU) study which will evaluate the association of the urinary metabolites of 18 parent VOCs from tobacco exposure with a comprehensive set of CV biomarkers representative of early disease and predictive of future CV events.[24]

METHODS AND DESIGN

Overall design

The CITU study is an investigator-initiated cross-sectional observational study of around 500 healthy participants 21 to 45 years of age who are never or current tobacco product users in two urban areas at Boston University (BU) and University of Louisville (UofL) (Boston, MA and Louisville, KY) designed to evaluate CV toxicity due to tobacco product use, with correlations to VOCs found in the tobacco products (**Figure 1**).

Participant Eligibility Criteria

The goal of the study is to examine the impact of tobacco products on healthy young adults who could be classified as a current tobacco product users (Defined in table 1), or never-users (does not have lifetime use of any tobacco product). Participants were self-reported to be healthy therefore we excluded participants if they had: 1) diagnosis of clinical cardiovascular disease including but not limited to known heart attack, peripheral artery disease, heart failure or stroke; 2) diagnosis of diabetes (HbA1c >7.0 or treatment for diabetes), hypertension (systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg), hypothyroidism or hyperthyroidism, inflammatory conditions such as lupus or inflammatory bowel disease, HIV/AIDS, hepatitis, liver disease, anemia, cancer of any type or another medical condition that might compromise the successful completion of the study; 2) recipients of organ transplant or renal replacement therapy; 3) individuals that are taking the following medications: immunosuppressant agents estrogen, testosterone, anti TNF agents, certain biologics, Procrit, statins, beta-blockers or other cardiovascular medicine; 4) individuals using nutraceuticals or anabolic steroids beyond the recommended daily allowance; 5) body weight less than 100 pounds; 6) pregnant women; 7) prisoners and other vulnerable populations; and 8) active illness or infection. Participants are rescheduled or considered screen-failures and excluded from the study if symptomatic of an acute illness, i.e. viral upper respiratory infection, on study date.

Table 1. Tobacco product use classifications

Classification	Qualification
Never	Does not meet lifetime limits for any tobacco use (see below)
Smoker	>100 lifetime cigarettes and current use for the past year
Smokeless Tobacco User	>20 lifetime dips or chews and current use for the past year
Cigar/Cigarillo User	>20 lifetime cigars or cigarillos and current use for the past year
Pipe User	>20 lifetime pipefuls and current use for the past year

ENDS User	>20 lifetime vape sessions and current use for the past year
Hookah User	>20 lifetime hookah sessions and current use for the past year

Study participants are screened prior to enrollment for current and past tobacco product use. Participants are characterized and assigned a use group based on self-reported patterns collected during the study visits.

Overall Study Procedure

Study participants fast for 8 h from food and 6 h from tobacco prior to the visit. All study visits occur before 11AM to limit effects due to circadian changes. All vascular function studies are completed after 10 min of supine positioning. All vascular studies are sent to the BU central lab for analysis. BU biologic samples have minimal processing and are shipped overnight to the UofL central laboratory at the completion of each study visit. Samples obtained at UofL are processed to a similar stage, then held overnight prior to analysis for standardization of time to measurement for all samples.

Study visits take approximately 90 minutes to complete and include a structured interview on demographics, socioeconomics, lifestyle, health, family history of heart disease, allergies, and tobacco use. (**Figure 2**) Participants were compensated appropriately for their time. All surveys are collected and kept in Research Electronic Data Capture (REDCap), a secure web application for building and managing online surveys and databases.

Exposure Variables

Tobacco Product Use & Particulate Matter Exposure

Comprehensive tobacco product exposure is assessed using a modified version of the National Health Interview survey on tobacco use [25]. The survey is modified to include detailed information on electronic nicotine devices (ENDs) and other new or

emerging tobacco products. Residential addresses are collected for assessment of ambient airborne particulate matter ($PM_{2.5}$) exposure and future correction of overall exposure. $PM_{2.5}$ data from the day of the study visit, and 3 and 5 days prior to the study is collected from publicly available data associated with EPA monitoring stations. Other exposure variables, including occupation, are collected through interview.

VOC Measurements

Standard clean catch urine specimens are obtained from participants. We have developed a robust Core Lab that utilizes mass spectrometry procedures adopted from the Centers for Disease Control and Prevention (CDC) protocols, to quantify 23 urinary metabolites of tobacco smoking related toxins (aldehydes and other VOCs), including acrolein[26] (**Table 2**). The concentration values of analytes are then normalized to urinary creatinine levels measured using Infinity Creatinine Reagent (Thermo Fisher Scientific, MA) on a COBAS MIRA-plus analyzer (Roche, NJ).

Table 2 Exposure Variables (Please see end of article)

Parent compound	***VOC metabolite***	***Common abbr.***
Acetaldehyde	Acetic acid/Acetate	ACETATE
Acrolein	N-Acetyl-S-(2-carboxyethyl)-L-cysteine	CEMA
	N-Acetyl-S-(3-hydroxypropyl)-L-cysteine	3HPMA
Acrylamide	N-Acetyl-S-(2-carbamoylethyl)-L-cysteine	AAMA
	N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	GAMA
Acrylonitrile	N-Acetyl-S-(2-cyanoethyl)-L-cysteine	CYMA

Acrylonitrile, vinyl chloride, ethylene oxide	N-Acetyl-S-(2-hydroxyethyl)-L-cysteine	HEMA
Anabasine	Anabasine (free)	ANB
Anatabine	Anatabine (free)	ANTB
Benzene	N-Acetyl-S-(phenyl)-L-cysteine	PMA
	trans, trans-Muconic acid	MU
1-Bromopropane	N-Acetyl-S-(n-propyl)-L-cysteine	BPMA
1,3-Butadiene	N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine	DHBMA
	N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine	MHBMA1
	N-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine	MHBMA2
	N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine	MHBMA3
Carbon-disulfide	2-Thioxothiazolidine-4-carboxylic acid	TTCA
Crotonaldehyde	N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine	HPMMA
Cyanide	2-Aminothiazoline-4-carboxylic acid	ATCA
N,N-Dimethylformamide	N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine	AMCC
Ethylbenzene, styrene	Phenylglyoxylic acid	PGA
Formaldehyde	Formate	FORMATE
Nicotine	Nicotine	NIC
	Cotinine	COT
	3-Hydroxycotinine	3HC
Propylene oxide	N-Acetyl-S-(2-hydroxypropyl)-L-cysteine	2HPMA
Styrene	N-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine +	PHEMA

	N-Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine	
	Mandelic acid	MA
Tetrachloroethylene	N-Acetyl-S-(trichlorovinyl)-L-cysteine	TCVMA
Toluene	N-Acetyl-S-(benzyl)-L-cysteine	BMA
Trichloroethylene	N-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine	1,2DCVMA
	N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine	2,2DCVMA
Xylene	N-Acetyl-S-(2,4-dimethylphenyl)-L-cysteine + N-Acetyl-S-(2,5-dimethylphenyl)-L-cysteine + N-Acetyl-S-(3,4-dimethylphenyl)-L-cysteine	DPMA
	2-Methylhippuric acid	2MHA
	3-Methylhippuric acid + 4-Methylhippuric acid	3MHA+ 4MHA

Urine is analyzed for 23 metabolites of 18 parent VOCs and tobacco alkaloids by UPLC-MS/MS. Analytes are listed as parent, metabolite and their common abbreviation.

Circulating Markers of Cardiovascular Injury

To assess tobacco product-induced cardiovascular toxicity, we examine endothelial function, inflammatory mediators, biomarkers, and thrombosis. CV risk is defined through measurements of circulating angiogenic cells, lipid profile, and glucose metabolism [24 27 28]. Plasma (BD367863 and BD366415) and serum (BD367814) samples are obtained from all participants for laboratory testing and long term biobanking. Whole blood (BD366415) is obtained for flow cytometry on fresh samples at UofL pathology core. BU biologic samples have minimal processing and are shipped

overnight to the UofL central laboratory at the completion of each study visit. Samples obtained at UofL are processed to a similar stage, then held overnight prior to analysis to standardize the time to measurement for all samples. The UofL central laboratory, as previously reported, will complete fasting and biomarker measurements (**Table 3**), with the exception of cytomics [12 29]. For cytomic measurements, mononuclear cells are labeled with the peripheral blood phenotyping panel kit (Fluidigm).Samples are shipped at 4 degree C to Core Lab facilities at the University of Rochester for Mass cytometric analysis.

Table 3 Blood analysis

Fasting Measurements
LDL cholesterol, HDL cholesterol, total cholesterol, triglycerides, glucose, uric acid, SAA and fibrinogen
Biomarkers
CAC (1-15)[1], Platelet-monocyte aggregates, MP (1-5)[1], PF4, t-PA, TxA2, Factor VII, IL-6, CRP, D-dimer, PAI-1, s-ICAM-1, s-VCAM, s-thrombomodulin, s-TNFR1, MMP-2, MMP-3, MMP-9, cytomics, endothelin, E-selectin and P-selectin
1: Fifteen different CAP subpopulations and 5 subtypes of microparticles were measured by flow cytometry.

All participants who complete the study visit will have blood samples taken and processed. Flow cytometric analysis is completed on fresh samples. All other analysis will be completed on biobanked samples in batches LDL= low density lipoprotein. HDL= high density lipoprotein. SAA= serum amyloid A. CAC= circulating angiogenic cells. MP= microparticles. PF4= Platelet factor 4. t-PA= tissue plasminogen activator. TxA2=Thromboxane A. IL-6= Interleukin 6. CRP= C-reactive protein. PAI-=- Plasminogen activator. s- ICAM- soluble intercellular adhesion protein inhibitor. s-VCAM= soluble vascular adhesion protein. TNFR1= Tumor necrosis factor receptor 1. MMP- Matrix metalloproteinase.

Non-Invasive Vascular Function Testing

Smoking, is associated with endothelial damage and vascular dysfunction [30 31]. Endothelial cells are exposed to circulating toxins and measures of endothelial function are reflective of cardiovascular injury [32]. Thus, we examine the non-invasive endothelial vasodilator function using flow-mediated vasodilation [33 34], arterial stiffness with carotid-femoral and carotid-radial pulse wave velocity [35], and peripheral vascular function with ankle brachial index. Flow mediated dilation was assessed with a 7.5MHZ ultrasound probe is used to image the brachial artery while a 10cm blood pressure cuff is attached

to the lower arm and a 3 lead ECG is attached to the patient. After baseline images and 10 cycles of Doppler images are captured using NIHEM R-wave triggered image capturing software, the blood pressure cuff is inflated to 200mmHg or 50mmHg higher than the systolic pressure. After the 5 minute occlusion, the cuff is released and the NIHEM software records two minutes of imaging. Images were analyzed by a single blinded analyzer using MIA vascular Research Tolls Brachial Analyzer for Research, version 6.8.5. All vascular imagers where trained at BU who have a previously reported reproducibility with intra- and inter-observer correlation coefficients of 0.98 and 0.99 for brachial diameter and 0.78 and 0.92 for FMD.[36] Similar equipment and software is used at both sites. All vascular studies are sent to the BU central lab for analysis.

Anthropometric measures

Anthropometric measures included height, weight, waist and hip circumference and body fat. All anthropometric measures are completed twice and the average recorded. Standing height measurements are completed on a fixed stadiometer. Weight measurements are completed on a digital scale to the nearest tenth of a pound. Waist circumference is measured at the level of the umbilicus to the nearest tenth of a centimeter. Hip circumference is measured at the maximal protrusion of the gluteal muscle to the nearest tenth of a centimeter. Body fat percentage is calculated by the bioelectrical impedance measured with the Omron fat loss monitor (HBF-306C).

DATA ANALYSIS

We expect that from this study we will be able to identify specific biomarkers of cardiovascular injury due to tobacco use and the relationship of these biomarkers to specific measures of tobacco exposure. For instance, we will identify which biomarkers

are affected by tobacco use, and which ones are most sensitive; including their dose-dependence. Additionally we will examine the extent to which biomarkers are associated with exposure to nicotine versus exposure to HPHC of tobacco like aldehydes.

All statistical analysis will be performed using SAS version 9.4 software (SAS Institute, Inc., Cary, North Carolina), and a two-sided p-value of <0.05 will be considered significant for any statistical test. Demographics and other baseline characteristics will be summarized according to product group. The primary outcomes will be analyzed using multiple regression techniques. Appropriate Interaction variables will be tested for in the regression models and subgroup analyses will be conducted according to the following factors: significant interactions, sex, age, race, tobacco product group. Multiple imputation method will be used for missing data where appropriate. Sensitivity analysis using different analytic approaches, such as generalized linear models, as well as considering different covariate adjustments, will be used to build concordant results.

The dose-dependence of the changes in biomarkers will be determined by analyzing the data obtained from individuals that are exposed to different doses of a single product (e.g. smoking 0, <15, 15-20 and >20 cigarettes per day) and by comparing between tobacco products that have different doses of HPHC constituents. In the US the average cigarettes per day is between15-20 [37] and therefore this dose range distribution is reflective of general population exposure. Comparisons of the effects of novel tobacco products and smoking will be informative of the relative toxicity of the two products.

We believe that the methods employed in the current project are exquisitely sensitive and responsive to even low dose insults such as ambient air pollution [12] allowing us to quantify tobacco product-induced changes with high precision. Moreover, levels of acrolein exposure vary between different individuals due to difference in puffing intensity and the time a cigarette is left smoldering. Thus, direct measurements of acrolein metabolites afford better estimates of acrolein exposure than machine yields. We expect to obtain wide variations in acrolein/crotonaldehyde exposure which will enable us to construct a dose-response relationship and identify which injury biomarkers are associated with aldehyde exposure and whether high levels of exposure are associated with high levels of injury, despite similar nicotine delivery.

Sample size

The sample size is justified in terms of the primary dependent measure, FMD, given the potential importance of this variable as a direct measure of the impact of tobacco exposure. The main comparisons are between non-tobacco users and tobacco users. Due to one control group, we will conservatively adjust our α (significance level) using a Bonferroni correction, and we will set α=0.01. Based on preliminary data for FMD, we have observed mean ± SD in smoker and nonsmoker groups to be 4.0 ±1.6 and 6.8 ±1.0, respectively. We consider at least 25% (mean FMD=3.0 from 4.0) reduction from smokers to non-smokers is meaningful. Using a two sample, one-sided t test with an α of 0.01 and 80% power (1-β), assuming a common SD of 1.3, we will need 34 evaluable subjects in each group. We will recruit a total of 120 tobacco using participants per site. This over sampling will allow us to look at multiple endpoints and for associations with VOCs.

ETHICS AND DISSEMINATION

The CITU study was approved at each institution by their institutional review board (BU #H-32613 and UofL #13.0590) and all participants provide written consent. No study related procedures will be completed until after participant consent.

Participants for the CITU study are being recruited in both Boston, MA and Louisville KY. The two populations show significant differences, therefore recruitment at two sites will ensure a range more reflective of the general population. Although overall racial and ethnic demographics for both cities show a clear majority of Caucasians (70%) and despite smokers typically male, we strive to, and currently are successful in, recruiting a population that was gender balanced and almost evenly split between Caucasian and African Americans. Despite this balanced recruitment, e-cigarette users have been reported as predominantly Caucasian and male[38], and thus far our recruitment mirrors these demographics. We expect very few Hispanic/Latino's to participate, due to data suggesting tobacco use, including ENDS, tends to be lower among Hispanic's/Latino's [38 39]. Thus we have also opted to only recruit English speakers. We have carefully develop our recruitment strategy and exclusion criteria to protect vulnerable populations, which is important since many report a lower socioeconomic status and educational level in smokers in addition to higher rates of reported alcohol and drug use [40 41].

Our study is an observational study where participants have already assumed the risk of using tobacco. Study procedures pose minimal risk. Given the known harms associated with smoking, we will provide information on tobacco treatment when

requested by the participant. Participant information is de-identified for analysis and reported in aggregate to protect privacy.

Completion of these studies will enable a greater understanding of the biological responses to use of a variety of tobacco products. Specifically, they will help to identify the constituents of these products; and how a panel of exposure and CV injury biomarkers are associated with these different constituents. This data will be available to the FDA and could help guide new policy measures to reduce or eliminate the harmful components of tobacco smoke and other nicotine products. The study is dedicated to the rapid dissemination of their rigorously characterized and well-controlled research findings to the public in the form of peer-reviewed publications. Subsequent to the initial full-length manuscript publications of the resources generated with funding from this program, the study will make them available to interested and qualified investigators upon written request. The study will provide relevant protocols of published data, upon request (presuming prior publication by the Center members). Participants will be provided a summary of the results as they become available. Finally press releases of relevant findings will inform the general population.

LIST OF ABBREVIATIONS

ABI- Ankle Brachial Index

CAC= circulating angiogenic cells

CRP= C-reactive protein

CVD- Cardiovascular disease

ENDS- Electronic nicotine Device (i.e. e-cigarette)

FACS- Fluorescence-activated cell sorting

FMD- Flow mediated dilation

HDL= high density lipoprotein

IL-6= Interleukin 6

MMP- Matrix metalloproteinase

MP= micoparticles

PAI-=- Plasminogen activator

PF4= Platelet factor 4

PWV- Pulse wave velocity

SAA= serum amyloid A

s-ICAM- soluble intercellular adhesion protein inhibitor

s-VCAM= soluble vascular adhesion protein

TNFR1= Tumor necrosis factor receptor 1

t-PA= tissue plasminogen activator

TxA2=Thromboxane A

VOC- Volatile organic compound

W:H- ratio: Waist to hip ratio

AUTHORS CONTRIBUTIONS

Rachel Keith- Study design, study recruitment, study visits, statistical analysis and manuscript preparation. Jessica Fetterman- study recruitment, study visits, manuscript preparation and editing. Dan Riggs- statistical analysis, manuscript preparation and editing. Tim O'Toole- Biomarker measurements, manuscript preparation and editing.

Jessica Nystoriak- study recruitment and study visits. Monica Holbrook- study recruitment and study visits. Pawel Lorkiewicz- VOC measurements and manuscript preparation. Aruni Bhatnagar- Study design, study funding and manuscript editing. Andrew DeFilippis- Human subject assessment planning, manuscript preparation and editing. Naomi M. Hamburg- Study design, study funding, vascular core, manuscript preparation and editing.

COMPETING INTERESTS

None declared

FUNDING

This work was supported by the National Institutes of Health and the FDA Center for Tobacco Products (CTP) grant number P50HL120163.

Acknowledgements

Study design reported in this publication was supported by the AHA Tobacco Regulation and Addiction Center (A-TRAC) and FDA Center for Tobacco Products (CTP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the Food and Drug Administration.

REFERENCES

1. Rostron B. Smoking-Attributable Mortality by Cause in the United States: Revising the CDC's Data and Estimates. *Nicotine Tob Res* 2013;15(1):238-46. doi: 10.1093/ntr/nts120
2. Morris PB, Ference BA, Jahangir E, et al. Cardiovascular Effects of Exposure to Cigarette Smoke and Electronic Cigarettes: Clinical Perspectives From the Prevention of Cardiovascular Disease Section Leadership Council and Early Career Councils of the American College of Cardiology. *J Am Coll Cardiol* 2015;66(12):1378-91. doi: 10.1016/j.jacc.2015.07.037 [published Online First: 2015/09/19]
3. Haussmann HJ. Use of hazard indices for a theoretical evaluation of cigarette smoke composition. *Chem Res Toxicol* 2012;25(4):794-810. doi: 10.1021/tx200536w [published Online First: 2012/02/23]
4. Ghilarducci DP, Tjeerdema RS. Fate and effects of acrolein. *Rev Environ Contam Toxicol* 1995;144:95-146. [published Online First: 1995/01/01]

5. Dong JZ, Moldoveanu SC. Gas chromatography-mass spectrometry of carbonyl compounds in cigarette mainstream smoke after derivatization with 2,4-dinitrophenylhydrazine. *J Chromatogr A* 2004;1027(1-2):25-35. [published Online First: 2004/02/20]
6. Stepanov I, Jensen J, Hatsukami D, et al. New and traditional smokeless tobacco: comparison of toxicant and carcinogen levels. *Nicotine Tob Res* 2008;10(12):1773-82. doi: 10.1080/14622200802443544 [published Online First: 2008/11/22]
7. Awe SO, Adeagbo AS, D'Souza SE, et al. Acrolein induces vasodilatation of rodent mesenteric bed via an EDHF-dependent mechanism. *Toxicol Appl Pharmacol* 2006;217(3):266-76. doi: 10.1016/j.taap.2006.08.008 [published Online First: 2006/10/31]
8. Conklin DJ, Barski OA, Lesgards JF, et al. Acrolein consumption induces systemic dyslipidemia and lipoprotein modification. *Toxicol Appl Pharmacol* 2010;243(1):1-12. doi: 10.1016/j.taap.2009.12.010 [published Online First: 2009/12/26]
9. Conklin DJ, Bhatnagar A, Cowley HR, et al. Acrolein generation stimulates hypercontraction in isolated human blood vessels. *Toxicol Appl Pharmacol* 2006;217(3):277-88. doi: 10.1016/j.taap.2006.09.009 [published Online First: 2006/11/11]
10. Conklin DJ, Haberzettl P, Prough RA, et al. Glutathione-S-transferase P protects against endothelial dysfunction induced by exposure to tobacco smoke. *Am J Physiol Heart Circ Physiol* 2009;296(5):H1586-97. doi: 10.1152/ajpheart.00867.2008 [published Online First: 2009/03/10]
11. Ismahil MA, Hamid T, Haberzettl P, et al. Chronic oral exposure to the aldehyde pollutant acrolein induces dilated cardiomyopathy. *Am J Physiol Heart Circ Physiol* 2011;301(5):H2050-60. doi: 10.1152/ajpheart.00120.2011 [published Online First: 2011/09/13]
12. O'Toole TE, Hellmann J, Wheat L, et al. Episodic exposure to fine particulate air pollution decreases circulating levels of endothelial progenitor cells. *Circ Res* 2010;107(2):200-3. doi: 10.1161/circresaha.110.222679 [published Online First: 2010/07/03]
13. O'Toole TE, Zheng YT, Hellmann J, et al. Acrolein activates matrix metalloproteinases by increasing reactive oxygen species in macrophages. *Toxicol Appl Pharmacol* 2009;236(2):194-201. doi: 10.1016/j.taap.2009.01.024 [published Online First: 2009/04/18]
14. Sithu SD, Srivastava S, Siddiqui MA, et al. Exposure to acrolein by inhalation causes platelet activation. *Toxicol Appl Pharmacol* 2010;248(2):100-10. doi: 10.1016/j.taap.2010.07.013 [published Online First: 2010/08/04]
15. Srivastava S, Sithu SD, Vladykovskaya E, et al. Oral exposure to acrolein exacerbates atherosclerosis in apoE-null mice. *Atherosclerosis* 2011;215(2):301-8. doi: 10.1016/j.atherosclerosis.2011.01.001 [published Online First: 2011/03/05]
16. Tsakadze NL, Srivastava S, Awe SO, et al. Acrolein-induced vasomotor responses of rat aorta. *Am J Physiol Heart Circ Physiol* 2003;285(2):H727-34. doi: 10.1152/ajpheart.00269.2003 [published Online First: 2003/05/06]
17. Wang GW, Guo Y, Vondriska TM, et al. Acrolein consumption exacerbates myocardial ischemic injury and blocks nitric oxide-induced PKCepsilon signaling and cardioprotection. *J Mol Cell Cardiol* 2008;44(6):1016-22. doi: 10.1016/j.yjmcc.2008.03.020 [published Online First: 2008/05/13]
18. Wheat LA, Haberzettl P, Hellmann J, et al. Acrolein inhalation prevents vascular endothelial growth factor-induced mobilization of Flk-1+/Sca-1+ cells in mice. *Arterioscler Thromb Vasc Biol* 2011;31(7):1598-606. doi: 10.1161/atvbaha.111.227124 [published Online First: 2011/04/30]
19. WHO Study Group on Tobacco Product Regulation. Report on the scientific basic of tobacco product regulation: fourth report of a WHO study group. *World Health Organ Tech Rep Ser* 2012(967):1-83, 1 p following 83. [published Online First: 2012/09/08]
20. De Woskin R, Greenberg M, Pepelko W, et al. Toxicological review of acrolein (cas no. 107-02-08) in support of summary information on the integrated risk information system (Iris). *Washington, DC: US Environmental Protection Agency* 2003

21. Kosmider L, Sobczak A, Fik M, et al. Carbonyl compounds in electronic cigarette vapors: effects of nicotine solvent and battery output voltage. *Nicotine Tob Res* 2014;16(10):1319-26. doi: 10.1093/ntr/ntu078 [published Online First: 2014/05/17]
22. Goniewicz ML, Knysak J, Gawron M, et al. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tob Control* 2014;23(2):133-9. doi: 10.1136/tobaccocontrol-2012-050859 [published Online First: 2013/03/08]
23. Wang P, Chen W, Liao J, et al. A Device-Independent Evaluation of Carbonyl Emissions from Heated Electronic Cigarette Solvents. *PLoS One* 2017;12(1):e0169811. doi: 10.1371/journal.pone.0169811 [published Online First: 2017/01/12]
24. Hill JM, Zalos G, Halcox JP, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *The New England journal of medicine* 2003;348(7):593-600. doi: 10.1056/NEJMoa022287 [published Online First: 2003/02/14]
25. Parsons VL, Moriarity C, Jonas K, et al. Design and estimation for the national health interview survey, 2006-2015. *Vital Health Stat 2* 2014(165):1-53. [published Online First: 2014/04/30]
26. Alwis KU, Blount BC, Britt AS, et al. Simultaneous analysis of 28 urinary VOC metabolites using ultra high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI/MSMS). *Anal Chim Acta* 2012;750:152-60. doi: 10.1016/j.aca.2012.04.009 [published Online First: 2012/10/16]
27. Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001;89(1):E1-7. [published Online First: 2001/07/07]
28. Werner N, Nickenig G. Influence of cardiovascular risk factors on endothelial progenitor cells: limitations for therapy? *Arterioscler Thromb Vasc Biol* 2006;26(2):257-66. doi: 10.1161/01.ATV.0000198239.41189.5d [published Online First: 2005/12/03]
29. DeJarnett N, Yeager R, Conklin DJ, et al. Residential Proximity to Major Roadways Is Associated With Increased Levels of AC133+ Circulating Angiogenic Cells. *Arteriosclerosis, thrombosis, and vascular biology* 2015;35(11):2468-77. doi: 10.1161/atvbaha.115.305724 [published Online First: 2015/08/22]
30. Poredos P, Orehek M, Tratnik E. Smoking is associated with dose-related increase of intima-media thickness and endothelial dysfunction. *Angiology* 1999;50(3):201-8. [published Online First: 1999/03/24]
31. Heiss C, Amabile N, Lee AC, et al. Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production. *J Am Coll Cardiol* 2008;51(18):1760-71. doi: 10.1016/j.jacc.2008.01.040 [published Online First: 2008/05/03]
32. Flammer AJ, Anderson T, Celermajer DS, et al. The assessment of endothelial function: from research into clinical practice. *Circulation* 2012;126(6):753-67. doi: 10.1161/circulationaha.112.093245 [published Online First: 2012/08/08]
33. Benjamin EJ, Larson MG, Keyes MJ, et al. Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. *Circulation* 2004;109(5):613-9. doi: 10.1161/01.cir.0000112565.60887.1e [published Online First: 2004/02/11]
34. Vita JA. Nitric oxide-dependent vasodilation in human subjects. *Methods Enzymol* 2002;359:186-200. [published Online First: 2002/12/17]
35. Mitchell GF, Guo CY, Benjamin EJ, et al. Cross-sectional correlates of increased aortic stiffness in the community: the Framingham Heart Study. *Circulation* 2007;115(20):2628-36. doi: 10.1161/circulationaha.106.667733 [published Online First: 2007/05/09]

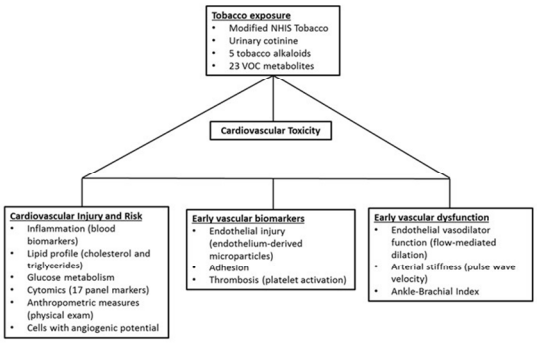
36. Duffy SJ, Keaney Jr JF, Holbrook M, et al. Short-and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation* 2001;104(2):151-56.
37. Benowitz NL, Dains KM, Dempsey D, et al. Racial differences in the relationship between number of cigarettes smoked and nicotine and carcinogen exposure. *Nicotine Tob Res* 2011;13(9):772-83. doi: 10.1093/ntr/ntr072 [published Online First: 2011/05/07]
38. Schoenborn CA, Gindi RM. Electronic Cigarette Use Among Adults: United States, 2014. *NCHS Data Brief* 2015(217):1-8. [published Online First: 2015/11/12]
39. Maher J, Boysun M, Rohde K, et al. Are Latinos really less likely to be smokers? Lessons from Oregon. *Nicotine & Tobacco Research* 2005;7(2):283-87. doi: 10.1080/14622200500056259
40. Barbeau EM, Krieger N, Soobader M-J. Working Class Matters: Socioeconomic Disadvantage, Race/Ethnicity, Gender, and Smoking in NHIS 2000. *Am J Public Health* 2004;94(2):269-78. doi: 10.2105/AJPH.94.2.269
41. Giskes K, Kunst AE, Benach J, et al. Trends in smoking behaviour between 1985 and 2000 in nine European countries by education. *Journal of Epidemiology and Community Health* 2005;59(5):395.

Figure 1. Cardiovascular Injury due to Tobacco Use

CITU is designed to assess how tobacco related VOC exposure contributes to cardiovascular risk factors. Our exposure measurements include a panel of 23 urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular phenotyping includes measures of injury, risk, vascular biomarkers and early vascular dysfunction. Tobacco use included use of traditional cigarettes, smokeless tobacco, waterpipe tobacco (hookah), electronic nicotine devices (ENDS), little cigars, cigarillos, pipes, cigars or any other form of tobacco that is available. Enrollment began in July 2014 and is ongoing.

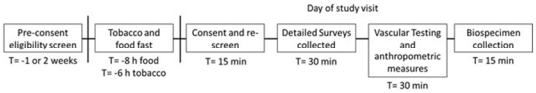
Figure 2. Study Visit Design

Study flow chart for interested participants from screening through study completion. Potential participants are pre-screened for eligibility prior to enrollment. Potential participants are asked to fast from tobacco for a minimum of 6 hours prior to the study visit. On the day of the visit the study lasts approximately 90 minute.



CITU is designed to assess how tobacco related VOC exposure contributes to cardiovascular risk factors. Our exposure measurements include a panel of 23 urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular phenotyping includes measures of injury, risk, vascular biomarkers and early vascular dysfunction. Tobacco use included use of traditional cigarettes, smokeless tobacco, waterpipe tobacco (hookah), electronic nicotine devices (ENDS), little cigars, cigarillos, pipes, cigars or any other form of tobacco that is available. Enrollment began in July 2014 and is ongoing.

338x190mm (96 x 96 DPI)



Study flow chart for interested participants from screening through study completion. Potential participants are pre-screened for eligibility prior to enrollment. Potential participants are asked to fast from tobacco for a minimum of 6 hours prior to the study visit. On the day of the visit the study lasts approximately 90 minute.

338x190mm (96 x 96 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2-3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3-4
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5, 7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5, 7
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6-7
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7-12
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*7-12*
Bias	9	Describe any efforts to address potential sources of bias	7
Study size	10	Explain how the study size was arrived at	14-16
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	12-14
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	12-14
		(*b*) Describe any methods used to examine subgroups and interactions	13
		(*c*) Explain how missing data were addressed	13
		(*d*) If applicable, explain how loss to follow-up was addressed	N/A (study protocol)
		(*e*) Describe any sensitivity analyses	13
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	N/A (study protocol)
		(b) Give reasons for non-participation at each stage	N/A (study protocol)
		(c) Consider use of a flow diagram	N/A (study protocol)
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	N/A (study protocol)
		(b) Indicate number of participants with missing data for each variable of interest	N/A (study protocol)
		(c) Summarise follow-up time (eg, average and total amount)	N/A (study protocol)
Outcome data	15*	Report numbers of outcome events or summary measures over time	N/A (study protocol)
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	N/A (study protocol)
		(*b*) Report category boundaries when continuous variables were categorized	N/A (study protocol)
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A (study protocol)
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N/A (study protocol)
Discussion			
Key results	18	Summarise key results with reference to study objectives	N/A (study protocol)
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	17
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Protocol to Assess the Impact of Tobacco-Induced Volatile Organic Compounds on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to Tobacco Study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-019850.R2
Article Type:	Protocol
Date Submitted by the Author:	14-Feb-2018

Complete List of Authors:	Keith, Rachel; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Fetterman, Jessica; Boston Medical Center, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center Riggs, Daniel; American Heart Association- Tobacco Regulation and Addiction Center; University of Louisville, Medicine O'Toole, Timothy; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Nystoriak, Jessica; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Holbrook, Monika; Boston Medical Center, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center Lorkiewicz, Pawel; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Bhatnagar, Aruni; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center DeFilippis, Andrew; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Hamburg, Naomi ; Boston University, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Public health
Keywords:	smoking, tobacco, electronic cigarette, cardiovascular risk, vascular injury, cigarettes

Protocol to Assess the Impact of Tobacco-Induced Volatile Organic Compounds on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to Tobacco Study

Rachel J. Keith, Jessica L. Fetterman, Daniel W. Riggs, Tim O'Toole, Jessica Nystoriak, Monica Holbrook, Pawel Lorkiewicz, Aruni Bhatnagar, Andrew DeFilippis*, Naomi M. Hamburg*

Rachel J. Keith-Division of Cardiovascular Medicine, University of Louisville School of Medicine 580 S. Preston St. Louisville KY, 40202 rachel.keith@louisville.edu 502-852-4211

Jessica L. Fetterman- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston University School of Medicine Evans Building, Boston, MA USA

Daniel W. Riggs- University of Louisville School of Medicine Louisville, KY USA

Timothy O-Toole- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Jessica L. Nystoriak- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Monika Holbrook- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston University School of Medicine Boston, MA USA

Pawel Lorkiewicz- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Aruni Bhatnagar- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA

Andrew P. DeFilippis- Division of Cardiovascular Medicine, University of Louisville School of Medicine Louisville, KY USA (co-senior author)

Naomi M. Hamburg- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston University School of Medicine Boston, MA USA (co-senior author)

Word Count: 2581

ABSTRACT

Introduction: Tobacco use leads to increased mortality, the majority of which is attributed to cardiovascular disease. Despite this knowledge, the early cardiovascular impact of tobacco product use is not well understood. Tobacco use increases exposure to harmful and potentially harmful constituents including volatile organic compounds (VOCs) such as acrolein and crotonaldehyde, which may contribute to cardiovascular risk. The link between exposure patterns, risk profiles and demographic distribution of tobacco product users, particularly users of new and emerging products, are not well known. Therefore, we designed the Cardiovascular Injury due to Tobacco Use (CITU) study to assess population characteristics, demographic features, exposure patterns and cardiovascular risk in relation to tobacco.

Methods and analysis: We present the design and methodology of the CITU study a cross-section observational tobacco study conducted in Boston MA and Louisville KY starting in 2014. Healthy participants 21 to 45 years of age who use tobacco products, including ENDS, or who never used tobacco are being recruited. The study aims to recruit an evenly split cohort of African Americans and Caucasians that is sex balanced

for evaluation of self-reported tobacco exposure, VOC exposure and tobacco-induced injury profiling. Detailed information about participant's demographics, health status and lifestyle is also collected.

Ethics and dissemination: The study protocol was approved institutional review boards at both participating universities. All study protocols will protect participant confidentiality. Results from the study will be disseminated via peer-reviewed journals and presented at scientific conferences.

Strengths and limitations

- Young age to allow for evaluation of early stage disease (e.g. inflammation, endothelial function) as opposed to end stage clinical consequence (e.g. myocardial infarction)
- Diverse tobacco product use allows for assessment of a wide range of tobacco-induced VOC exposure
- All study visits are in English introducing selection bias
- Data will inform regulatory agencies on the cardiovascular health effects of multiple tobacco products and the contribution of HPHCs

Keywords: Tobacco, smoking, electronic cigarette, vascular injury, cardiovascular risk, cigarettes.

INTRODUCTION

Tobacco product use and smoking are the leading causes of preventable deaths throughout the world. Of those deaths, one-third are attributed to cardiovascular disease (CVD) [1]. The cardiovascular (CV) effects of tobacco exposure can include atherogenesis, vascular injury, thrombosis, arrhythmias and inflammation [2] and may be attributable to the many different harmful and potentially harmful constituents (HPHCs) present in tobacco products.

The HPHCs found in tobacco products include volatile organic compounds (VOCs) of which reactive aldehydes, such as acrolein and crotonaldehyde, are likely the most significant contributors to CV toxicity [3]. High levels of aldehydes are present in cigarette smoke [4 5] as well as smokeless tobacco (ST) [6]. Risk assessments, using the prevalence of each individual chemical weighed by its potency, suggest that the non-cancer risk of smoking is dominated by acrolein, which contributes 40-100 times more to risk than any other chemical present in cigarette smoke [3].

Although HPHCs, including VOC reactive aldehydes, have been suspected to be major contributors to the toxicity of cigarette smoke for over 4 decades, their contribution to CV injury and early CVD risk has not been rigorously evaluated. Experimental studies in animal models suggest that because of low aldehyde-metabolizing capacity, CV tissues are highly sensitive to aldehydes and exposure to low levels of aldehydes can induce CV injury and accelerate CVD [7-18]. The WHO Study Group on Tobacco Product Regulation (TobReg) has marked acrolein, a VOC, along with 8 other cigarette constituents for monitoring and regulation [19] and the U.S. Environmental Protection Agency lists Acrolein as one of most hazardous air pollutants[20]. Nevertheless, the contribution of tobacco induced VOCs, including acrolein

or other aldehydes, toward CV toxicity in humans has not been fully assessed. Greater understanding of how aldehydes affect cardiovascular health and disease will provide new avenues for evaluating the toxicity of cigarette smoke and for assessing the injurious potential of new and emerging tobacco products, such as ENDS, which may also contain VOCs including acrolein [21-23].

The latency period between tobacco exposure and the development of major clinical adverse health effects is long, therefore biomarkers that provide information over a shorter period allow for the identification of harm decades before clinical outcome data is available. Thus, in this paper we present the design and methodology of the Cardiovascular Injury due To Tobacco Use (CITU) study which will evaluate the association of the urinary metabolites of 18 parent VOCs from tobacco exposure with a comprehensive set of CV biomarkers representative of early disease and predictive of future CV events.[24]

METHODS AND DESIGN

Overall design

The CITU study is an investigator-initiated cross-sectional observational study of around 500 healthy participants 21 to 45 years of age who are never or current tobacco product users in two urban areas at Boston University (BU) and University of Louisville (UofL) (Boston, MA and Louisville, KY) designed to evaluate CV toxicity due to tobacco product use, with correlations to VOCs found in the tobacco products (**Figure 1**).

Participant Eligibility Criteria

The goal of the study is to examine the impact of tobacco products on healthy young adults who could be classified as a current tobacco product users (Defined in table 1), or never-users (does not have lifetime use of any tobacco product). Participants were self-reported to be healthy therefore we excluded participants if they had: 1) diagnosis of clinical cardiovascular disease including but not limited to known heart attack, peripheral artery disease, heart failure or stroke; 2) diagnosis of diabetes (HbA1c >7.0 or treatment for diabetes), hypertension (systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg), hypothyroidism or hyperthyroidism, inflammatory conditions such as lupus or inflammatory bowel disease, HIV/AIDS, hepatitis, liver disease, anemia, cancer of any type or another medical condition that might compromise the successful completion of the study; 2) recipients of organ transplant or renal replacement therapy; 3) individuals that are taking the following medications: immunosuppressant agents estrogen, testosterone, anti TNF agents, certain biologics, Procrit, statins, beta-blockers or other cardiovascular medicine; 4) individuals using nutraceuticals or anabolic steroids beyond the recommended daily allowance; 5) body weight less than 100 pounds; 6) pregnant women; 7) prisoners and other vulnerable populations; and 8) active illness or infection. Participants are rescheduled or considered screen-failures and excluded from the study if symptomatic of an acute illness, i.e. viral upper respiratory infection, on study date.

Table 1. Tobacco product use classifications

Classification	Qualification
Never	Does not meet lifetime limits for any tobacco use (see below)
Smoker	>100 lifetime cigarettes and current use for the past year
Smokeless Tobacco User	>20 lifetime dips or chews and current use for the past year
Cigar/Cigarillo User	>20 lifetime cigars or cigarillos and current use for the past year
Pipe User	>20 lifetime pipefuls and current use for the past year

ENDS User	>20 lifetime vape sessions and current use for the past year
Hookah User	>20 lifetime hookah sessions and current use for the past year

Study participants are screened prior to enrollment for current and past tobacco product use. Participants are characterized and assigned a use group based on self-reported patterns collected during the study visits.

Overall Study Procedure

Study participants fast for 8 h from food and 6 h from tobacco prior to the visit. All study visits occur before 11AM to limit effects due to circadian changes. All vascular function studies are completed after 10 min of supine positioning. All vascular studies are sent to the BU central lab for analysis. BU biologic samples have minimal processing and are shipped overnight to the UofL central laboratory at the completion of each study visit. Samples obtained at UofL are processed to a similar stage, then held overnight prior to analysis for standardization of time to measurement for all samples.

Study visits take approximately 90 minutes to complete and include a structured interview on demographics, socioeconomics, lifestyle, health, family history of heart disease, allergies, and tobacco use. (**Figure 2**) Participants were compensated appropriately for their time. All surveys are collected and kept in Research Electronic Data Capture (REDCap), a secure web application for building and managing online surveys and databases.

Exposure Variables

Tobacco Product Use & Particulate Matter Exposure

Comprehensive tobacco product exposure is assessed using a modified version of the National Health Interview survey on tobacco use [25]. The survey is modified to include detailed information on electronic nicotine devices (ENDs) and other new or

emerging tobacco products. Residential addresses are collected for assessment of ambient airborne particulate matter ($PM_{2.5}$) exposure and future correction of overall exposure. $PM_{2.5}$ data from the day of the study visit, and 3 and 5 days prior to the study is collected from publicly available data associated with EPA monitoring stations. Other exposure variables, including occupation, are collected through interview.

VOC Measurements

Humans are exposed to VOCs from a variety of sources including indoor and outdoor environments as well as diet. The most significant sources of ambient exposure ambient are air pollution, car exhaust, household products, personal hygiene products, and solvents[26 27]. Although concurrent exposures from multiple sources could confound attribution to smoking, the levels of urinary metabolites of these VOCs in smokers far exceeds those measured in non-smokers exposed to typical sources of VOCs [28].

Standard clean catch urine specimens are obtained from participants. Though only a single urine time point is collected, previous studies show spot urine measurements correlate well with 24-hour urine collections[29]. Many VOC metabolites have relatively short half-lives that range from 2 - 25.2h, [30 31] but given the constant pattern of tobacco product use by most users, spot collection reflects recurrent use. Moreover, even though some VOC metabolites, such as HPMA, are known vary with time of day,[29] synchronizing the study visits and requiring a tobacco fast is likely to minimize diurnal variations in metabolism. Our past work has shown that spot-urine collected at the same time of day reliably reflects daily VOC exposure and is correlated to CVD risk[32].

We have developed a robust Core Lab that utilizes mass spectrometry procedures adopted from the Centers for Disease Control and Prevention (CDC) protocols, to quantify 23 urinary metabolites of tobacco smoking related toxins (aldehydes and other VOCs), including acrolein[33] (**Table 2**). The concentration values of analytes are then normalized to urinary creatinine levels measured using Infinity Creatinine Reagent (Thermo Fisher Scientific, MA) on a COBAS MIRA-plus analyzer (Roche, NJ).

Table 2 Exposure Variables (Please see end of article)

Parent compound	***VOC metabolite***	***Common abbr.***
Acetaldehyde	Acetic acid/Acetate	ACETATE
Acrolein	N-Acetyl-S-(2-carboxyethyl)-L-cysteine	CEMA
	N-Acetyl-S-(3-hydroxypropyl)-L-cysteine	3HPMA
Acrylamide	N-Acetyl-S-(2-carbamoylethyl)-L-cysteine	AAMA
	N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	GAMA
Acrylonitrile	N-Acetyl-S-(2-cyanoethyl)-L-cysteine	CYMA
Acrylonitrile, vinyl chloride, ethylene oxide	N-Acetyl-S-(2-hydroxyethyl)-L-cysteine	HEMA
Anabasine	Anabasine (free)	ANB
Anatabine	Anatabine (free)	ANTB
Benzene	N-Acetyl-S-(phenyl)-L-cysteine	PMA
	trans, trans-Muconic acid	MU

1-Bromopropane	N-Acetyl-S-(n-propyl)-L-cysteine	BPMA
1,3-Butadiene	N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine	DHBMA
	N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine	MHBMA1
	N-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine	MHBMA2
	N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine	MHBMA3
Carbon-disulfide	2-Thioxothiazolidine-4-carboxylic acid	TTCA
Crotonaldehyde	N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine	HPMMA
Cyanide	2-Aminothiazoline-4-carboxylic acid	ATCA
N,N-Dimethylformamide	N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine	AMCC
Ethylbenzene, styrene	Phenylglyoxylic acid	PGA
Formaldehyde	Formate	FORMATE
Nicotine	Nicotine	NIC
	Cotinine	COT
	3-Hydroxycotinine	3HC
Propylene oxide	N-Acetyl-S-(2-hydroxypropyl)-L-cysteine	2HPMA
Styrene	N-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine +	PHEMA
	N-Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine	
	Mandelic acid	MA
Tetrachloroethylene	N-Acetyl-S-(trichlorovinyl)-L-cysteine	TCVMA
Toluene	N-Acetyl-S-(benzyl)-L-cysteine	BMA
Trichloroethylene	N-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine	1,2DCVMA
	N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine	2,2DCVMA

Xylene	N-Acetyl-S-(2,4-dimethylphenyl)-L-cysteine + N-Acetyl-S-(2,5-dimethylphenyl)-L-cysteine + N-Acetyl-S-(3,4-dimethylphenyl)-L-cysteine	DPMA
	2-Methylhippuric acid	2MHA
	3-Methylhippuric acid + 4-Methylhippuric acid	3MHA+ 4MHA

Urine is analyzed for 23 metabolites of 18 parent VOCs and tobacco alkaloids by UPLC-MS/MS. Analytes are listed as parent, metabolite and their common abbreviation.

Circulating Markers of Cardiovascular Injury

To assess tobacco product-induced cardiovascular toxicity, we examine endothelial function, inflammatory mediators, biomarkers, and thrombosis. CV risk is defined through measurements of circulating angiogenic cells, lipid profile, and glucose metabolism [24 34 35]. Plasma (BD367863 and BD366415) and serum (BD367814) samples are obtained from all participants for laboratory testing and long term biobanking. Whole blood (BD366415) is obtained for flow cytometry on fresh samples at UofL pathology core. BU biologic samples have minimal processing and are shipped overnight to the UofL central laboratory at the completion of each study visit. Samples obtained at UofL are processed to a similar stage, then held overnight prior to analysis to standardize the time to measurement for all samples. The UofL central laboratory, as previously reported, will complete fasting and biomarker measurements (**Table 3**), with the exception of cytomics [12 36]. For cytomic measurements, mononuclear cells are labeled with the peripheral blood phenotyping panel kit (Fluidigm).Samples are shipped

at 4 degree C to Core Lab facilities at the University of Rochester for Mass cytometric analysis.

Table 3 Blood analysis

Fasting Measurements
LDL cholesterol, HDL cholesterol, total cholesterol, triglycerides, glucose, uric acid, SAA and fibrinogen
Biomarkers
CAC (1-15)[1], Platelet-monocyte aggregates, MP (1-5)[1], PF4, t-PA, TxA2, Factor VII, IL-6, CRP, D-dimer, PAI-1, s-ICAM-1, s-VCAM, s-thrombomodulin, s-TNFR1, MMP-2, MMP-3, MMP-9, cytomics, endothelin, E-selectin and P-selectin
1: Fifteen different CAP subpopulations and 5 subtypes of microparticles were measured by flow cytometry.

All participants who complete the study visit will have blood samples taken and processed. Flow cytometric analysis is completed on fresh samples. All other analysis will be completed on biobanked samples in batches LDL= low density lipoprotein. HDL= high density lipoprotein. SAA= serum amyloid A. CAC= circulating angiogenic cells. MP= microparticles. PF4= Platelet factor 4. t-PA= tissue plasminogen activator. TxA2=Thromboxane A. IL-6= Interleukin 6. CRP= C-reactive protein. PAI-=-Plasminogen activator. s- ICAM- soluble intercellular adhesion protein inhibitor. s-VCAM= soluble vascular adhesion protein. TNFR1= Tumor necrosis factor receptor 1. MMP- Matrix metalloproteinase.

Non-Invasive Vascular Function Testing

Smoking, is associated with endothelial damage and vascular dysfunction [37 38]. Endothelial cells are exposed to circulating toxins and measures of endothelial function are reflective of cardiovascular injury [39]. Thus, we examine the non-invasive endothelial vasodilator function using flow-mediated vasodilation [40 41], arterial stiffness with carotid-femoral and carotid-radial pulse wave velocity [42], and peripheral vascular function with

ankle brachial index. Flow mediated dilation was assessed with a 7.5MHZ ultrasound probe is used to image the brachial artery while a 10cm blood pressure cuff is attached to the lower arm and a 3 lead ECG is attached to the patient. After baseline images and 10 cycles of Doppler images are captured using NIHEM R-wave triggered image capturing software, the blood pressure cuff is inflated to 200mmHg or 50mmHg higher than the systolic pressure. After the 5 minute occlusion, the cuff is released and the NIHEM software records two minutes of imaging. Images were analyzed by a single blinded analyzer using MIA vascular Research Tolls Brachial Analyzer for Research, version 6.8.5. All vascular imagers where trained at BU who have a previously reported reproducibility with intra- and inter-observer correlation coefficients of 0.98 and 0.99 for brachial diameter and 0.78 and 0.92 for FMD.[43] Similar equipment and software is used at both sites. All vascular studies are sent to the BU central lab for analysis.

Anthropometric measures

Anthropometric measures included height, weight, waist and hip circumference and body fat. All anthropometric measures are completed twice and the average recorded. Standing height measurements are completed on a fixed stadiometer. Weight measurements are completed on a digital scale to the nearest tenth of a pound. Waist circumference is measured at the level of the umbilicus to the nearest tenth of a centimeter. Hip circumference is measured at the maximal protrusion of the gluteal muscle to the nearest tenth of a centimeter. Body fat percentage is calculated by the bioelectrical impedance measured with the Omron fat loss monitor (HBF-306C).

DATA ANALYSIS

We expect that from this study we will be able to identify specific biomarkers of cardiovascular injury due to tobacco use and the relationship of these biomarkers to specific measures of tobacco exposure. For instance, we will identify which biomarkers are affected by tobacco use, and which ones are most sensitive; including their dose-dependence. Additionally we will examine the extent to which biomarkers are associated with exposure to nicotine versus exposure to HPHC of tobacco like aldehydes.

Sample size

The sample size is justified in terms of the primary dependent measure, FMD, given the potential importance of this variable as a direct measure of the impact of tobacco exposure. The main comparisons are between non-tobacco users and tobacco users. Due to one control group, we will conservatively adjust our α (significance level) using a Bonferroni correction, and we will set α=0.01. Based on preliminary data for FMD, we have observed mean ± SD in smoker and nonsmoker groups to be 4.0 ±1.6 and 6.8 ±1.0, respectively. We consider at least 25% (mean FMD=3.0 from 4.0) reduction from smokers to non-smokers is meaningful. Using a two sample, one-sided *t* test with an α of 0.01 and 80% power (1-β), assuming a common SD of 1.3, we will need 34 evaluable subjects in each group. We will recruit a total of 120 tobacco using participants per site. This over sampling will allow us to look at multiple endpoints and for associations with VOCs.

Analysis Plan

All statistical analysis will be performed using SAS version 9.4 software (SAS Institute, Inc., Cary, North Carolina), and a two-sided p-value of <0.05 will be considered

significant for any statistical test. Demographics and other baseline characteristics will be summarized according to product group. Differences in VOC's between product groups will be tested using ANOVA for normally distributed data or Kruskal-Wallis test for non-normal data. The association between primary outcomes of vascular function as well as circulating markers of cardiovascular injury with individual VOC levels will be analyzed using multiple regression models, adjusting for appropriate confounders. Additionally, because we have multiple VOC's, which are highly correlated, we will use methods such as LASSO to identify the VOC's that are most associated with the outcomes of interest. Multipollutant approaches, such as principal component analysis (PCA), will be used to test whether overall VOC exposure is associated with the health outcomes. Interaction variables will be tested for in the regression models and subgroup analyses will be conducted according to the following factors: significant interactions, sex, age, race, tobacco product group. Multiple imputation method will be used for missing data where appropriate. Sensitivity analysis using different analytic approaches, such as generalized linear models, as well as considering different covariate adjustments, will be used to build concordant results.

The dose-dependence of the changes in biomarkers will be determined by analyzing the data obtained from individuals that are exposed to different doses of a single product (e.g. smoking 0, <10, 10-20 and >20 cigarettes per day) and by comparing between tobacco products that have different doses of HPHC constituents. In the US the average cigarettes per day is between10-20 [44] and therefore this dose range distribution is reflective of general population exposure. Comparisons of the

effects of novel tobacco products and smoking will be informative of the relative toxicity of the two products.

We believe that the methods employed in the current project are exquisitely sensitive and responsive to even low dose insults such as ambient air pollution [12] allowing us to quantify tobacco product-induced changes with high precision. Moreover, levels of acrolein exposure vary between different individuals due to difference in puffing intensity and the time a cigarette is left smoldering. Thus, direct measurements of acrolein metabolites afford better estimates of acrolein exposure than machine yields. We expect to obtain wide variations in acrolein/crotonaldehyde exposure which will enable us to construct a dose-response relationship and identify which injury biomarkers are associated with aldehyde exposure and whether high levels of exposure are associated with high levels of injury, despite similar nicotine delivery.

ETHICS AND DISSEMINATION

The CITU study was approved at each institution by their institutional review board (BU #H-32613 and UofL #13.0590) and all participants provide written consent. No study related procedures will be completed until after participant consent.

Participants for the CITU study are being recruited in both Boston, MA and Louisville KY. The two populations show significant differences, therefore recruitment at two sites will ensure a range more reflective of the general population. Although overall racial and ethnic demographics for both cities show a clear majority of Caucasians (70%) and despite smokers typically male, we strive to, and currently are successful in, recruiting a population that was gender balanced and almost evenly split between Caucasian and African Americans. Despite this balanced recruitment, e-cigarette users

have been reported as predominantly Caucasian and male[45], and thus far our recruitment mirrors these demographics. We expect very few Hispanic/Latino's to participate, due to data suggesting tobacco use, including ENDS, tends to be lower among Hispanic's/Latino's [45 46]. Thus we have also opted to only recruit English speakers. We have carefully develop our recruitment strategy and exclusion criteria to protect vulnerable populations, which is important since many report a lower socioeconomic status and educational level in smokers in addition to higher rates of reported alcohol and drug use [47 48].

Our study is an observational study where participants have already assumed the risk of using tobacco. Study procedures pose minimal risk. Given the known harms associated with smoking, we will provide information on tobacco treatment when requested by the participant. Participant information is de-identified for analysis and reported in aggregate to protect privacy.

Completion of these studies will enable a greater understanding of the biological responses to use of a variety of tobacco products. Specifically, they will help to identify the constituents of these products; and how a panel of exposure and CV injury biomarkers are associated with these different constituents. This data will be available to the FDA and could help guide new policy measures to reduce or eliminate the harmful components of tobacco smoke and other nicotine products. The study is dedicated to the rapid dissemination of their rigorously characterized and well-controlled research findings to the public in the form of peer-reviewed publications. Subsequent to the initial full-length manuscript publications of the resources generated with funding from this program, the study will make them available to interested and qualified

investigators upon written request. The study will provide relevant protocols of published data, upon request (presuming prior publication by the Center members). Participants will be provided a summary of the results as they become available. Finally press releases of relevant findings will inform the general population.

LIST OF ABBREVIATIONS

ABI- Ankle Brachial Index

CAC= circulating angiogenic cells

CRP= C-reactive protein

CVD- Cardiovascular disease

ENDS- Electronic nicotine Device (i.e. e-cigarette)

FACS- Fluorescence-activated cell sorting

FMD- Flow mediated dilation

HDL= high density lipoprotein

IL-6= Interleukin 6

MMP- Matrix metalloproteinase

MP= micoparticles

PAI-=- Plasminogen activator

PF4= Platelet factor 4

PWV- Pulse wave velocity

SAA= serum amyloid A

s-ICAM- soluble intercellular adhesion protein inhibitor

s-VCAM= soluble vascular adhesion protein

TNFR1= Tumor necrosis factor receptor 1

t-PA= tissue plasminogen activator

TxA2=Thromboxane A

VOC- Volatile organic compound

W:H- ratio: Waist to hip ratio

AUTHORS CONTRIBUTIONS

Rachel Keith- Study design, study recruitment, study visits, statistical analysis and manuscript preparation. Jessica Fetterman- study recruitment, study visits, manuscript preparation and editing. Dan Riggs- statistical analysis, manuscript preparation and editing. Tim O'Toole- Biomarker measurements, manuscript preparation and editing. Jessica Nystoriak- study recruitment and study visits. Monica Holbrook- study recruitment and study visits. Pawel Lorkiewicz- VOC measurements and manuscript preparation. Aruni Bhatnagar- Study design, study funding and manuscript editing. Andrew DeFilippis- Human subject assessment planning, manuscript preparation and editing. Naomi M. Hamburg- Study design, study funding, vascular core, manuscript preparation and editing.

COMPETING INTERESTS

None declared

FUNDING

This work was supported by the National Institutes of Health and the FDA Center for Tobacco Products (CTP) grant number P50HL120163.

Acknowledgements

Study design reported in this publication was supported by the AHA Tobacco Regulation and Addiction Center (A-TRAC) and FDA Center for Tobacco Products (CTP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the Food and Drug Administration.

REFERENCES

1. Rostron B. Smoking-Attributable Mortality by Cause in the United States: Revising the CDC's Data and Estimates. *Nicotine Tob Res* 2013;15(1):238-46. doi: 10.1093/ntr/nts120
2. Morris PB, Ference BA, Jahangir E, et al. Cardiovascular Effects of Exposure to Cigarette Smoke and Electronic Cigarettes: Clinical Perspectives From the Prevention of Cardiovascular Disease Section Leadership Council and Early Career Councils of the American College of Cardiology. *J Am Coll Cardiol* 2015;66(12):1378-91. doi: 10.1016/j.jacc.2015.07.037 [published Online First: 2015/09/19]
3. Haussmann HJ. Use of hazard indices for a theoretical evaluation of cigarette smoke composition. *Chem Res Toxicol* 2012;25(4):794-810. doi: 10.1021/tx200536w [published Online First: 2012/02/23]
4. Ghilarducci DP, Tjeerdema RS. Fate and effects of acrolein. *Rev Environ Contam Toxicol* 1995;144:95-146. [published Online First: 1995/01/01]
5. Dong JZ, Moldoveanu SC. Gas chromatography-mass spectrometry of carbonyl compounds in cigarette mainstream smoke after derivatization with 2,4-dinitrophenylhydrazine. *J Chromatogr A* 2004;1027(1-2):25-35. [published Online First: 2004/02/20]
6. Stepanov I, Jensen J, Hatsukami D, et al. New and traditional smokeless tobacco: comparison of toxicant and carcinogen levels. *Nicotine Tob Res* 2008;10(12):1773-82. doi: 10.1080/14622200802443544 [published Online First: 2008/11/22]
7. Awe SO, Adeagbo AS, D'Souza SE, et al. Acrolein induces vasodilatation of rodent mesenteric bed via an EDHF-dependent mechanism. *Toxicol Appl Pharmacol* 2006;217(3):266-76. doi: 10.1016/j.taap.2006.08.008 [published Online First: 2006/10/31]
8. Conklin DJ, Barski OA, Lesgards JF, et al. Acrolein consumption induces systemic dyslipidemia and lipoprotein modification. *Toxicol Appl Pharmacol* 2010;243(1):1-12. doi: 10.1016/j.taap.2009.12.010 [published Online First: 2009/12/26]
9. Conklin DJ, Bhatnagar A, Cowley HR, et al. Acrolein generation stimulates hypercontraction in isolated human blood vessels. *Toxicol Appl Pharmacol* 2006;217(3):277-88. doi: 10.1016/j.taap.2006.09.009 [published Online First: 2006/11/11]
10. Conklin DJ, Haberzettl P, Prough RA, et al. Glutathione-S-transferase P protects against endothelial dysfunction induced by exposure to tobacco smoke. *Am J Physiol Heart Circ Physiol* 2009;296(5):H1586-97. doi: 10.1152/ajpheart.00867.2008 [published Online First: 2009/03/10]
11. Ismahil MA, Hamid T, Haberzettl P, et al. Chronic oral exposure to the aldehyde pollutant acrolein induces dilated cardiomyopathy. *Am J Physiol Heart Circ Physiol* 2011;301(5):H2050-60. doi: 10.1152/ajpheart.00120.2011 [published Online First: 2011/09/13]
12. O'Toole TE, Hellmann J, Wheat L, et al. Episodic exposure to fine particulate air pollution decreases circulating levels of endothelial progenitor cells. *Circ Res* 2010;107(2):200-3. doi: 10.1161/circresaha.110.222679 [published Online First: 2010/07/03]

13. O'Toole TE, Zheng YT, Hellmann J, et al. Acrolein activates matrix metalloproteinases by increasing reactive oxygen species in macrophages. *Toxicol Appl Pharmacol* 2009;236(2):194-201. doi: 10.1016/j.taap.2009.01.024 [published Online First: 2009/04/18]
14. Sithu SD, Srivastava S, Siddiqui MA, et al. Exposure to acrolein by inhalation causes platelet activation. *Toxicol Appl Pharmacol* 2010;248(2):100-10. doi: 10.1016/j.taap.2010.07.013 [published Online First: 2010/08/04]
15. Srivastava S, Sithu SD, Vladykovskaya E, et al. Oral exposure to acrolein exacerbates atherosclerosis in apoE-null mice. *Atherosclerosis* 2011;215(2):301-8. doi: 10.1016/j.atherosclerosis.2011.01.001 [published Online First: 2011/03/05]
16. Tsakadze NL, Srivastava S, Awe SO, et al. Acrolein-induced vasomotor responses of rat aorta. *Am J Physiol Heart Circ Physiol* 2003;285(2):H727-34. doi: 10.1152/ajpheart.00269.2003 [published Online First: 2003/05/06]
17. Wang GW, Guo Y, Vondriska TM, et al. Acrolein consumption exacerbates myocardial ischemic injury and blocks nitric oxide-induced PKCepsilon signaling and cardioprotection. *J Mol Cell Cardiol* 2008;44(6):1016-22. doi: 10.1016/j.yjmcc.2008.03.020 [published Online First: 2008/05/13]
18. Wheat LA, Haberzettl P, Hellmann J, et al. Acrolein inhalation prevents vascular endothelial growth factor-induced mobilization of Flk-1+/Sca-1+ cells in mice. *Arterioscler Thromb Vasc Biol* 2011;31(7):1598-606. doi: 10.1161/atvbaha.111.227124 [published Online First: 2011/04/30]
19. WHO Study Group on Tobacco Product Regulation. Report on the scientific basic of tobacco product regulation: fourth report of a WHO study group. *World Health Organ Tech Rep Ser* 2012(967):1-83, 1 p following 83. [published Online First: 2012/09/08]
20. De Woskin R, Greenberg M, Pepelko W, et al. Toxicological review of acrolein (cas no. 107-02-08) in support of summary information on the integrated risk information system (Iris). *Washington, DC: US Environmental Protection Agency* 2003
21. Kosmider L, Sobczak A, Fik M, et al. Carbonyl compounds in electronic cigarette vapors: effects of nicotine solvent and battery output voltage. *Nicotine Tob Res* 2014;16(10):1319-26. doi: 10.1093/ntr/ntu078 [published Online First: 2014/05/17]
22. Goniewicz ML, Knysak J, Gawron M, et al. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tob Control* 2014;23(2):133-9. doi: 10.1136/tobaccocontrol-2012-050859 [published Online First: 2013/03/08]
23. Wang P, Chen W, Liao J, et al. A Device-Independent Evaluation of Carbonyl Emissions from Heated Electronic Cigarette Solvents. *PLoS One* 2017;12(1):e0169811. doi: 10.1371/journal.pone.0169811 [published Online First: 2017/01/12]
24. Hill JM, Zalos G, Halcox JP, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *The New England journal of medicine* 2003;348(7):593-600. doi: 10.1056/NEJMoa022287 [published Online First: 2003/02/14]
25. Parsons VL, Moriarity C, Jonas K, et al. Design and estimation for the national health interview survey, 2006-2015. *Vital Health Stat 2* 2014(165):1-53. [published Online First: 2014/04/30]
26. Raysoni AU, Stock TH, Sarnat JA, et al. Evaluation of VOC concentrations in indoor and outdoor microenvironments at near-road schools. *Environ Pollut* 2017;231(Pt 1):681-93. doi: 10.1016/j.envpol.2017.08.065 [published Online First: 2017/08/30]
27. Boyle EB, Viet SM, Wright DJ, et al. Assessment of Exposure to VOCs among Pregnant Women in the National Children's Study. *Int J Environ Res Public Health* 2016;13(4):376. doi: 10.3390/ijerph13040376 [published Online First: 2016/04/05]
28. Jain RB. Distributions of selected urinary metabolites of volatile organic compounds by age, gender, race/ethnicity, and smoking status in a representative sample of U.S. adults. *Environ Toxicol Pharmacol* 2015;40(2):471-9. doi: 10.1016/j.etap.2015.07.018 [published Online First: 2015/08/19]

29. Sarkar M, Muhammad-Kah R, Liang Q, et al. Evaluation of spot urine as an alternative to 24h urine collection for determination of biomarkers of exposure to cigarette smoke in adult smokers. *Environ Toxicol Pharmacol* 2013;36(1):108-14. doi: 10.1016/j.etap.2013.03.001 [published Online First: 2013/04/23]
30. Benowitz NL, Jacob P. Nicotine and cotinine elimination pharmacokinetics in smokers and nonsmokers. *Clin Pharmacol Ther* 1993;53(3):316-23.
31. Fuhr U, Boettcher MI, Kinzig-Schippers M, et al. Toxicokinetics of acrylamide in humans after ingestion of a defined dose in a test meal to improve risk assessment for acrylamide carcinogenicity. *Cancer Epidemiol Biomarkers Prev* 2006;15(2):266-71. doi: 10.1158/1055-9965.epi-05-0647 [published Online First: 2006/02/24]
32. DeJarnett N, Conklin DJ, Riggs DW, et al. Acrolein exposure is associated with increased cardiovascular disease risk. *J Am Heart Assoc* 2014;3(4) doi: 10.1161/jaha.114.000934 [published Online First: 2014/08/08]
33. Alwis KU, Blount BC, Britt AS, et al. Simultaneous analysis of 28 urinary VOC metabolites using ultra high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI/MSMS). *Anal Chim Acta* 2012;750:152-60. doi: 10.1016/j.aca.2012.04.009 [published Online First: 2012/10/16]
34. Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001;89(1):E1-7. [published Online First: 2001/07/07]
35. Werner N, Nickenig G. Influence of cardiovascular risk factors on endothelial progenitor cells: limitations for therapy? *Arterioscler Thromb Vasc Biol* 2006;26(2):257-66. doi: 10.1161/01.ATV.0000198239.41189.5d [published Online First: 2005/12/03]
36. DeJarnett N, Yeager R, Conklin DJ, et al. Residential Proximity to Major Roadways Is Associated With Increased Levels of AC133+ Circulating Angiogenic Cells. *Arteriosclerosis, thrombosis, and vascular biology* 2015;35(11):2468-77. doi: 10.1161/atvbaha.115.305724 [published Online First: 2015/08/22]
37. Poredos P, Orehek M, Tratnik E. Smoking is associated with dose-related increase of intima-media thickness and endothelial dysfunction. *Angiology* 1999;50(3):201-8. [published Online First: 1999/03/24]
38. Heiss C, Amabile N, Lee AC, et al. Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production. *J Am Coll Cardiol* 2008;51(18):1760-71. doi: 10.1016/j.jacc.2008.01.040 [published Online First: 2008/05/03]
39. Flammer AJ, Anderson T, Celermajer DS, et al. The assessment of endothelial function: from research into clinical practice. *Circulation* 2012;126(6):753-67. doi: 10.1161/circulationaha.112.093245 [published Online First: 2012/08/08]
40. Benjamin EJ, Larson MG, Keyes MJ, et al. Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. *Circulation* 2004;109(5):613-9. doi: 10.1161/01.cir.0000112565.60887.1e [published Online First: 2004/02/11]
41. Vita JA. Nitric oxide-dependent vasodilation in human subjects. *Methods Enzymol* 2002;359:186-200. [published Online First: 2002/12/17]
42. Mitchell GF, Guo CY, Benjamin EJ, et al. Cross-sectional correlates of increased aortic stiffness in the community: the Framingham Heart Study. *Circulation* 2007;115(20):2628-36. doi: 10.1161/circulationaha.106.667733 [published Online First: 2007/05/09]
43. Duffy SJ, Keaney Jr JF, Holbrook M, et al. Short-and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation* 2001;104(2):151-56.

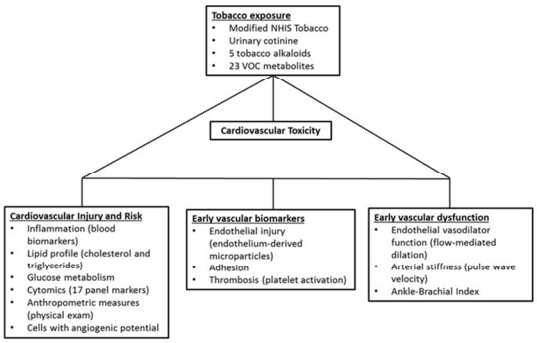
44. Benowitz NL, Dains KM, Dempsey D, et al. Racial differences in the relationship between number of cigarettes smoked and nicotine and carcinogen exposure. *Nicotine Tob Res* 2011;13(9):772-83. doi: 10.1093/ntr/ntr072 [published Online First: 2011/05/07]
45. Schoenborn CA, Gindi RM. Electronic Cigarette Use Among Adults: United States, 2014. *NCHS Data Brief* 2015(217):1-8. [published Online First: 2015/11/12]
46. Maher J, Boysun M, Rohde K, et al. Are Latinos really less likely to be smokers? Lessons from Oregon. *Nicotine & Tobacco Research* 2005;7(2):283-87. doi: 10.1080/14622200500056259
47. Barbeau EM, Krieger N, Soobader M-J. Working Class Matters: Socioeconomic Disadvantage, Race/Ethnicity, Gender, and Smoking in NHIS 2000. *Am J Public Health* 2004;94(2):269-78. doi: 10.2105/AJPH.94.2.269
48. Giskes K, Kunst AE, Benach J, et al. Trends in smoking behaviour between 1985 and 2000 in nine European countries by education. *Journal of Epidemiology and Community Health* 2005;59(5):395.

Figure 1. Cardiovascular Injury due to Tobacco Use

CITU is designed to assess how tobacco related VOC exposure contributes to cardiovascular risk factors. Our exposure measurements include a panel of 23 urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular phenotyping includes measures of injury, risk, vascular biomarkers and early vascular dysfunction. Tobacco use included use of traditional cigarettes, smokeless tobacco, waterpipe tobacco (hookah), electronic nicotine devices (ENDS), little cigars, cigarillos, pipes, cigars or any other form of tobacco that is available. Enrollment began in July 2014 and is ongoing.

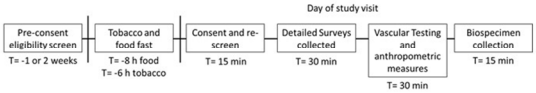
Figure 2. Study Visit Design

Study flow chart for interested participants from screening through study completion. Potential participants are pre-screened for eligibility prior to enrollment. Potential participants are asked to fast from tobacco for a minimum of 6 hours prior to the study visit. On the day of the visit the study lasts approximately 90 minute.



CITU is designed to assess how tobacco related VOC exposure contributes to cardiovascular risk factors. Our exposure measurements include a panel of 23 urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular phenotyping includes measures of injury, risk, vascular biomarkers and early vascular dysfunction. Tobacco use included use of traditional cigarettes, smokeless tobacco, waterpipe tobacco (hookah), electronic nicotine devices (ENDS), little cigars, cigarillos, pipes, cigars or any other form of tobacco that is available. Enrollment began in July 2014 and is ongoing.

108x60mm (300 x 300 DPI)



Study flow chart for interested participants from screening through study completion. Potential participants are pre-screened for eligibility prior to enrollment. Potential participants are asked to fast from tobacco for a minimum of 6 hours prior to the study visit. On the day of the visit the study lasts approximately 90 minute.

108x60mm (300 x 300 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2-3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3-4
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5, 7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5, 7
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6-7
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7-12
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*7-12*
Bias	9	Describe any efforts to address potential sources of bias	7
Study size	10	Explain how the study size was arrived at	14-16
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	12-14
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	12-14
		(*b*) Describe any methods used to examine subgroups and interactions	13
		(*c*) Explain how missing data were addressed	13
		(*d*) If applicable, explain how loss to follow-up was addressed	N/A (study protocol)
		(*e*) Describe any sensitivity analyses	13
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	N/A (study protocol)
		(b) Give reasons for non-participation at each stage	N/A (study protocol)
		(c) Consider use of a flow diagram	N/A (study protocol)
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	N/A (study protocol)
		(b) Indicate number of participants with missing data for each variable of interest	N/A (study protocol)
		(c) Summarise follow-up time (eg, average and total amount)	N/A (study protocol)
Outcome data	15*	Report numbers of outcome events or summary measures over time	N/A (study protocol)
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	N/A (study protocol)
		(*b*) Report category boundaries when continuous variables were categorized	N/A (study protocol)
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A (study protocol)
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N/A (study protocol)
Discussion			
Key results	18	Summarise key results with reference to study objectives	N/A (study protocol)
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	17
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.