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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

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| Keywords: | smoking, tobacco, electronic cigarette, cardiovascular risk, vascular injury, cigarettes |

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| 3 4 | 1 | Assessing the Impact of Tobacco-Induced Volatile Organic Compounds on |
| 5 6 | 2 | Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to |
| 7 8 9 | 3 | Tobacco Study |
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28 Word Count: 2581

30 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

45 Americans and Caucasians that is sex balanced for evaluation of self-reported tobacco

| 1 | | |
|----------------|----|--|
| 2 3 4 | 46 | exposure, VOC exposure and tobacco-induced injury profiling. Detailed information |
| 5 6 7 | 47 | about participant's demographics, health status and lifestyle is also collected. |
| 7 8 9 | 48 | Ethics and dissemination: The study protocol was approved institutional review |
| 10 11 | 49 | boards at both participating universities. All study protocols will protect participant |
| 12 13 | 50 | confidentiality. Results from the study will be disseminated via peer-reviewed journals |
| 14 15 16 | 51 | and presented at scientific conferences. |
| 17 18 | 52 | |
| 19 20 | 53 | Strengths and limitations |
| 21 22 | 54 | Young age to allow for evaluation of early stage disease (e.g. inflammation, |
| 23 24 25 | 55 | endothelial function) as opposed to end stage clinical consequence (e.g. |
| 26 27 | 56 | myocardial infarction) |
| 28 29 | 57 | Diverse tobacco product use allows for assessment of a wide range of tobacco- |
| 30 31 32 | 58 | induced VOC exposure |
| 33 34 | 59 | All study visits are in English introducing selection bias |
| 35 36 | 60 | Data will inform regulatory agencies on the cardiovascular health effects of |
| 37 38 39 | 61 | multiple tobacco products and the contribution of HPHCs |
| 40 41 | 62 | |
| 42 43 | 63 | Keywords: Tobacco, smoking, electronic cigarette, vascular injury, cardiovascular risk, |
| 44 45 | 64 | cigarettes. |
| 46 47 48 | 65 | |
| 48 49 50 | 66 | INTRODUCTION |
| 51 52 | 67 | Tobacco product use and smoking are the leading causes of preventable deaths |
| 53 54 55 | 68 | throughout the world. Of those deaths, one-third are attributed to cardiovascular disease |
| 56 57 | | 3 |
| 58 59 | | |
| 60 | | For peer review only - http://bmjopen.bmj.com/site/about/guidelines.xhtml |

(CVD)¹. The cardiovascular (CV) effects of tobacco exposure can include atherogenesis, vascular injury, thrombosis, arrhythmias and inflammation² 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 toxicity³. High levels of aldehydes are present in cigarette smoke ⁴⁵ 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 pollutants²¹. 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

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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

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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; 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.

| | 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 | >20 lifetime dips or chews and current use for the past year |
| | User | |
| | 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 |
| 139 | Study participants are | e screened prior to enrollment for current and past tobacco product |
| 140 | use. Participants are | characterized and assigned a use group based on self-reported |
| 141 | patterns collected dur | ing the study visits. |
| 142 | Overall Study Proce | dure |
| 143 | Study participa | nts fast for 8 h from food and 6 h from tobacco prior to the visit. Al |
| 144 | study visits occur befo | ore 11AM to limit effects due to circadian changes. All vascular |
| 145 | function studies are c | ompleted after 10 min of supine positioning. All vascular studies |
| 146 | are sent to the BU cer | ntral lab for analysis. BU biologic samples have minimal |
| 147 | processing and are sh | nipped overnight to the UofL central laboratory at the completion or |
| 148 | each study visit. Sam | ples obtained at UofL are processed to a similar stage, then held |
| 149 | overnight prior to anal | lysis for standardization of time to measurement for all samples. |
| 150 | • | clude a structured interview on demographics, socioeconomics, |
| 151 | | history of heart disease, allergies, and tobacco use. All surveys |
| 152 | | t in Research Electronic Data Capture (REDCap), a secure web |
| 153 | | g and managing online surveys and databases. |
| 154 | Exposure Variables | |
| 155 | Tobacco Product Use | & Particulate Matter Exposure |
| | | 7 |

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| 156 | Comprehensi | ive tobacco product exposure is assessed using a modified ve | ersion |
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| 157 | of the National Heal | th Interview survey on tobacco use ²⁶ . The survey is modified | to |
| 158 | include detailed info | rmation on electronic nicotine devices (ENDs) and other new | or |
| 159 | emerging tobacco p | roducts. Residential addresses are collected for assessment | of |
| 160 | ambient airborne pa | rticulate matter ($PM_{2.5}$) exposure and future correction of ove | rall |
| 161 | exposure. PM _{2.5} data | a from the day of the study visit, and 3 and 5 days prior to the | study |
| 162 | is collected from put | blicly available data associated with EPA monitoring stations. | Other |
| 163 | exposure variables, | including occupation, are collected through interview. | |
| 164 | VOC Measurements | | |
| 165 | Standard clea | an catch urine specimens are obtained from participants. We | have |
| 166 | developed a robust | Core Lab that utilizes mass spectrometry procedures adopted | l from |
| 167 | the Centers for Dise | ase Control and Prevention (CDC) protocols, to quantify 23 u | rinary |
| 168 | metabolites of tobac | co smoking related toxins (aldehydes and other VOCs), inclu | ding |
| 169 | acrolein ²⁷ (Table 2). The concentration values of analytes are then normalized to | | |
| 170 | urinary creatinine levels measured using Infinity Creatinine Reagent (Thermo Fisher | | her |
| 171 | Scientific, MA) on a COBAS MIRA-plus analyzer (Roche, NJ). | | |
| 172 | | | |
| Parer | nt compound | VOC metabolite | Common |
| | • | | abbr. |
| Aceta | ldehyde | Acetic acid/Acetate | ACETATE |
| Acrole | ain | N-Acetyl-S-(2-carboxyethyl)-L-cysteine | CEMA |
| ACIDIE | 2111 | | |

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Acrylamide

8

N-Acetyl-S-(3-hydroxypropyl)-L-cysteine

N-Acetyl-S-(2-carbamoylethyl)-L-cysteine

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| | N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine | GAMA |
|---|--|---------|
| Acrylonitrile | N-Acetyl-S-(2-cyanoethyl)-L-cysteine | СҮМА |
| Acrylonitrile, vinyl chloride ethylene oxide | , N-Acetyl-S-(2-hydroxyethyl)-L-cysteine | НЕМА |
| 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 |
| | N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine | DHBMA |
| 1,3-Butadiene | 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 | МНВМАЗ |
| Carbon-disulfide | 2-Thioxothiazolidine-4-carboxylic acid | TTCA |
| Crotonaldehyde | N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine | НРММА |
| 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 | NIC |
| Nicotine | Cotinine | СОТ |
| | 3-Hydroxycotinine | ЗНС |
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| Propylene oxide | N-Acetyl-S-(2-hydroxypropyl)-L-cysteine | 2HPMA | |
|--|--|----------|--|
| | N-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine + | | |
| Styrene | N-Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine | PHEMA | |
| | Mandelic acid | MA | Prote |
| Tetrachloroethylene | N-Acetyl-S-(trichlorovinyl)-L-cysteine | TCVMA | cted by |
| Toluene | N-Acetyl-S-(benzyl)-L-cysteine | BMA | y dobài |
| | N-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine | 1,2DCVMA | rotected by dopyright, including for uses related to |
| Trichloroethylene | N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine | 2,2DCVMA | ocluding |
| | N-Acetyl-S-(2,4-dimethylphenyl)-L-cysteine + | | g for u |
| | N-Acetyl-S-(2,5-dimethylphenyl)-L-cysteine + | DPMA | ses rel |
| Xylene | N-Acetyl-S-(3,4-dimethylphenyl)-L-cysteine | | ated to |
| | 2-Methylhippuric acid | 2MHA | text an |
| | 3-Methylhippuric acid + 4-Methylhippuric acid | 3MHA+ 4M | |
| 173 | | | mining |
| 174 Urine is analyzed | d for 23 metabolites of 18 parent VOCs and tobacco alkaloids by | y UPLC- | |
| 175 MS/MS. Analytes | Urine is analyzed for 23 metabolites of 18 parent VOCs and tobacco alkaloids by UPLC- Arring and similar technologies 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 10 | | |
| 176 | | | and si |
| 177 Circulating Mark | Circulating Markers of Cardiovascular Injury | | |
| | To assess tobacco product-induced cardiovascular toxicity, we examine | | |
| 178 To assess | endothelial function, inflammatory mediators, biomarkers, and thrombosis. CV risk is | | |
| | ion, inflammatory mediators, biomarkers, and thrombosis. CV ris | SK IS | ŝ. |
| 179 endothelial function | ion, inflammatory mediators, biomarkers, and thrombosis. CV ris measurements of circulating angiogenic cells, lipid profile, and g | | ës. |
| 179 endothelial function180 defined through n | | | vs. |
| endothelial function defined through n metabolism ^{25 28 2} | measurements of circulating angiogenic cells, lipid profile, and g | | S. |

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| 2 3 | 183 | biobanking. Whole blood (BD366415) is obtained for flow cytometry on fresh samples at |
| 4 5 | | |
| 6 7 | 184 | UofL pathology core. BU biologic samples have minimal processing and are shipped |
| 8 9 | 185 | overnight to the UofL central laboratory at the completion of each study visit. Samples |
| 10 11 | 186 | obtained at UofL are processed to a similar stage, then held overnight prior to analysis |
| 12 13 | 187 | to standardize the time to measurement for all samples. The UofL central laboratory, as |
| 14 15 | 188 | previously reported, will complete fasting and biomarker measurements (Table 3), with |
| 16 17 | 189 | the exception of cytomics ^{13 30} . For cytomic measurements, mononuclear cells are |
| 18 19 20 | 190 | labeled with the peripheral blood phenotyping panel kit (Fluidigm).Samples are shipped |
| 21 22 | 191 | at 4 degree C to Core Lab facilities at the University of Rochester for Mass cytometric |
| 23 24 | 192 | analysis. |
| 25 26 | 193 | Table 3 Blood analysis |
| 27 28 | | |
| 29 | | Fasting Measurements |
| 30 31 | | LDL cholesterol, HDL cholesterol, total cholesterol, triglycerides, glucose, uric acid, |
| 32 | | SAA and fibrinogen |
| 33 34 | | <u>Biomarkers</u> |
| 35 | | CAC (1-15) ¹ , Platelet-monocyte aggregates, MP (1-5) ¹ , PF4, t-PA, TxA2, Factor VII, |
| 36 37 38 | | 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 |
| 39 | | 1: Fifteen different CAP subpopulations and 5 subtypes of microparticles were |
| 40 | | measured by flow cytometry. |
| 41 42 | 194 | All participants who complete the study visit will have blood samples taken and |
| 43 44 45 | 195 | processed. Flow cytometric analysis is completed on fresh samples. All other analysis |
| 46 47 | 196 | will be completed on biobanked samples in batches LDL= low density lipoprotein. HDL= |
| 48 49 | | |
| 50 51 | 197 | high density lipoprotein. SAA= serum amyloid A. CAC= circulating angiogenic cells. |
| 52 53 | 198 | MP= microparticles. PF4= Platelet factor 4. t-PA= tissue plasminogen activator. |
| 54 55 | 199 | TxA2=Thromboxane A. IL-6= Interleukin 6. CRP= C-reactive protein. PAI-=- |
| 56 57 58 | 200 | Plasminogen activator. s- ICAM- soluble intercellular adhesion protein inhibitor. s- 11 |
| 59 60 | | For peer review only - http://bmjopen.bmj.com/site/about/guidelines.xhtml |

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

203 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.

29 212 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).

221 DATA ANALYSIS

222 We expect that from this study we will be able to identify specific biomarkers of 223 cardiovascular injury due to tobacco use and the relationship of these biomarkers to

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specific measures of tobacco exposure. For instance, we will identify which biomarkers 24 are affected by tobacco use, and which ones are most sensitive; including their dose-25 dependence. Additionally we will examine the extent to which biomarkers are 26 associated with exposure to nicotine versus exposure to HPHC of tobacco like 27 aldehydes. 28

All statistical analysis will be performed using SAS version 9.4 software (SAS 29 Institute, Inc., Cary, North Carolina), and a two-sided p-value of <0.05 will be considered 30 significant for any statistical test. Demographics and other baseline characteristics will 31 32 be summarized according to product group. The primary outcomes will be analyzed using multiple regression techniques. Appropriate Interaction variables will be tested for 33 in the regression models and subgroup analyses will be conducted according to the 34 following factors: significant interactions, sex, age, race, tobacco product group. 35 Multiple imputation method will be used for missing data where appropriate. Sensitivity 36 analysis using different analytic approaches, such as generalized linear models, as well 37 as considering different covariate adjustments, will be used to build concordant results. 38 The dose-dependence of the changes in biomarkers will be determined by 39 analyzing the data obtained from individuals that are exposed to different doses of a 40 single product (e.g. smoking 0, <15, 15-20 and >20 cigarettes per day) and by 41 comparing between tobacco products that have different doses of HPHC constituents. 42 In the US the average cigarettes per day is between 15-20³⁷ and therefore this dose 43 range distribution is reflective of general population exposure. Comparisons of the 44 45 effects of novel tobacco products and smoking will be informative of the relative toxicity 46 of the two products.

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|----------------|-----|---|
| 2 3 4 | 247 | We believe that the methods employed in the current project are exquisitely |
| 5 6 | 248 | sensitive and responsive to even low dose insults such as ambient air pollution 13 |
| 7 8 9 | 249 | allowing us to quantify tobacco product-induced changes with high precision. Moreover, |
| 9 10 11 | 250 | levels of acrolein exposure vary between different individuals due to difference in puffing |
| 12 13 | 251 | intensity and the time a cigarette is left smoldering. Thus, direct measurements of |
| 14 15 | 252 | acrolein metabolites afford better estimates of acrolein exposure than machine yields. |
| 16 17 18 | 253 | We expect to obtain wide variations in acrolein/crotonaldehyde exposure which will |
| 19 20 | 254 | enable us to construct a dose-response relationship and identify which injury |
| 21 22 | 255 | biomarkers are associated with aldehyde exposure and whether high levels of exposure |
| 23 24 25 | 256 | are associated with high levels of injury, despite similar nicotine delivery. |
| 26 27 | 257 | We consider three major factors for balancing sample selection: age, gender, |
| 28 29 | 258 | and race. Given that very few females use e-cigarette, only males will be enrolled in |
| 30 31 32 | 259 | this group. With the balanced design to determine the main effects and interactions in |
| 33 34 | 260 | selected scenarios, we justify the sample size. The analysis plan is primarily based on |
| 35 36 | 261 | evaluating the effect of tobacco exposure on endothelial function (FMD), and the main |
| 37 38 | 262 | biomarkers, EPCs, and platelet-monocyte aggregates (PMA). The sample size is |
| 39 40 41 | 263 | justified in terms of the primary dependent measure, FMD, given the potential |
| 42 43 | 264 | importance of this variable as a direct measure of the impact of tobacco exposure. The |
| 44 45 | 265 | main comparisons are between non-tobacco users and tobacco users. Due to one |
| 46 47 48 | 266 | control group, we will conservatively adjust our α (significance level) using a Bonferroni |
| 49 50 | 267 | correction, and we will set α =0.01. Based on preliminary data for FMD, we have |
| 51 52 | 268 | observed mean \pm SD in smoker and nonsmoker groups to be 4.0 \pm 1.6 and 6.8 \pm 1.0, |
| 53 54 55 | 269 | respectively. We consider at least 25% (mean FMD=3.0 from 4.0) reduction from |
| 56 57 | | 14 |
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270smokers to non-smokers is meaningful. Using a two sample, one-sided t test with an α 271of 0.01 and 80% power (1- β), assuming a common SD of 1.3, we will need 34 evaluable272subjects in each group. To examine dose response, smokers will be recruited in 3273groups (<15, 15-20 and >20 CPD). We will recruit 40 participants in each group; total274group size = 120 participants. In **Table 4** we provide estimable effect size for different275outcome measures.

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

| Variable | Non-smokers | Smokers | n | р | Ref | MØØ | |
|------------------|-------------------|------------------|----|----------|-----|--------------------|---------|
| Primary Fur | nctional Outcome | | | | | 278 | |
| FMD | 6.8 ± 1% | 4.0 ± 1.6% | 10 | <0.05 | 32 | 1.0 ²⁷⁹ | |
| Primary Bio | markers | | | | | 280 | |
| EPC | 25 ± 5 cell/ml | 10 ± 3 cells/ml | 24 | 0.037 | 38 | 3.1 ²⁸¹ | |
| PMA | 19.7 ± 8.6% | 26.6 ± 9% | 25 | 0.02 | 39 | 7.0 ²⁸² | |
| EMP | 1.1 ± 0.4 | 0.5 ± 0.2 | 32 | <0.05 | 40 | 0.23 ⁸³ | |
| Other Bioch | nemical Variables | 1 | | 0 | | 284 | PMA: |
| PF4 | 3.9 ± 1.2 IU/ml | 5.0 ± 2.6 IU/ml | 12 | <0.05 | 41 | 2.0 ²⁸⁵ | Platele |
| tPA | 3.0 ± 0.6 ng/ml | 4.3 ± 2.0 ng/ml | 20 | <0.05 | 42 | 1.6 ²⁸⁶ | _ |
| TxA ₂ | 2.2 ± 0.1 pg/ml | 3.3 ± 0.02 pg/ml | 12 | <0.05 | 43 | 0.016 | monod |
| | 1 | | I | <u> </u> | I | 288 | yte |

aggregates; EMP: Endothelial microparticles (CD62+/CD31+); MDD: minimal detectable

290 difference. Values are mean ± SD

291 ETHICS AND DISSEMINATION

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292 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. 293 No study related procedures will be completed until after participant consent. 294 Participants for the CITU study are being recruited in both Boston, MA and 295 Louisville KY. The two populations show significant differences, therefore recruitment at 296 two sites will ensure a range more reflective of the general population. Although overall 297 racial and ethnic demographics for both cities show a clear majority of Caucasians 298 (70%) and despite smokers typically male, we strive to, and currently are successful in, 299 300 recruiting a population that was gender balanced and almost evenly split between Caucasian and African Americans. Despite this balanced recruitment, e-cigarette users 301 have been reported as predominantly Caucasian and male⁴⁴, and thus far our 302 recruitment mirrors these demographics. We expect very few Hispanic/Latino's to 303 participate, due to data suggesting tobacco use, including ENDS, tends to be lower 304 among Hispanic's/Latino's ^{44 45}. Thus we have also opted to only recruit English 305 speakers. We have carefully develop our recruitment strategy and exclusion criteria to 306 protect vulnerable populations, which is important since many report a lower 307 socioeconomic status and educational level in smokers in addition to higher rates of 308 reported alcohol and drug use ^{46 47}. 309

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.

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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 gualified 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.

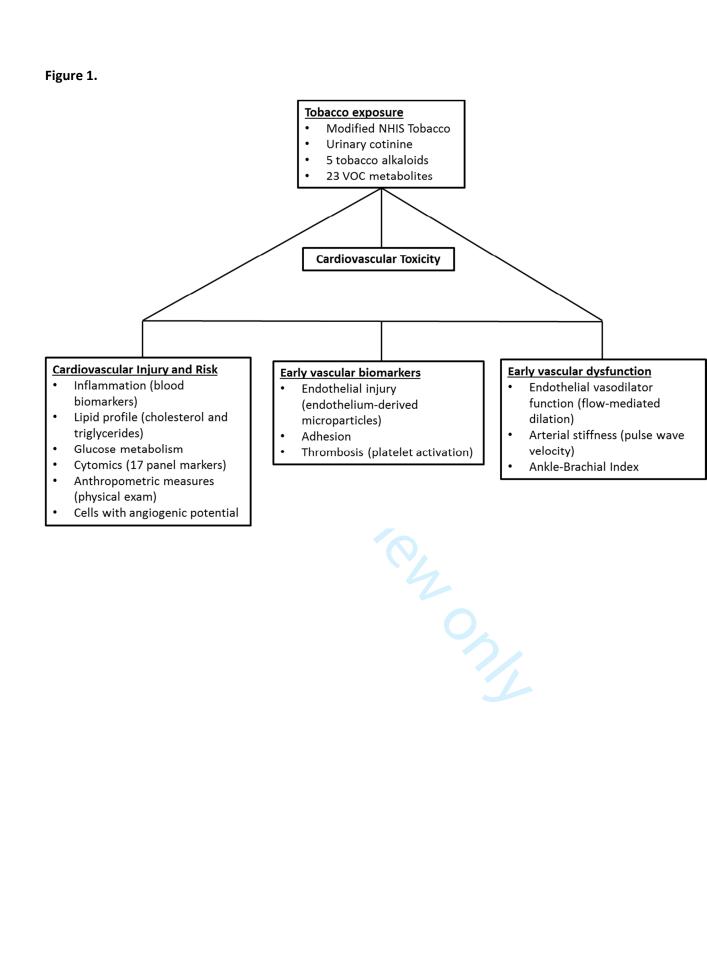
- 330 LIST OF ABBREVIATIONS
- 0 331 ABI- Ankle Brachial Index
- $\frac{2}{2}$ 332 CAC= circulating angiogenic cells
- ¹⁷ 333 CRP= C-reactive protein
- 47 334 CVD- Cardiovascular disease
- ⁴⁹ 335 ENDS- Electronic nicotine Device (i.e. e-cigarette)
- ⁵¹ 336 FACS- Fluorescence-activated cell sorting
- 337 FMD- Flow mediated dilation

| 338 | HDL= high density lipoprotein |
|-----|--|
| 339 | IL-6= Interleukin 6 |
| 340 | MMP- Matrix metalloproteinase |
| 341 | MP= micoparticles |
| 342 | PAI-=- Plasminogen activator |
| 343 | PF4= Platelet factor 4 |
| 344 | PWV- Pulse wave velocity |
| 345 | SAA= serum amyloid A |
| 346 | s-ICAM- soluble intercellular adhesion protein inhibitor |
| 347 | s-VCAM= soluble vascular adhesion protein |
| 348 | TNFR1= Tumor necrosis factor receptor 1 |
| 349 | t-PA= tissue plasminogen activator |
| 350 | TxA2=Thromboxane A |
| 351 | VOC- Volatile organic compound |
| 352 | W:H- ratio: Waist to hip ratio |
| 353 | |
| 354 | AUTHORS CONTRIBUTIONS |
| 355 | Rachel Keith- Study design, study recruitment, study visits, statistical analysis and |
| 356 | manuscript preparation. Jessica Fetterman- study recruitment, study visits, manuscript |
| 357 | preparation and editing. Dan Riggs- statistical analysis, manuscript preparation and |
| 358 | editing. Tim O'Toole- Biomarker measurements, manuscript preparation and editing. |
| 359 | Jessica Nystoriak- study recruitment and study visits. Monica Holbrook- study |
| 360 | recruitment and study visits. Pawel Lorkiewicz- VOC measurements and manuscript |
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| - 3 4 | 361 | preparation. Aruni Bhatnagar- Study design, study funding and manuscript editing. |
| 5 6 | 362 | Andrew DeFilippis- Human subject assessment planning, manuscript preparation and |
| 7 8 | 363 | editing. Naomi M. Hamburg- Study design, study funding, vascular core, manuscript |
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| Section/Topic | ltem # | 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 protoco |
| | | (e) Describe any sensitivity analyses | 13 |

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

*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

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| Manuscript ID | bmjopen-2017-019850.R1 |
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| Primary Subject Heading : | Cardiovascular medicine |
| Secondary Subject Heading: | Public health |
| Keywords: | smoking, tobacco, electronic cigarette, cardiovascular risk, vascular injury, cigarettes |
| | |

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SCHOLARONE[™] Manuscripts

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| 3 4 | 1 | Protocol to Assess the Impact of Tobacco-Induced Volatile Organic Compounds |
| 5 6 | 2 | on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to |
| 7 8 9 | 3 | Tobacco Study |
| 9 10 11 | 4 | Rachel J. Keith, Jessica L. Fetterman, Daniel W. Riggs, Tim O'Toole, Jessica Nystoriak, |
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28 Word Count: 2581

30 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

45 recruit an evenly split cohort of African Americans and Caucasians that is sex balanced

| 1 | | |
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| 2 3 4 | 46 | for evaluation of self-reported tobacco exposure, VOC exposure and tobacco-induced |
| 5 6 7 | 47 | injury profiling. Detailed information about participant's demographics, health status and |
| 7 8 9 | 48 | lifestyle is also collected. |
| 10 11 | 49 | Ethics and dissemination: The study protocol was approved institutional review |
| 12 13 14 | 50 | boards at both participating universities. All study protocols will protect participant |
| 14 15 16 | 51 | confidentiality. Results from the study will be disseminated via peer-reviewed journals |
| 17 18 | 52 | and presented at scientific conferences. |
| 19 20 | 53 | |
| 21 22 | 54 | Strengths and limitations |
| 23 24 25 | 55 | • Young age to allow for evaluation of early stage disease (e.g. inflammation, |
| 26 27 | 56 | endothelial function) as opposed to end stage clinical consequence (e.g. |
| 28 29 30 | 57 | myocardial infarction) |
| 30 31 32 | 58 | Diverse tobacco product use allows for assessment of a wide range of tobacco- |
| 33 34 | 59 | induced VOC exposure |
| 35 36 37 | 60 | All study visits are in English introducing selection bias |
| 38 39 | 61 | Data will inform regulatory agencies on the cardiovascular health effects of |
| 40 41 | 62 | multiple tobacco products and the contribution of HPHCs |
| 42 43 44 | 63 | |
| 45 46 | 64 | Keywords: Tobacco, smoking, electronic cigarette, vascular injury, cardiovascular risk, |
| 47 48 | 65 | cigarettes. |
| 49 50 51 | 66 | |
| 52 53 54 55 | 67 | INTRODUCTION |
| 56 57 58 | | 3 |
| 59 60 | | For peer review only - http://bmjopen.bmj.com/site/about/guidelines.xhtml |

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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 inflammation² 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 noncancer risk of smoking is dominated by acrolein, which contributes 40-100 times more
to risk than any other chemical present in cigarette smoke ³.

81 Although HPHCs, including VOC reactive aldehydes, have been suspected to be

82 major contributors to the toxicity of cigarette smoke for over 4 decades, their

83 contribution to CV injury and early CVD risk has not been rigorously evaluated.

84 Experimental studies in animal models suggest that because of low aldehyde-

85 metabolizing capacity, CV tissues are highly sensitive to aldehydes and exposure to low

86 levels of aldehydes can induce CV injury and accelerate CVD ⁷⁻¹⁸. The WHO Study

87 Group on Tobacco Product Regulation (TobReg) has marked acrolein, a VOC, along

88 with 8 other cigarette constituents for monitoring and regulation ¹⁹ and the U.S.

89 Environmental Protection Agency lists Acrolein as one of most hazardous air

90 pollutants²⁰. Nevertheless, the contribution of tobacco induced VOCs, including acrolein

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BMJ Open

| 1 2 | | |
|----------------|-----|---|
| 2 3 4 | 91 | or other aldehydes, toward CV toxicity in humans has not been fully assessed. Greater |
| 5 6 | 92 | understanding of how aldehydes affect cardiovascular health and disease will provide |
| 7 8 9 | 93 | new avenues for evaluating the toxicity of cigarette smoke and for assessing the |
| 9 10 11 | 94 | injurious potential of new and emerging tobacco products, such as ENDS, which may |
| 12 13 | 95 | also contain VOCs including acrolein ²¹⁻²³ . |
| 14 15 | 96 | The latency period between tobacco exposure and the development of major |
| 16 17 18 | 97 | clinical adverse health effects is long, therefore biomarkers that provide information over |
| 19 20 | 98 | a shorter period allow for the identification of harm decades before clinical outcome data |
| 21 22 | 99 | is available. Thus, in this paper we present the design and methodology of the |
| 23 24 25 | 100 | Cardiovascular Injury due To Tobacco Use (CITU) study which will evaluate the |
| 25 26 27 | 101 | association of the urinary metabolites of 18 parent VOCs from tobacco exposure with a |
| 28 29 | 102 | comprehensive set of CV biomarkers representative of early disease and predictive of |
| 30 31 32 | 103 | future CV events. ²⁴ |
| 32 33 34 | 104 | future CV events. ²⁴ METHODS AND DESIGN Overall design |
| 35 36 | 105 | Overall design |
| 37 38 | 106 | The CITU study is an investigator-initiated cross-sectional observational study of |
| 39 40 41 | 107 | around 500 healthy participants 21 to 45 years of age who are never or current tobacco |
| 42 43 | 108 | product users in two urban areas at Boston University (BU) and University of Louisville |
| 44 45 | 109 | (UofL) (Boston, MA and Louisville, KY) designed to evaluate CV toxicity due to tobacco |
| 46 47 48 | 110 | product use, with correlations to VOCs found in the tobacco products (Figure 1). |
| 48 49 50 | 111 | |
| 51 52 | 112 | |
| 53 54 | 113 | Participant Eligibility Criteria |
| 55 56 57 | | <u>_</u> |
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| | 114 | The goal of the study is to examine the impact of tobacco products on healthy |
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| | 115 | young adults who could be classified as a current tobacco product users (Defined in |
| | 116 | table 1), or never-users (does not have lifetime use of any tobacco product). |
|) I | 117 | Participants were self-reported to be healthy therefore we excluded participants if they |
| 2 3 | 118 | had: 1) diagnosis of clinical cardiovascular disease including but not limited to known |
| 4 5 | 119 | heart attack, peripheral artery disease, heart failure or stroke; 2) diagnosis of diabetes |
| 5 7 3 | 120 | (HbA1c >7.0 or treatment for diabetes), hypertension (systolic blood pressure >140 mm |
|)) | 121 | Hg or diastolic blood pressure >90 mm Hg), hypothyroidism or hyperthyroidism, |
| 1 2 | 122 | inflammatory conditions such as lupus or inflammatory bowel disease, HIV/AIDS, |
| 3 1 5 | 123 | hepatitis, liver disease, anemia, cancer of any type or another medical condition that |
| 5 7 | 124 | might compromise the successful completion of the study; 2) recipients of organ |
| 3 | 125 | transplant or renal replacement therapy; 3) individuals that are taking the following |
|) > | 126 | medications: immunosuppressant agents estrogen, testosterone, anti TNF agents, |
| <u>2</u> 3 1 | 127 | certain biologics, Procrit, statins, beta-blockers or other cardiovascular medicine; 4) |
| 5 | 128 | individuals using nutraceuticals or anabolic steroids beyond the recommended daily |
| 7 3 | 129 | allowance; 5) body weight less than 100 pounds; 6) pregnant women; 7) prisoners and |
| 9) | 130 | other vulnerable populations; and 8) active illness or infection. Participants are |
| <u>2</u> 3 | 131 | rescheduled or considered screen-failures and excluded from the study if symptomatic |
| 4 5 | 132 | of an acute illness, i.e. viral upper respiratory infection, on study date. |
| 5 7 | 133 | 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 |

| 1 2 | | | |
|----------------|-----|--------------------------|---|
| 3 4 | | ENDS User | >20 lifetime vape sessions and current use for the past year |
| 5 | | Hookah User | >20 lifetime hookah sessions and current use for the past year |
| 6 | 134 | Study participants are | screened prior to enrollment for current and past tobacco product |
| 7 8 9 | 135 | use. Participants are c | haracterized and assigned a use group based on self-reported |
| 10 11 | 136 | patterns collected duri | ng the study visits. |
| 12 13 14 | 137 | Overall Study Procee | lure |
| 15 16 | 138 | Study participar | nts fast for 8 h from food and 6 h from tobacco prior to the visit. All |
| 17 18 | 139 | study visits occur befo | re 11AM to limit effects due to circadian changes. All vascular |
| 19 20 21 | 140 | function studies are co | mpleted after 10 min of supine positioning. All vascular studies |
| 21 22 23 | 141 | are sent to the BU cen | tral lab for analysis. BU biologic samples have minimal |
| 24 25 | 142 | processing and are sh | ipped overnight to the UofL central laboratory at the completion of |
| 26 27 | 143 | each study visit. Samp | les obtained at UofL are processed to a similar stage, then held |
| 28 29 30 | 144 | overnight prior to analy | vsis for standardization of time to measurement for all samples. |
| 31 32 | 145 | Study visits tak | e approximately 90 minutes to complete and include a structured |
| 33 34 | 146 | interview on demograp | phics, socioeconomics, lifestyle, health, family history of heart |
| 35 36 37 | 147 | disease, allergies, and | tobacco use. (Figure 2) Participants were compensated |
| 37 38 39 | 148 | appropriately for their | time. All surveys are collected and kept in Research Electronic |
| 40 41 | 149 | Data Capture (REDCa | p), a secure web application for building and managing online |
| 42 43 | 150 | surveys and database | S. |
| 44 45 46 | 151 | Exposure Variables | |
| 47 48 | 152 | Tobacco Product Use | & Particulate Matter Exposure |
| 49 50 | 153 | Comprehensive | tobacco product exposure is assessed using a modified version |
| 51 52 53 | 154 | of the National Health | Interview survey on tobacco use ²⁵ . The survey is modified to |
| 55 54 55 | 155 | include detailed inform | ation on electronic nicotine devices (ENDs) and other new or |
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| 58 59 60 | | For peer re | eview only - http://bmjopen.bmj.com/site/about/guidelines.xhtml |
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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.

.61 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
acrolein²⁶ (**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)

| | | Common |
|-----------------|--|---------|
| Parent compound | VOC metabolite | 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 | СҮМА |

| Anabasine Anatabine Benzene 1-Bromopropane 1,3-Butadiene | Anabasine (free) Anatabine (free) N-Acetyl-S-(phenyl)-L-cysteine trans, trans-Muconic acid N-Acetyl-S-(n-propyl)-L-cysteine N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine | ANB ANTB PMA MU BPMA DHBMA MHBMA1 |
|--|---|---|
| Benzene 1-Bromopropane | N-Acetyl-S-(phenyl)-L-cysteine trans, trans-Muconic acid N-Acetyl-S-(n-propyl)-L-cysteine N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine | PMA MU BPMA DHBMA |
| 1-Bromopropane | trans, trans-Muconic acid N-Acetyl-S-(n-propyl)-L-cysteine N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine | MU BPMA DHBMA |
| 1-Bromopropane | N-Acetyl-S-(n-propyl)-L-cysteine N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine | BPMA DHBMA |
| | N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine | DHBMA |
| 1,3-Butadiene | N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine | |
| 1,3-Butadiene | | MHBMA1 |
| r,o-Dutadiche | | |
| · | 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 | NIC |
| Nicotine | Cotinine | СОТ |
| | 3-Hydroxycotinine | 3HC |
| Propylene oxide | N-Acetyl-S-(2-hydroxypropyl)-L-cysteine | 2HPMA |
| Styrene | N-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine + | PHEMA |

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| | 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 | |
| T (| N-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine | 1,2DCVMA | |
| Trichloroethylene | N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine | 2,2DCVMA | |
| | N-Acetyl-S-(2,4-dimethylphenyl)-L-cysteine + | | |
| | N-Acetyl-S-(2,5-dimethylphenyl)-L-cysteine + | DPMA | |
| Xylene | N-Acetyl-S-(3,4-dimethylphenyl)-L-cysteine | | |
| | 2-Methylhippuric acid | 2MHA | |
| | 3-Methylhippuric acid + 4-Methylhippuric acid | 3MHA+ 4Mł | |
| 170 | | | |
| 171 Urine is analyzed | for 23 metabolites of 18 parent VOCs and tobacco alkalo | ids by UPLC- | |
| 172 MS/MS. Analytes | are listed as parent, metabolite and their common abbrev | iation. | |
| 173 | | | |
| 174 Circulating Mark | ers of Cardiovascular Injury | | |
| 175 To assess | تع To assess tobacco product-induced cardiovascular toxicity, we examine | | |
| 176 endothelial function | endothelial function, inflammatory mediators, biomarkers, and thrombosis. CV risk is | | |
| 177 defined through m | defined through measurements of circulating angiogenic cells, lipid profile, and glucose | | |
| 178 metabolism ²⁴ ²⁷ ²⁶ | 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) | | |
| 179 samples are obta | م samples are obtained from all participants for laboratory testing and long term | | |
| 180 biobanking. Whole | biobanking. Whole blood (BD366415) is obtained for flow cytometry on fresh samples at | | |
| 181 UofL pathology co | UofL pathology core. BU biologic samples have minimal processing and are shipped | | |
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| 1 2 | | |
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| 2 3 4 | 182 | overnight to the UofL central laboratory at the completion of each study visit. Samples |
| 5 6 | 183 | obtained at UofL are processed to a similar stage, then held overnight prior to analysis |
| 7 8 9 | 184 | to standardize the time to measurement for all samples. The UofL central laboratory, as |
| 10 11 | 185 | previously reported, will complete fasting and biomarker measurements (Table 3), with |
| 12 13 | 186 | the exception of cytomics ^{12 29} . For cytomic measurements, mononuclear cells are |
| 14 15 16 | 187 | labeled with the peripheral blood phenotyping panel kit (Fluidigm). Samples are shipped |
| 16 17 18 | 188 | at 4 degree C to Core Lab facilities at the University of Rochester for Mass cytometric |
| 19 20 | 189 | at 4 degree C to Core Lab facilities at the University of Rochester for Mass cytometric analysis. |
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190 Table 3 Blood analysis

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Fasting Measurements

LDL cholesterol, HDL cholesterol, total cholesterol, triglycerides, glucose, uric acid, SAA and fibrinogen

<u>Biomarkers</u>

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.

²¹ 192 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=
 will be completed on biobanked samples in batches LDL= low density lipoprotein. HDL=
- $\frac{26}{27}$ 194 high density lipoprotein. SAA= serum amyloid A. CAC= circulating angiogenic cells.
- $^{28}_{29}$ 195 *MP= microparticles. PF4= Platelet factor 4. t-PA= tissue plasminogen activator.*
- ³⁰ ³¹ 196 *TxA2=Thromboxane A. IL-6= Interleukin 6. CRP= C-reactive protein. PAI-=-*
- ³³ 197 Plasminogen activator. s- ICAM- soluble intercellular adhesion protein inhibitor. s-
- $_{36}^{35}$ 198 VCAM= soluble vascular adhesion protein. TNFR1= Tumor necrosis factor receptor 1.
- ³⁷
 ³⁸ 199 *MMP- Matrix metalloproteinase.*³⁹
- 40 200 Non-Invasive Vascular Function Testing
 41

42 Smoking, is associated with endothelial damage and vascular dysfunction ^{30 31}. 201 43 44 Endothelial cells are exposed to circulating toxins and measures of endothelial function 202 45 46 are reflective of cardiovascular injury ³². Thus, we examine the non-invasive endothelial 47 203 48 vasodilator function using flow-mediated vasodilation ^{33 34}, arterial stiffness with carotid-49 204 50 51 femoral and carotid-radial pulse wave velocity ³⁵, and peripheral vascular function with 205 52 53 ankle brachial index. Flow mediated dilation was assessed with a 7.5MHZ ultrasound 206 54 55 56 probe is used to image the brachial artery while a 10cm blood pressure cuff is attached 207 57 12 58

¹⁹ 191 All participants who complete the study visit will have blood samples taken and

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208 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 209 capturing software, the blood pressure cuff is inflated to 200mmHg or 50mmHg higher 210 than the systolic pressure. After the 5 minute occlusion, the cuff is released and the 211 NIHEM software records two minutes of imaging. Images were analyzed by a single 212 blinded analyzer using MIA vascular Research Tolls Brachial Analyzer for Research, 213 version 6.8.5. All vascular imagers where trained at BU who have a previously reported 214 reproducibility with intra- and inter-observer correlation coefficients of 0.98 and 0.99 for 215 brachial diameter and 0.78 and 0.92 for FMD.³⁶ Similar equipment and software is used 216 at both sites. All vascular studies are sent to the BU central lab for analysis. 217 Anthropometric measures 218

Anthropometric measures included height, weight, waist and hip circumference 219 and body fat. All anthropometric measures are completed twice and the average 220 recorded. Standing height measurements are completed on a fixed stadiometer. Weight 221 measurements are completed on a digital scale to the nearest tenth of a pound. Waist 222 circumference is measured at the level of the umbilicus to the nearest tenth of a 223 centimeter. Hip circumference is measured at the maximal protrusion of the gluteal 224 muscle to the nearest tenth of a centimeter. Body fat percentage is calculated by the 225 bioelectrical impedance measured with the Omron fat loss monitor (HBF-306C). 226

227 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

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data mining, AI training, and similar technologies

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are affected by tobacco use, and which ones are most sensitive; including their dosedependence. 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 between 15-20³⁷ 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.

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| 3 4 | 253 | We believe that the methods employed in the current project are exquisitely |
| 5 6 | 254 | sensitive and responsive to even low dose insults such as ambient air pollution 12 |
| 7 8 | 255 | allowing us to quantify tobacco product-induced changes with high precision. Moreover, |
| 9 10 11 | 256 | levels of acrolein exposure vary between different individuals due to difference in puffing |
| 12 13 | 257 | intensity and the time a cigarette is left smoldering. Thus, direct measurements of |
| 14 15 | 258 | acrolein metabolites afford better estimates of acrolein exposure than machine yields. |
| 16 17 | 259 | We expect to obtain wide variations in acrolein/crotonaldehyde exposure which will |
| 18 19 20 | 260 | enable us to construct a dose-response relationship and identify which injury |
| 20 21 22 | 261 | biomarkers are associated with aldehyde exposure and whether high levels of exposure |
| 23 24 | 262 | are associated with high levels of injury, despite similar nicotine delivery. |
| 25 26 | 263 | Sample size |
| 27 28 29 | 264 | The sample size is justified in terms of the primary dependent measure, FMD, |
| 30 31 | 265 | given the potential importance of this variable as a direct measure of the impact of |
| 32 33 | 266 | tobacco exposure. The main comparisons are between non-tobacco users and tobacco |
| 34 35 36 | 267 | users. Due to one control group, we will conservatively adjust our α (significance level) |
| 37 38 | 268 | using a Bonferroni correction, and we will set α =0.01. Based on preliminary data for |
| 39 40 | 269 | FMD, we have observed mean \pm SD in smoker and nonsmoker groups to be 4.0 \pm 1.6 |
| 41 42 | 209 | This, we have observed mean ± 05 in shoker and honsmoker groups to be 4.0 ±1.0 |
| 43 44 | 270 | and 6.8 ±1.0, respectively. We consider at least 25% (mean FMD=3.0 from 4.0) |
| 45 46 | 271 | reduction from smokers to non-smokers is meaningful. Using a two sample, one-sided t |
| 47 48 | 272 | test with an α of 0.01 and 80% power (1- β), assuming a common SD of 1.3, we will |
| 49 50 | 273 | need 34 evaluable subjects in each group. We will recruit a total of 120 tobacco using |
| 51 52 | 274 | participants per site. This over sampling will allow us to look at multiple endpoints and |
| 53 54 55 | 275 | for associations with VOCs. |
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276 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 male³⁸, 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

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requested by the participant. Participant information is de-identified for analysis and 98 reported in aggregate to protect privacy. 99

Completion of these studies will enable a greater understanding of the biological 00 responses to use of a variety of tobacco products. Specifically, they will help to identify 01 the constituents of these products; and how a panel of exposure and CV injury 02 biomarkers are associated with these different constituents. This data will be available 03 to the FDA and could help guide new policy measures to reduce or eliminate the 04 harmful components of tobacco smoke and other nicotine products. The study is 05 06 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 07 the initial full-length manuscript publications of the resources generated with funding 808 from this program, the study will make them available to interested and qualified 09 investigators upon written request. The study will provide relevant protocols of published 10 data, upon request (presuming prior publication by the Center members). Participants 11 will be provided a summary of the results as they become available. Finally press 12 releases of relevant findings will inform the general population. 13 14 LIST OF ABBREVIATIONS 15 ABI- Ankle Brachial Index 16 17 CAC= circulating angiogenic cells CRP= C-reactive protein 18

- 19 CVD- Cardiovascular disease
- 20 ENDS- Electronic nicotine Device (i.e. e-cigarette)

| 1 2 | | |
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| 2 3 4 | 321 | FACS- Fluorescence-activated cell sorting |
| 5 6 7 8 | 322 | FMD- Flow mediated dilation |
| | 323 | HDL= high density lipoprotein |
| 9 10 11 | 324 | IL-6= Interleukin 6 |
| 12 13 | 325 | MMP- Matrix metalloproteinase |
| 14 15 | 326 | MP= micoparticles |
| 16 17 18 | 327 | PAI-=- Plasminogen activator |
| 19 20 | 328 | PF4= Platelet factor 4 |
| 21 22 | 329 | PWV- Pulse wave velocity |
| 23 24 | 330 | SAA= serum amyloid A |
| 25 26 27 | 331 | s-ICAM- soluble intercellular adhesion protein inhibitor |
| 28 29 | 332 | s-VCAM= soluble vascular adhesion protein |
| 30 31 | 333 | TNFR1= Tumor necrosis factor receptor 1 |
| 32 33 34 | 334 | t-PA= tissue plasminogen activator |
| 34 35 36 37 38 39 40 41 | 335 | TxA2=Thromboxane A |
| | 336 | VOC- Volatile organic compound |
| | 337 | W:H- ratio: Waist to hip ratio |
| 42 43 | 338 | |
| 44 45 | 339 | AUTHORS CONTRIBUTIONS |
| 46 47 | 340 | Rachel Keith- Study design, study recruitment, study visits, statistical analysis and |
| 48 49 50 51 52 53 54 | 341 | manuscript preparation. Jessica Fetterman- study recruitment, study visits, manuscript |
| | 342 | preparation and editing. Dan Riggs- statistical analysis, manuscript preparation and |
| | 343 | editing. Tim O'Toole- Biomarker measurements, manuscript preparation and editing. |
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| 3 4 | 344 | Jessica Nystoriak- study recruitment and study visits. Monica Holbrook- study |
| 5 6 | 345 | recruitment and study visits. Pawel Lorkiewicz- VOC measurements and manuscript |
| 7 8 9 | 346 | preparation. Aruni Bhatnagar- Study design, study funding and manuscript editing. |
| 10 11 | 347 | Andrew DeFilippis- Human subject assessment planning, manuscript preparation and |
| 12 13 | 348 | editing. Naomi M. Hamburg- Study design, study funding, vascular core, manuscript |
| 14 15 16 | 349 | preparation and editing. |
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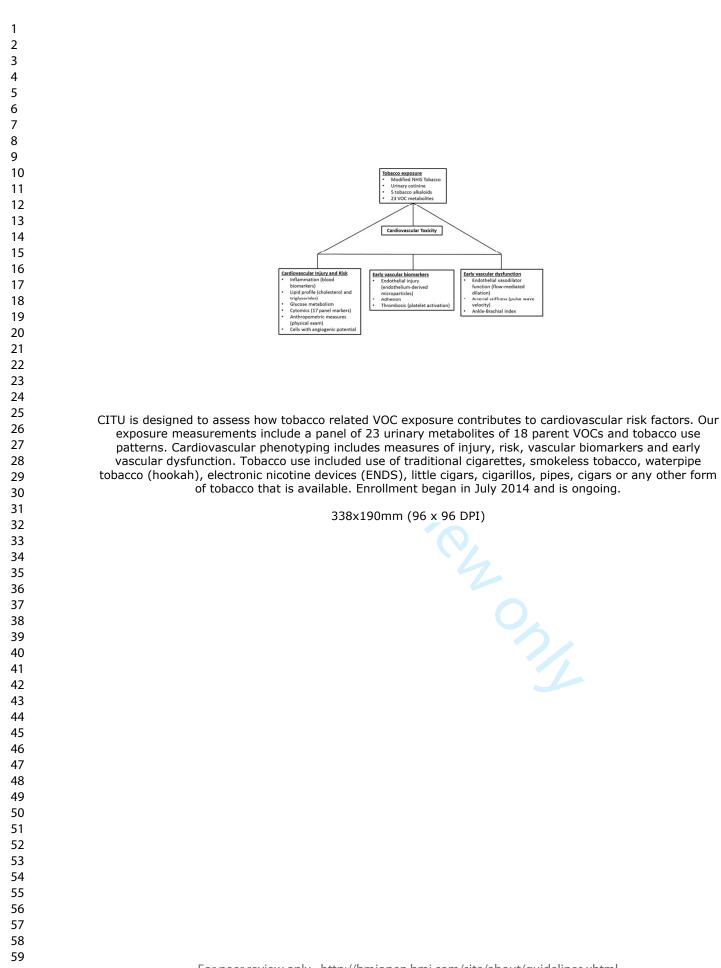
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Figure 1. Cardiovascular Injury due to Tobacco Use Figure 1. Cardiovascular Injury due to Tobacco Use

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.

33 34 492 Figure 2. Study Visit Design

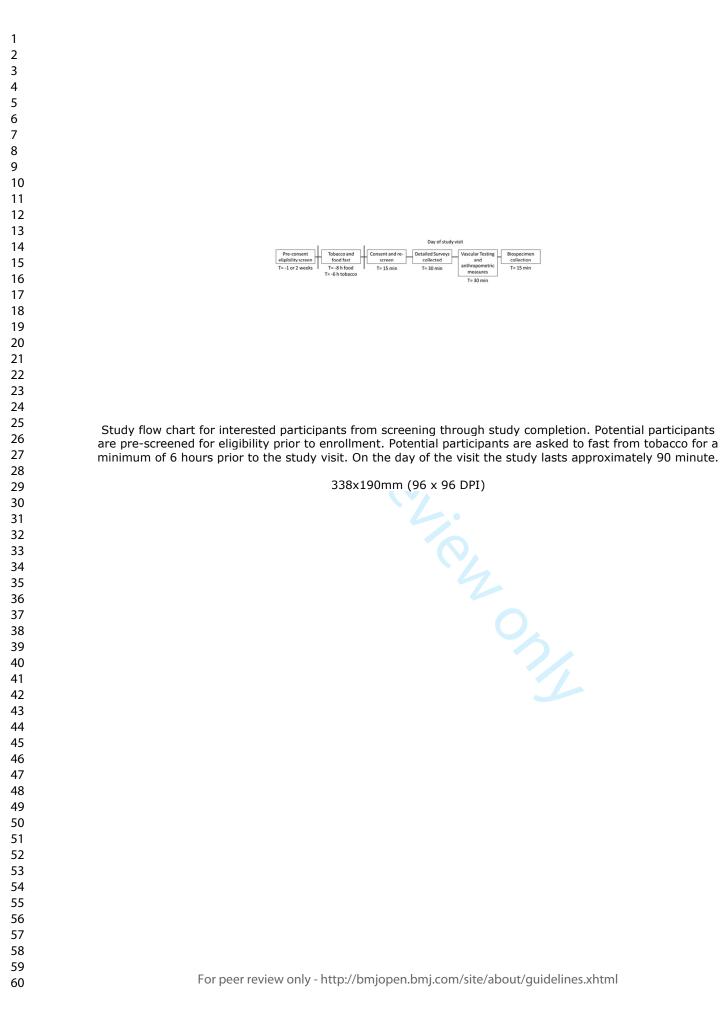
- Study flow chart for interested participants from screening through study completion.
 Study flow chart for interested participants from screening through study completion.
 Potential participants are pre-screened for eligibility prior to enrollment. Potential participants are asked to fast from tobacco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



Page 24 of 26

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| Section/Topic | ltem # | 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 | Sgit Present iter paper 5 Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data 5 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 | | 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 1 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 protoco |
| | | (e) Describe any sensitivity analyses | 13 |

| Page 2 | 6 of 26 |
|--------|---------|
|--------|---------|

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

*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.

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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

| | 1 |
|--------------------------------------|---|
| 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 BeFilippis, Andrew; 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 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 |
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| 3 4 | 1 | Protocol to Assess the Impact of Tobacco-Induced Volatile Organic Compounds |
| 5 6 | 2 | on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to |
| 7 8 9 | 3 | Tobacco Study |
| 9 10 11 | 4 | Rachel J. Keith, Jessica L. Fetterman, Daniel W. Riggs, Tim O'Toole, Jessica Nystoriak, |
| 12 13 | 5 | Monica Holbrook, Pawel Lorkiewicz, Aruni Bhatnagar, Andrew DeFilippis*, Naomi M. |
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26 Boston University School of Medicine Boston, MA USA (co-senior author)

28 Word Count: 2581

30 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

45 recruit an evenly split cohort of African Americans and Caucasians that is sex balanced

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| 2 3 | | for evolveting of ealf responsed to be an even we have a set to be and to be and to be a set in durand |
| 4 | 46 | for evaluation of self-reported tobacco exposure, VOC exposure and tobacco-induced |
| 5 6 7 | 47 | injury profiling. Detailed information about participant's demographics, health status and |
| 7 8 9 | 48 | lifestyle is also collected. |
| 10 11 | 49 | Ethics and dissemination: The study protocol was approved institutional review |
| 12 13 14 | 50 | boards at both participating universities. All study protocols will protect participant |
| 15 16 | 51 | confidentiality. Results from the study will be disseminated via peer-reviewed journals |
| 17 18 | 52 | and presented at scientific conferences. |
| 19 20 | 53 | |
| 21 22 | 54 | Strengths and limitations |
| 23 24 25 | 55 | Young age to allow for evaluation of early stage disease (e.g. inflammation, |
| 26 27 | 56 | endothelial function) as opposed to end stage clinical consequence (e.g. |
| 28 29 30 | 57 | myocardial infarction) |
| 31 32 | 58 | Diverse tobacco product use allows for assessment of a wide range of tobacco- |
| 33 34 | 59 | induced VOC exposure |
| 35 36 37 | 60 | All study visits are in English introducing selection bias |
| 38 39 | 61 | Data will inform regulatory agencies on the cardiovascular health effects of |
| 40 41 42 | 62 | multiple tobacco products and the contribution of HPHCs |
| 43 | 63 | |
| 44 45 46 | 64 | Keywords: Tobacco, smoking, electronic cigarette, vascular injury, cardiovascular risk, |
| 47 48 | 65 | cigarettes. |
| 49 50 51 | 66 | |
| 52 53 54 55 | 67 | INTRODUCTION |
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| 59 60 | | For peer review only - http://bmjopen.bmj.com/site/about/guidelines.xhtml |
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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 noncancer risk of smoking is dominated by acrolein, which contributes 40-100 times more
to risk than any other chemical present in cigarette smoke ³.

81 Although HPHCs, including VOC reactive aldehydes, have been suspected to be

82 major contributors to the toxicity of cigarette smoke for over 4 decades, their

83 contribution to CV injury and early CVD risk has not been rigorously evaluated.

84 Experimental studies in animal models suggest that because of low aldehyde-

85 metabolizing capacity, CV tissues are highly sensitive to aldehydes and exposure to low

86 levels of aldehydes can induce CV injury and accelerate CVD ⁷⁻¹⁸. The WHO Study

- 87 Group on Tobacco Product Regulation (TobReg) has marked acrolein, a VOC, along
- 88 with 8 other cigarette constituents for monitoring and regulation ¹⁹ and the U.S.
- 89 Environmental Protection Agency lists Acrolein as one of most hazardous air

90 pollutants²⁰. Nevertheless, the contribution of tobacco induced VOCs, including acrolein

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| | J Ope |
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| or other aldehydes, toward CV toxicity in humans has not been fully assessed. Greater | J Open: first published as 10.1136/bmjopen-2017-019850 on Protected by copyright, including |
| understanding of how aldehydes affect cardiovascular health and disease will provide | t publi |
| new avenues for evaluating the toxicity of cigarette smoke and for assessing the | shed a |
| injurious potential of new and emerging tobacco products, such as ENDS, which may | Prot |
| also contain VOCs including acrolein ²¹⁻²³ . | ected |
| The latency period between tobacco exposure and the development of major | hjopen by cop |
| clinical adverse health effects is long, therefore biomarkers that provide information over | -2017- yright, |
| a shorter period allow for the identification of harm decades before clinical outcome data | incluc |
| is available. Thus, in this paper we present the design and methodology of the | Protected by copyright, including for |
| Cardiovascular Injury due To Tobacco Use (CITU) study which will evaluate the | Ensei r uses r |
| association of the urinary metabolites of 18 parent VOCs from tobacco exposure with a | relatec |
| comprehensive set of CV biomarkers representative of early disease and predictive of | to text |
| future CV events. ²⁴ | t Superieu text and |
| METHODS AND DESIGN | data m |
| Overall design | ining, , |
| The CITU study is an investigator-initiated cross-sectional observational study of | Al trair |
| around 500 healthy participants 21 to 45 years of age who are never or current tobacco | aining, and similar technologies |
| product users in two urban areas at Boston University (BU) and University of Louisville | nd sim |
| (UofL) (Boston, MA and Louisville, KY) designed to evaluate CV toxicity due to tobacco | ilar teo |
| product use, with correlations to VOCs found in the tobacco products (Figure 1). | chnolo |
| | gies. |
| | Al training, and similar technologies. |
| Participant Eligibility Criteria | |
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|----------------|-----|--|
| - 3 1 | 114 | The goal of the study is to examine the impact of tobacco products on healthy |
| 5 | 115 | young adults who could be classified as a current tobacco product users (Defined in |
| 7 3 | 116 | table 1), or never-users (does not have lifetime use of any tobacco product). |
| 9 0 1 | 117 | Participants were self-reported to be healthy therefore we excluded participants if they |
| 2 3 | 118 | had: 1) diagnosis of clinical cardiovascular disease including but not limited to known |
| 4 5 | 119 | heart attack, peripheral artery disease, heart failure or stroke; 2) diagnosis of diabetes |
| 6 7 8 | 120 | (HbA1c >7.0 or treatment for diabetes), hypertension (systolic blood pressure >140 mm |
| 19 20 | 121 | Hg or diastolic blood pressure >90 mm Hg), hypothyroidism or hyperthyroidism, |
| 21 22 | 122 | inflammatory conditions such as lupus or inflammatory bowel disease, HIV/AIDS, |
| 23 24 25 | 123 | hepatitis, liver disease, anemia, cancer of any type or another medical condition that |
| 26 27 | 124 | might compromise the successful completion of the study; 2) recipients of organ |
| 28 29 | 125 | transplant or renal replacement therapy; 3) individuals that are taking the following |
| 30 31 | 126 | medications: immunosuppressant agents estrogen, testosterone, anti TNF agents, |
| 32 33 34 | 127 | certain biologics, Procrit, statins, beta-blockers or other cardiovascular medicine; 4) |
| 85 86 | 128 | individuals using nutraceuticals or anabolic steroids beyond the recommended daily |
| 37 38 | 129 | allowance; 5) body weight less than 100 pounds; 6) pregnant women; 7) prisoners and |
| 39 10 | 130 | other vulnerable populations; and 8) active illness or infection. Participants are |
| 41 42 43 | 131 | rescheduled or considered screen-failures and excluded from the study if symptomatic |
| 14 15 | 132 | of an acute illness, i.e. viral upper respiratory infection, on study date. |
| 16 17 | 133 | Table 1. Tobacco product use classifications |
| 10 | | |

| 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 |

| 1 2 | | | |
|--|-----|---------------------------|---|
| 3 4 | | ENDS User | >20 lifetime vape sessions and current use for the past year |
| 5 | | Hookah User | >20 lifetime hookah sessions and current use for the past year |
| 6 | 134 | Study participants are | screened prior to enrollment for current and past tobacco product |
| 7 8 9 | 135 | use. Participants are c | haracterized and assigned a use group based on self-reported |
| 10 11 | 136 | patterns collected duri | ng the study visits. |
| 12 13 14 | 137 | Overall Study Proced | ure |
| 15 16 | 138 | Study participar | ts fast for 8 h from food and 6 h from tobacco prior to the visit. All |
| 17 18 | 139 | study visits occur befor | e 11AM to limit effects due to circadian changes. All vascular |
| 19 20 21 | 140 | function studies are co | mpleted after 10 min of supine positioning. All vascular studies |
| 21 22 23 | 141 | are sent to the BU cen | tral lab for analysis. BU biologic samples have minimal |
| 24 25 | 142 | processing and are shi | pped overnight to the UofL central laboratory at the completion of |
| 26 27 28 29 30 31 32 | 143 | each study visit. Samp | les obtained at UofL are processed to a similar stage, then held |
| | 144 | overnight prior to analy | rsis for standardization of time to measurement for all samples. |
| | 145 | Study visits tak | e approximately 90 minutes to complete and include a structured |
| 33 34 | 146 | interview on demograp | hics, socioeconomics, lifestyle, health, family history of heart |
| 35 36 37 | 147 | disease, allergies, and | tobacco use. (Figure 2) Participants were compensated |
| 38 39 | 148 | appropriately for their t | ime. All surveys are collected and kept in Research Electronic |
| 40 41 | 149 | Data Capture (REDCa | p), a secure web application for building and managing online |
| 42 43 44 | 150 | surveys and databases | 6. |
| 44 45 46 | 151 | Exposure Variables | |
| 47 48 | 152 | Tobacco Product Use | & Particulate Matter Exposure |
| 49 50 | 153 | Comprehensive | tobacco product exposure is assessed using a modified version |
| 51 52 53 | 154 | of the National Health | Interview survey on tobacco use ²⁵ . The survey is modified to |
| 54 55 | 155 | include detailed inform | ation on electronic nicotine devices (ENDs) and other new or |
| 56 57 58 | | | 7 |
| 59 60 | | For peer re | view only - http://bmjopen.bmj.com/site/about/guidelines.xhtml |

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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.

161 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 VOCs²⁸. 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 collections²⁹. 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 risk³².

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ACETATE

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| 470 | Ma have de | valanad a robust Care Lab that utilizes made an attended | |
|--------------------------------|---|---|---------|
| 178 | We have developed a robust Core Lab that utilizes mass spectrometry | | |
| 179 | procedures adopte | d from the Centers for Disease Control and Prevention (Cl | DC) |
| 180 | protocols, to quant | ify 23 urinary metabolites of tobacco smoking related toxin | S |
| 181 | (aldehydes and oth | ner VOCs), including acrolein ³³ (Table 2). The concentration | on valu |
| 182 | analytes are then r | normalized to urinary creatinine levels measured using Infir | nity |
| 183 | Creatinine Reagen | t (Thermo Fisher Scientific, MA) on a COBAS MIRA-plus a | analyz |
| 184 | (Roche, NJ). | | |
| 185 | Table 2 Exposure | Variables (Please see end of article) | |
| Daras | at compound | VOC motobalita | (|
| Paren | nt compound | VOC metabolite | ê |
| Aceta | ldehyde | Acetic acid/Acetate | ŀ |
| Acrole | ain | N-Acetyl-S-(2-carboxyethyl)-L-cysteine | (|
| | 511 | N-Acetyl-S-(3-hydroxypropyl)-L-cysteine | 3 |
| Acryla | amide | N-Acetyl-S-(2-carbamoylethyl)-L-cysteine | ŀ |
| | annue | N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine | (|
| Acrylc | onitrile | N-Acetyl-S-(2-cyanoethyl)-L-cysteine | (|
| Acrylonitrile, vinyl chloride, | | , N-Acetyl-S-(2-hydroxyethyl)-L-cysteine | ŀ |
| ethyle | ene oxide | | ľ |
| Anabasine | | Anabasine (free) | ŀ |
| Anatabine | | Anatabine (free) | ŀ |
| 5 | | N-Acetyl-S-(phenyl)-L-cysteine | F |
| Benze | | trans, trans-Muconic acid | P |

| I-Bromopropane | N-Acetyl-S-(n-propyl)-L-cysteine | BPMA |
|-----------------------|--|---|
| | N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine | DHBMA |
| I,3-Butadiene | N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine | MHBMA1 |
| , 3-Dulaulene | 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 | MHBMA2 MHBMA3 TTCA HPMMA ATCA AMCC |
| Ethylbenzene, styrene | Phenylglyoxylic acid | PGA |
| Formaldehyde | Formate | FORMATE |
| | Nicotine | NIC |
| Nicotine | Cotinine | СОТ |
| | 3-Hydroxycotinine | 3HC |
| Propylene oxide | N-Acetyl-S-(2-hydroxypropyl)-L-cysteine | 2HPMA |
| | N-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine + | PHEMA |
| Styrene | N-Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine | |
| | Mandelic acid | MA |
| Fetrachloroethylene | N-Acetyl-S-(trichlorovinyl)-L-cysteine | TCVMA |
| Foluene | N-Acetyl-S-(benzyl)-L-cysteine | BMA |
| Frichloroothydoro | N-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine | 1,2DCVMA |
| Frichloroethylene | N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine | 2,2DCVMA |

| | | N-Acetyl-S-(2,4-dimethylphenyl)-L-cysteine + | | |
|-------|---|--|----------------------|--|
| | | N-Acetyl-S-(2,5-dimethylphenyl)-L-cysteine + | DPMA | |
| Xyler | e | N-Acetyl-S-(3,4-dimethylphenyl)-L-cysteine | | |
| | | 2-Methylhippuric acid | 2MHA | |
| | | 3-Methylhippuric acid + 4-Methylhippuric acid | 3MHA+ 4MH | |
| 186 | | | | |
| 187 | Urine is analyzed f | or 23 metabolites of 18 parent VOCs and tobacc | o alkaloids by UPLC- | |
| 188 | MS/MS. Analytes a | re listed as parent, metabolite and their commo | n abbreviation. | |
| 189 | | | c | |
| 190 | Circulating Marke | rs of Cardiovascular Injury | | |
| 191 | To assess to | bacco product-induced cardiovascular toxicity, | we examine | |
| 192 | endothelial functior | , inflammatory mediators, biomarkers, and thror | nbosis. CV risk is | |
| 193 | defined through me | asurements of circulating angiogenic cells, lipid | profile, and glucose | |
| 194 | metabolism ^{24 34 35} . | Plasma (BD367863 and BD366415) and serum | (BD367814) | |
| 195 | samples are obtained from all participants for laboratory testing and long term | | | |
| 196 | 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 | | | |
| 197 | UofL pathology cor | ية UofL pathology core. BU biologic samples have minimal processing and are shipped ي | | |
| 198 | overnight to the UofL central laboratory at the completion of each study visit. Samples | | | |
| 199 | ब obtained at UofL are processed to a similar stage, then held overnight prior to analysis | | | |
| 200 | to standardize the | to standardize the time to measurement for all samples. The UofL central laboratory, as | | |
| 201 | previously reported | previously reported, will complete fasting and biomarker measurements (Table 3), with | | |
| 202 | the exception of cytomics ^{12 36} . For cytomic measurements, mononuclear cells are | | | |
| 203 | labeled with the pe | ipheral blood phenotyping panel kit (Fluidigm).S | Samples are shipped | |
| | | 11 | | |

| 2 | | |
|----------------------------|-----|---|
| 3 4 | 204 | at 4 degree C to Core Lab facilities at the University of Rochester for Mass cytometric |
| 5 6 | 205 | analysis. |
| 7 8 9 | 206 | Table 3 Blood analysis |
| 10 11 | | Fasting Measurements |
| 12 13 14 | | LDL cholesterol, HDL cholesterol, total cholesterol, triglycerides, glucose, uric acid, SAA and fibrinogen |
| 15 | | Biomarkers |
| 16 17 18 19 20 | | 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 |
| 21 22 23 | | 1: Fifteen different CAP subpopulations and 5 subtypes of microparticles were measured by flow cytometry. |
| 25 24 25 | 207 | All participants who complete the study visit will have blood samples taken and |
| 26 27 | 208 | processed. Flow cytometric analysis is completed on fresh samples. All other analysis |
| 28 29 20 | 209 | will be completed on biobanked samples in batches LDL= low density lipoprotein. HDL= |
| 30 31 32 | 210 | high density lipoprotein. SAA= serum amyloid A. CAC= circulating angiogenic cells. |
| 33 34 | 211 | MP= microparticles. PF4= Platelet factor 4. t-PA= tissue plasminogen activator. |
| 35 36 | 212 | TxA2=Thromboxane A. IL-6= Interleukin 6. CRP= C-reactive protein. PAI-=- |
| 37 38 20 | 213 | Plasminogen activator. s- ICAM- soluble intercellular adhesion protein inhibitor. s- |
| 39 40 41 | 214 | VCAM= soluble vascular adhesion protein. TNFR1= Tumor necrosis factor receptor 1. |
| 42 43 | 215 | MMP- Matrix metalloproteinase. |
| 44 45 | 216 | Non-Invasive Vascular Function Testing |
| 46 47 48 | 217 | Smoking, is associated with endothelial damage and vascular dysfunction ^{37 38} . |
| 49 50 | 218 | Endothelial cells are exposed to circulating toxins and measures of endothelial function |
| 51 52 | 219 | are reflective of cardiovascular injury ³⁹ . Thus, we examine the non-invasive endothelial |
| 53 54 55 | 220 | vasodilator function using flow-mediated vasodilation ^{40 41} , arterial stiffness with carotid- |
| 56 57 58 | 221 | femoral and carotid-radial pulse wave velocity ⁴² , and peripheral vascular function with 12 |
| 59 60 | | For peer review only - http://bmjopen.bmj.com/site/about/guidelines.xhtml |

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222 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 223 to the lower arm and a 3 lead ECG is attached to the patient. After baseline images and 224 10 cycles of Doppler images are captured using NIHEM R-wave triggered image 225 capturing software, the blood pressure cuff is inflated to 200mmHg or 50mmHg higher 226 than the systolic pressure. After the 5 minute occlusion, the cuff is released and the 227 NIHEM software records two minutes of imaging. Images were analyzed by a single 228 blinded analyzer using MIA vascular Research Tolls Brachial Analyzer for Research, 229 230 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 231 brachial diameter and 0.78 and 0.92 for FMD.⁴³ Similar equipment and software is used 232 at both sites. All vascular studies are sent to the BU central lab for analysis. 233 Anthropometric measures 234 Anthropometric measures included height, weight, waist and hip circumference 235 and body fat. All anthropometric measures are completed twice and the average 236 recorded. Standing height measurements are completed on a fixed stadiometer. Weight 237

238 measurements are completed on a digital scale to the nearest tenth of a pound. Waist

239 circumference is measured at the level of the umbilicus to the nearest tenth of a

240 centimeter. Hip circumference is measured at the maximal protrusion of the gluteal

241 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).

243 DATA ANALYSIS

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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 dosedependence. Additionally we will examine the extent to which biomarkers are associated with exposure to nicotine versus exposure to HPHC of tobacco like

250 aldehydes.

251 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 \pm SD in smoker and nonsmoker groups to be 4.0 \pm 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.

264 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 Page 15 of 27

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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 between 10-20⁴⁴ and therefore this dose range distribution is reflective of general population exposure. Comparisons of the

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2 289 effects of novel tobacco products and smoking will be informative of the relative toxicity
 2 290 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

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| 1 2 | | |
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| 2 3 4 | 312 | have been reported as predominantly Caucasian and male ⁴⁵ , and thus far our |
| 5 6 | 313 | recruitment mirrors these demographics. We expect very few Hispanic/Latino's to |
| 7 8 9 | 314 | participate, due to data suggesting tobacco use, including ENDS, tends to be lower |
| 9 10 11 | 315 | among Hispanic's/Latino's ^{45 46} . Thus we have also opted to only recruit English |
| 12 13 | 316 | speakers. We have carefully develop our recruitment strategy and exclusion criteria to |
| 14 15 | 317 | protect vulnerable populations, which is important since many report a lower |
| 16 17 18 | 318 | socioeconomic status and educational level in smokers in addition to higher rates of |
| 19 20 | 319 | reported alcohol and drug use ^{47 48} . |
| 21 22 | 320 | Our study is an observational study where participants have already assumed |
| 23 24 25 | 321 | the risk of using tobacco. Study procedures pose minimal risk. Given the known harms |
| 26 27 | 322 | associated with smoking, we will provide information on tobacco treatment when |
| 28 29 | 323 | requested by the participant. Participant information is de-identified for analysis and |
| 30 31 32 | 324 | reported in aggregate to protect privacy. |
| 32 33 34 | 325 | Completion of these studies will enable a greater understanding of the biological |
| 35 36 | 326 | responses to use of a variety of tobacco products. Specifically, they will help to identify |
| 37 38 | 327 | the constituents of these products; and how a panel of exposure and CV injury |
| 39 40 41 | 328 | biomarkers are associated with these different constituents. This data will be available |
| 42 43 | 329 | to the FDA and could help guide new policy measures to reduce or eliminate the |
| 44 45 | 330 | harmful components of tobacco smoke and other nicotine products. The study is |
| 46 47 48 | 331 | dedicated to the rapid dissemination of their rigorously characterized and well-controlled |
| 49 50 | 332 | research findings to the public in the form of peer-reviewed publications. Subsequent to |
| 51 52 | 333 | the initial full-length manuscript publications of the resources generated with funding |
| 53 54 55 | 334 | from this program, the study will make them available to interested and qualified |
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335 investigators upon written request. The study will provide relevant protocols of published

data, upon request (presuming prior publication by the Center members). Participants 336

will be provided a summary of the results as they become available. Finally press 337

releases of relevant findings will inform the general population. 338

339

LIST OF ABBREVIATIONS 340

- ABI- Ankle Brachial Index 341
- CAC= circulating angiogenic cells 342
- 343 CRP= C-reactive protein
- CVD- Cardiovascular disease 344
- ENDS- Electronic nicotine Device (i.e. e-cigarette) 345
- FACS- Fluorescence-activated cell sorting 346
- FMD- Flow mediated dilation 347
- HDL= high density lipoprotein 348
- IL-6= Interleukin 6 349
- MMP- Matrix metalloproteinase 350
- MP= micoparticles 351
- PAI-=- Plasminogen activator 352
- PF4= Platelet factor 4 353
- 354 PWV- Pulse wave velocity
- SAA= serum amyloid A 355
- 356 s-ICAM- soluble intercellular adhesion protein inhibitor
- 357 s-VCAM= soluble vascular adhesion protein

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| 1 2 | | |
|----------------|-----|--|
| 2 3 4 | 358 | TNFR1= Tumor necrosis factor receptor 1 |
| 5 6 | 359 | t-PA= tissue plasminogen activator |
| 7 8 9 | 360 | TxA2=Thromboxane A |
| 9 10 11 | 361 | VOC- Volatile organic compound |
| 12 13 | 362 | W:H- ratio: Waist to hip ratio |
| 14 15 16 | 363 | |
| 16 17 18 | 364 | AUTHORS CONTRIBUTIONS |
| 19 20 | 365 | Rachel Keith- Study design, study recruitment, study visits, statistical analysis and |
| 21 22 | 366 | manuscript preparation. Jessica Fetterman- study recruitment, study visits, manuscript |
| 23 24 25 | 367 | preparation and editing. Dan Riggs- statistical analysis, manuscript preparation and |
| 26 27 | 368 | editing. Tim O'Toole- Biomarker measurements, manuscript preparation and editing. |
| 28 29 | 369 | Jessica Nystoriak- study recruitment and study visits. Monica Holbrook- study |
| 30 31 32 | 370 | recruitment and study visits. Pawel Lorkiewicz- VOC measurements and manuscript |
| 33 34 | 371 | preparation. Aruni Bhatnagar- Study design, study funding and manuscript editing. |
| 35 36 | 372 | Andrew DeFilippis- Human subject assessment planning, manuscript preparation and |
| 37 38 | 373 | editing. Naomi M. Hamburg- Study design, study funding, vascular core, manuscript |
| 39 40 41 | 374 | preparation and editing. |
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| 4 5 | | |
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| 7 8 9 | 383 | (CTP). The content is solely the responsibility of the authors and does not necessarily |
|) 10 11 | 384 | represent the official views of the NIH or the Food and Drug Administration. |
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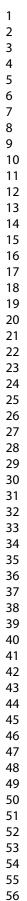
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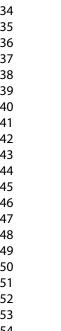
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| 18 19 | 531 | Figure 1. Cardiovascular Injury due to Tobacco Use |
| 20 | 532 | CITU is designed to assess how tobacco related VOC exposure contributes to |
| 21 22 | 532 533 | cardiovascular risk factors. Our exposure measurements include a panel of 23 |
| 22 | 535 534 | urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular |
| 24 | 535 535 | phenotyping includes measures of injury, risk, vascular biomarkers and early |
| 25 | 535 536 | vascular dysfunction. Tobacco use included use of traditional cigarettes, |
| 26 | 530 537 | smokeless tobacco, waterpipe tobacco (hookah), electronic nicotine devices |
| 27 | | (ENDS), little cigars, cigarillos, pipes, cigars or any other form of tobacco that is |
| 28 | 538 539 | available. Enrollment began in July 2014 and is ongoing. |
| 29 30 | 223 | available. Enrollment began in buly 2014 and is ongoing. |
| 31 | 540 | Figure 2. Study Visit Design |
| 32 | 541 | Study flow chart for interested participants from screening through study completion. |
| 33 34 | 542 | Potential participants are pre-screened for eligibility prior to enrollment. Potential |
| 34 35 | 543 | participants are asked to fast from tobacco for a minimum of 6 hours prior to the |
| 36 | 544 | study visit. On the day of the visit the study lasts approximately 90 minute. |
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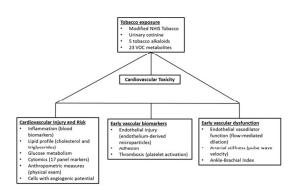




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CITU is designed to assess how tobacco related VOC exposure contributes to cardiovascular risk factors. Our exposure measurements include a panel of 23 urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular phenotyping includes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

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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.

 Tobacco and food fast
 Consent and rescreen
 Detailed Surveys
 Vascul release

 T = 8 h food
 T = 15 min
 T = 30 min
 T = 30 min

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

| Section/Topic | ltem # | 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 |

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| 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 | (<i>a</i>) 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.