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

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Manuscripts

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3 1 **Assessing the Impact of Tobacco-Induced Volatile Organic Compounds on**
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5 2 **Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to**
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7 **Tobacco Study**
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10 4 Rachel J. Keith, Jessica L. Fetterman, Daniel W. Riggs, Tim O'Toole, Jessica Nystoriak,
11
12 5 Monica Holbrook, Pawel Lorkiewicz, Aruni Bhatnagar, Andrew DeFilippis*, Naomi M.
13
14 6 Hamburg*

15
16
17 7 Rachel J. Keith-Division of Cardiovascular Medicine, University of Louisville School of
18
19 8 Medicine 580 S. Preston St. Louisville KY, 40202 rachel.keith@louisville.edu 502-852-
20
21 9 4211

22
23
24 10 Jessica L. Fetterman- Vascular Biology Section, Whitaker Cardiovascular Institute,
25
26 11 Boston University School of Medicine Evans Building, Boston, MA USA

27
28 12 Jasmit Shah- University of Louisville School of Medicine Louisville, KY USA

29
30
31 13 Timothy O-Toole- Division of Cardiovascular Medicine, University of Louisville School of
32
33 14 Medicine Louisville, KY USA

34
35 15 Jessica L. Nystoriak- Division of Cardiovascular Medicine, University of Louisville
36
37 16 School of Medicine Louisville, KY USA

38
39
40 17 Monika Holbrook- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston
41
42 18 University School of Medicine Boston, MA USA

43
44 19 Pawel Lorkiewicz- Division of Cardiovascular Medicine, University of Louisville School
45
46 20 of Medicine Louisville, KY USA

47
48
49 21 Aruni Bhatnagar- Division of Cardiovascular Medicine, University of Louisville School of
50
51 22 Medicine Louisville, KY USA

1
2
3 23 Andrew P. DeFilippis- Division of Cardiovascular Medicine, University of Louisville
4
5 24 School of Medicine Louisville, KY USA (co-senior author)
6

7
8 25 Naomi M. Hamburg- Vascular Biology Section, Whitaker Cardiovascular Institute,
9
10 26 Boston University School of Medicine Boston, MA USA (co-senior author)
11
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15 28 **Word Count: 2581**
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18

19 30 **ABSTRACT**

20
21 31 **Introduction:** Tobacco use leads to increased mortality, the majority of which is
22
23 32 attributed to cardiovascular disease. Despite this knowledge, the early cardiovascular
24
25 33 impact of tobacco product use is not well understood. Tobacco use increases exposure
26
27 34 to harmful and potentially harmful constituents including volatile organic compounds
28
29 35 (VOCs) such as acrolein and crotonaldehyde, which may contribute to cardiovascular
30
31 36 risk. The link between exposure patterns, risk profiles and demographic distribution of
32
33 37 tobacco product users, particularly users of new and emerging products, are not well
34
35 38 known. Therefore, we designed the Cardiovascular Injury due to Tobacco Use (CITU)
36
37 39 study to assess population characteristics, demographic features, exposure patterns
38
39 40 and cardiovascular risk in relation to tobacco.
40
41
42
43

44 41 **Methods and analysis:** This is a cross-section observational study conducted in
45
46 42 Boston MA and Louisville KY from 2014 through 2018. Healthy participants 21 to 45
47
48 43 years of age who use tobacco products, including ENDS, or who never used tobacco
49
50 44 are being recruited. The study aims to recruit an evenly split cohort of African
51
52 45 Americans and Caucasians that is sex balanced for evaluation of self-reported tobacco
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1
2
3 46 exposure, VOC exposure and tobacco-induced injury profiling. Detailed information
4
5 47 about participant's demographics, health status and lifestyle is also collected.
6

7
8 48 **Ethics and dissemination:** The study protocol was approved institutional review
9
10 49 boards at both participating universities. All study protocols will protect participant
11
12 50 confidentiality. Results from the study will be disseminated via peer-reviewed journals
13
14 51 and presented at scientific conferences.
15
16

17 52 18 19 53 **Strengths and limitations**

- 20
21 54 • Young age to allow for evaluation of early stage disease (e.g. inflammation,
22
23 55 endothelial function) as opposed to end stage clinical consequence (e.g.
24
25 56 myocardial infarction)
- 26
27 57 • Diverse tobacco product use allows for assessment of a wide range of tobacco-
28
29 58 induced VOC exposure
- 30
31 59 • All study visits are in English introducing selection bias
- 32
33 60 • Data will inform regulatory agencies on the cardiovascular health effects of
34
35 61 multiple tobacco products and the contribution of HPHCs
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43 63 **Keywords:** Tobacco, smoking, electronic cigarette, vascular injury, cardiovascular risk,
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45 64 cigarettes.
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49 66 **INTRODUCTION**

50
51
52 67 Tobacco product use and smoking are the leading causes of preventable deaths
53
54 68 throughout the world. Of those deaths, one-third are attributed to cardiovascular disease
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2
3 69 (CVD)¹. The cardiovascular (CV) effects of tobacco exposure can include
4
5 70 atherogenesis, vascular injury, thrombosis, arrhythmias and inflammation² and may be
6
7 71 attributable to the many different harmful and potentially harmful constituents (HPHCs)
8
9
10 72 present in tobacco products.

11
12 73 The HPHCs found in tobacco products include volatile organic compounds
13
14 74 (VOCs) of which reactive aldehydes, such as acrolein and crotonaldehyde, are likely the
15
16 75 most significant contributors to CV toxicity³. High levels of aldehydes are present in
17
18 76 cigarette smoke^{4 5} as well as smokeless tobacco (ST)⁶. Risk assessments, using the
19
20 77 prevalence of each individual chemical weighed by its potency, suggest that the non-
21
22 78 cancer risk of smoking is dominated by acrolein, which contributes 40-100 times more
23
24 79 to risk than any other chemical present in cigarette smoke³.

25
26
27
28 80 Although HPHCs, including VOC reactive aldehydes, have been suspected to be
29
30 81 major contributors to the toxicity of cigarette smoke for over 4 decades, their
31
32 82 contribution to CV injury and early CVD risk has not been rigorously evaluated.
33
34 83 Experimental studies in animal models suggest that because of low aldehyde-
35
36 84 metabolizing capacity, CV tissues are highly sensitive to aldehydes and exposure to low
37
38 85 levels of aldehydes can induce CV injury and accelerate CVD⁷⁻¹⁹. The WHO Study
39
40 86 Group on Tobacco Product Regulation (TobReg) has marked acrolein, a VOC, along
41
42 87 with 8 other cigarette constituents for monitoring and regulation²⁰ and the U.S.
43
44 88 Environmental Protection Agency lists Acrolein as one of most hazardous air
45
46 89 pollutants²¹. Nevertheless, the contribution of tobacco induced VOCs, including acrolein
47
48 90 or other aldehydes, toward CV toxicity in humans has not been fully assessed. Greater
49
50 91 understanding of how aldehydes affect cardiovascular health and disease will provide
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3 92 new avenues for evaluating the toxicity of cigarette smoke and for assessing the
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5 93 injurious potential of new and emerging tobacco products, such as ENDS, which may
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7 94 also contain VOCs including acrolein²²⁻²⁴.
8
9

10 95 The latency period between tobacco exposure and the development of major
11
12 96 clinical adverse health effects is long, therefore biomarkers that provide information over
13
14 97 a shorter period allow for the identification of harm decades before clinical outcome data
15
16 98 is available. Thus, the Cardiovascular Injury due To Tobacco Use (CITU) study
17
18 99 evaluates the association of the urinary metabolites of 18 parent VOCs from tobacco
19
20 100 exposure with a comprehensive set of CV biomarkers representative of early disease
21
22 101 and predictive of future CV events.²⁵
23
24
25

26 102 **METHODS AND DESIGN**

27 103 **Overall design**

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29
30 104 The CITU study is an investigator-initiated cross-sectional observational study of
31
32 105 around 500 healthy participants 21 to 45 years of age who are never or current tobacco
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34 106 product users in two urban areas at Boston University (BU) and University of Louisville
35
36 107 (UofL) (Boston, MA and Louisville, KY) designed to evaluate CV toxicity due to tobacco
37
38 108 product use, with correlations to VOCs found in the tobacco products (Figure 1).
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44 110 **Figure 1. Cardiovascular Injury due to Tobacco Use**

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49 112 *CITU is designed to assess how tobacco related VOC exposure contributes to*
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51 113 *cardiovascular risk factors. Our exposure measurements include a panel of 23 urinary*
52
53 114 *metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular phenotyping*
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3 115 *includes measures of injury, risk, vascular biomarkers and early vascular dysfunction.*
4
5 116 *Tobacco use included use of traditional cigarettes, smokeless tobacco, waterpipe*
6
7 117 *tobacco (hookah), electronic nicotine devices (ENDS), little cigars, cigarillos, pipes,*
8
9 118 *cigars or any other form of tobacco that is available. Enrollment began in July 2014 and*
10
11 119 *is ongoing.*
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13

14 120 **Participant Eligibility Criteria**

17 121 The goal of the study is to examine the impact of tobacco products on healthy
18
19 122 young adults who could be classified as a current tobacco product users (Defined in
20
21 123 table 1), or never-users (does not have lifetime use of any tobacco product); therefore
22
23 124 we excluded participants if they had: 1) diagnosis of diabetes (HbA1c >7.0 or treatment
24
25 125 for diabetes), hypertension (systolic blood pressure >140 mm Hg or diastolic blood
26
27 126 pressure >90 mm Hg), hypothyroidism or hyperthyroidism, inflammatory conditions such
28
29 127 as lupus or inflammatory bowel disease, HIV/AIDS, hepatitis, liver disease, anemia,
30
31 128 cancer of any type or another medical condition that might compromise the successful
32
33 129 completion of the study; 2) recipients of organ transplant or renal replacement therapy;
34
35 130 3) individuals that are taking the following medications: immunosuppressant agents
36
37 131 estrogen, testosterone, anti TNF agents, certain biologics, Procrit, statins, beta-blockers
38
39 132 or other cardiovascular medicine; 4) individuals using nutraceuticals or anabolic steroids
40
41 133 beyond the recommended daily allowance; 5) body weight less than 100 pounds; 6)
42
43 134 pregnant women; 7) prisoners and other vulnerable populations; and 8) active illness or
44
45 135 infection. Participants are rescheduled or considered screen-failures and excluded from
46
47 136 the study if symptomatic of an acute illness, i.e. viral upper respiratory infection, on
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49 137 study date.
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138 **Table 1. Tobacco product use classifications**

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

139 *Study participants are 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 during the study visits.*

142 Overall Study Procedure

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

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

154 Exposure Variables

155 Tobacco Product Use & Particulate Matter Exposure

1
2
3 156 Comprehensive tobacco product exposure is assessed using a modified version
4
5 157 of the National Health Interview survey on tobacco use²⁶. The survey is modified to
6
7 158 include detailed information on electronic nicotine devices (ENDs) and other new or
8
9 159 emerging tobacco products. Residential addresses are collected for assessment of
10
11 160 ambient airborne particulate matter (PM_{2.5}) exposure and future correction of overall
12
13 161 exposure. PM_{2.5} data from the day of the study visit, and 3 and 5 days prior to the study
14
15 162 is collected from publicly available data associated with EPA monitoring stations. Other
16
17 163 exposure variables, including occupation, are collected through interview.
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22 164 VOC Measurements

23
24 165 Standard clean catch urine specimens are obtained from participants. We have
25
26 166 developed a robust Core Lab that utilizes mass spectrometry procedures adopted from
27
28 167 the Centers for Disease Control and Prevention (CDC) protocols, to quantify 23 urinary
29
30 168 metabolites of tobacco smoking related toxins (aldehydes and other VOCs), including
31
32 169 acrolein²⁷ (**Table 2**). The concentration values of analytes are then normalized to
33
34 170 urinary creatinine levels measured using Infinity Creatinine Reagent (Thermo Fisher
35
36 171 Scientific, MA) on a COBAS MIRA-plus analyzer (Roche, NJ).
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38
39

40 172 **Table 2 Exposure Variables (Please see end of article)**

<i>Parent compound</i>	<i>VOC metabolite</i>	<i>Common abbr.</i>
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-carbamoyl-ethyl)-L-cysteine	AAMA

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

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

173

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

176

177 **Circulating Markers of Cardiovascular Injury**

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

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

193 **Table 3 Blood analysis**

Fasting Measurements

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

Biomarkers

CAC (1-15)¹, Platelet-monocyte aggregates, MP (1-5)¹, PF4, t-PA, TxA2, Factor VII, IL-6, CRP, D-dimer, PAI-1, s-ICAM-1, s-VCAM, s-thrombomodulin, s-TNFR1, MMP-2, MMP-3, MMP-9, cytomics, endothelin, E-selectin and P-selectin

1: Fifteen different CAP subpopulations and 5 subtypes of microparticles were measured by flow cytometry.

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

201 *VCAM= soluble vascular adhesion protein. TNFR1= Tumor necrosis factor receptor 1.*

202 *MMP- Matrix metalloproteinase.*

203 **Non-Invasive Vascular Function Testing**

204 Smoking, is associated with endothelial damage and vascular dysfunction^{31 32}.

205 Endothelial cells are exposed to circulating toxins and measures of endothelial function
206 are reflective of cardiovascular injury³³. Thus, we examine the non-invasive endothelial
207 vasodilator function using flow-mediated vasodilation^{34 35}, arterial stiffness with carotid-
208 femoral and carotid-radial pulse wave velocity³⁶, and peripheral vascular function with
209 ankle brachial index. All vascular imagers were trained at BU. Similar equipment and
210 software is used at both sites. All vascular studies are sent to the BU central lab for
211 analysis.

212 **Anthropometric measures**

213 Anthropometric measures included height, weight, waist and hip circumference
214 and body fat. All anthropometric measures are completed twice and the average
215 recorded. Standing height measurements are completed on a fixed stadiometer. Weight
216 measurements are completed on a digital scale to the nearest tenth of a pound. Waist
217 circumference is measured at the level of the umbilicus to the nearest tenth of a
218 centimeter. Hip circumference is measured at the maximal protrusion of the gluteal
219 muscle to the nearest tenth of a centimeter. Body fat percentage is calculated by the
220 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

1
2
3 224 specific measures of tobacco exposure. For instance, we will identify which biomarkers
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5 225 are affected by tobacco use, and which ones are most sensitive; including their dose-
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7 226 dependence. Additionally we will examine the extent to which biomarkers are
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10 227 associated with exposure to nicotine versus exposure to HPHC of tobacco like
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12 228 aldehydes.

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14 229 All statistical analysis will be performed using SAS version 9.4 software (SAS
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16
17 230 Institute, Inc., Cary, North Carolina), and a two-sided p-value of <0.05 will be considered
18
19 231 significant for any statistical test. Demographics and other baseline characteristics will
20
21 232 be summarized according to product group. The primary outcomes will be analyzed
22
23 233 using multiple regression techniques. Appropriate Interaction variables will be tested for
24
25
26 234 in the regression models and subgroup analyses will be conducted according to the
27
28 235 following factors: significant interactions, sex, age, race, tobacco product group.
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30 236 Multiple imputation method will be used for missing data where appropriate. Sensitivity
31
32 237 analysis using different analytic approaches, such as generalized linear models, as well
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34 238 as considering different covariate adjustments, will be used to build concordant results.

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36
37 239 The dose-dependence of the changes in biomarkers will be determined by
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39 240 analyzing the data obtained from individuals that are exposed to different doses of a
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41 241 single product (e.g. smoking 0, <15, 15-20 and >20 cigarettes per day) and by
42
43 242 comparing between tobacco products that have different doses of HPHC constituents.
44
45 243 In the US the average cigarettes per day is between 15-20³⁷ and therefore this dose
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47 244 range distribution is reflective of general population exposure. Comparisons of the
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49 245 effects of novel tobacco products and smoking will be informative of the relative toxicity
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51 246 of the two products.
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3 247 We believe that the methods employed in the current project are exquisitely
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5 248 sensitive and responsive to even low dose insults such as ambient air pollution¹³
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8 249 allowing us to quantify tobacco product-induced changes with high precision. Moreover,
9
10 250 levels of acrolein exposure vary between different individuals due to difference in puffing
11
12 251 intensity and the time a cigarette is left smoldering. Thus, direct measurements of
13
14 252 acrolein metabolites afford better estimates of acrolein exposure than machine yields.
15
16
17 253 We expect to obtain wide variations in acrolein/crotonaldehyde exposure which will
18
19 254 enable us to construct a dose-response relationship and identify which injury
20
21 255 biomarkers are associated with aldehyde exposure and whether high levels of exposure
22
23 256 are associated with high levels of injury, despite similar nicotine delivery.
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25

26 257 We consider three major factors for balancing sample selection: age, gender,
27
28 258 and race. Given that very few females use e-cigarette, only males will be enrolled in
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30
31 259 this group. With the balanced design to determine the main effects and interactions in
32
33 260 selected scenarios, we justify the sample size. The analysis plan is primarily based on
34
35 261 evaluating the effect of tobacco exposure on endothelial function (FMD), and the main
36
37 262 biomarkers, EPCs, and platelet-monocyte aggregates (PMA). The sample size is
38
39
40 263 justified in terms of the primary dependent measure, FMD, given the potential
41
42 264 importance of this variable as a direct measure of the impact of tobacco exposure. The
43
44 265 main comparisons are between non-tobacco users and tobacco users. Due to one
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46
47 266 control group, we will conservatively adjust our α (significance level) using a Bonferroni
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49 267 correction, and we will set $\alpha=0.01$. Based on preliminary data for FMD, we have
50
51 268 observed mean \pm SD in smoker and nonsmoker groups to be 4.0 ± 1.6 and 6.8 ± 1.0 ,
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53
54 269 respectively. We consider at least 25% (mean FMD=3.0 from 4.0) reduction from
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smokers to non-smokers is meaningful. Using a two sample, one-sided t test with an α of 0.01 and 80% power ($1-\beta$), assuming a common SD of 1.3, we will need 34 evaluable subjects in each group. To examine dose response, smokers will be recruited in 3 groups (<15, 15-20 and >20 CPD). We will recruit 40 participants in each group; total group size = 120 participants. In **Table 4** we provide estimable effect size for different outcome measures.

Table 4 Minimal Detectable Differences in Endpoints at $\alpha=0.01$ and Power=80%

Variable	Non-smokers	Smokers	n	p	Ref	MDD
Primary Functional Outcome						278
FMD	6.8 ± 1%	4.0 ± 1.6%	10	<0.05	³²	1.0 ²⁷⁹
Primary Biomarkers						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	0.2 ²⁸³
Other Biochemical Variables						284
PF4	3.9 ± 1.2 IU/ml	5.0 ± 2.6 IU/ml	12	<0.05	⁴¹	2.0 ²⁸⁵
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	0.016 ²⁸⁷

PMA: Platelet
- monoc
yte

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

ETHICS AND DISSEMINATION

1
2
3 292 The CITU study was approved at each institution by their institutional review
4
5 293 board (BU #H-32613 and UofL #13.0590) and all participants provide written consent.
6
7
8 294 No study related procedures will be completed until after participant consent.
9

10 295 Participants for the CITU study are being recruited in both Boston, MA and
11
12 296 Louisville KY. The two populations show significant differences, therefore recruitment at
13
14 297 two sites will ensure a range more reflective of the general population. Although overall
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16 298 racial and ethnic demographics for both cities show a clear majority of Caucasians
17
18 299 (70%) and despite smokers typically male, we strive to, and currently are successful in,
19
20 300 recruiting a population that was gender balanced and almost evenly split between
21
22 301 Caucasian and African Americans. Despite this balanced recruitment, e-cigarette users
23
24 302 have been reported as predominantly Caucasian and male⁴⁴, and thus far our
25
26 303 recruitment mirrors these demographics. We expect very few Hispanic/Latino's to
27
28 304 participate, due to data suggesting tobacco use, including ENDS, tends to be lower
29
30 305 among Hispanic's/Latino's ^{44 45}. Thus we have also opted to only recruit English
31
32 306 speakers. We have carefully develop our recruitment strategy and exclusion criteria to
33
34 307 protect vulnerable populations, which is important since many report a lower
35
36 308 socioeconomic status and educational level in smokers in addition to higher rates of
37
38 309 reported alcohol and drug use ^{46 47}.
39

40 310 Our study is an observational study where participants have already assumed
41
42 311 the risk of using tobacco. Study procedures pose minimal risk. Given the known harms
43
44 312 associated with smoking, we will provide information on tobacco treatment when
45
46 313 requested by the participant. Participant information is de-identified for analysis and
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48 314 reported in aggregate to protect privacy.
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3 315 Completion of these studies will enable a greater understanding of the biological
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5 316 responses to use of a variety of tobacco products. Specifically, they will help to identify
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7 317 the constituents of these products; and how a panel of exposure and CV injury
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9 318 biomarkers are associated with these different constituents. This data will be available
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11 319 to the FDA and could help guide new policy measures to reduce or eliminate the
12
13 320 harmful components of tobacco smoke and other nicotine products. The study is
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15 321 dedicated to the rapid dissemination of their rigorously characterized and well-controlled
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17 322 research findings to the public in the form of peer-reviewed publications. Subsequent to
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19 323 the initial full-length manuscript publications of the resources generated with funding
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21 324 from this program, the study will make them available to interested and qualified
22
23 325 investigators upon written request. The study will provide relevant protocols of published
24
25 326 data, upon request (presuming prior publication by the Center members). Participants
26
27 327 will be provided a summary of the results as they become available. Finally press
28
29 328 releases of relevant findings will inform the general population.
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330 LIST OF ABBREVIATIONS

331 ABI- Ankle Brachial Index
332 CAC= circulating angiogenic cells
333 CRP= C-reactive protein
334 CVD- Cardiovascular disease
335 ENDS- Electronic nicotine Device (i.e. e-cigarette)
336 FACS- Fluorescence-activated cell sorting
337 FMD- Flow mediated dilation

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2
3 338 HDL= high density lipoprotein
4

5 339 IL-6= Interleukin 6
6

7 340 MMP- Matrix metalloproteinase
8

9 341 MP= micoparticles
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11 342 PAI=- Plasminogen activator
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13 343 PF4= Platelet factor 4
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15 344 PWV- Pulse wave velocity
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17 345 SAA= serum amyloid A
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19 346 s-ICAM- soluble intercellular adhesion protein inhibitor
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21 347 s-VCAM= soluble vascular adhesion protein
22

23 348 TNFR1= Tumor necrosis factor receptor 1
24

25 349 t-PA= tissue plasminogen activator
26

27 350 TxA2=Thromboxane A
28

29 351 VOC- Volatile organic compound
30

31 352 W:H- ratio: Waist to hip ratio
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40 **AUTHORS CONTRIBUTIONS**

41
42 355 Rachel Keith- Study design, study recruitment, study visits, statistical analysis and
43

44 356 manuscript preparation. Jessica Fetterman- study recruitment, study visits, manuscript
45

46 357 preparation and editing. Dan Riggs- statistical analysis, manuscript preparation and
47

48 358 editing. Tim O'Toole- Biomarker measurements, manuscript preparation and editing.
49

50 359 Jessica Nystoriak- study recruitment and study visits. Monica Holbrook- study
51

52 360 recruitment and study visits. Pawel Lorkiewicz- VOC measurements and manuscript
53
54
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1
2
3 361 preparation. Aruni Bhatnagar- Study design, study funding and manuscript editing.
4
5 362 Andrew DeFilippis- Human subject assessment planning, manuscript preparation and
6
7 363 editing. Naomi M. Hamburg- Study design, study funding, vascular core, manuscript
8
9 364 preparation and editing.
10
11

12 365 **COMPETING INTERESTS**

13
14 366 None declared
15

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18
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22

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31
32 374 represent the official views of the NIH or the Food and Drug Administration.
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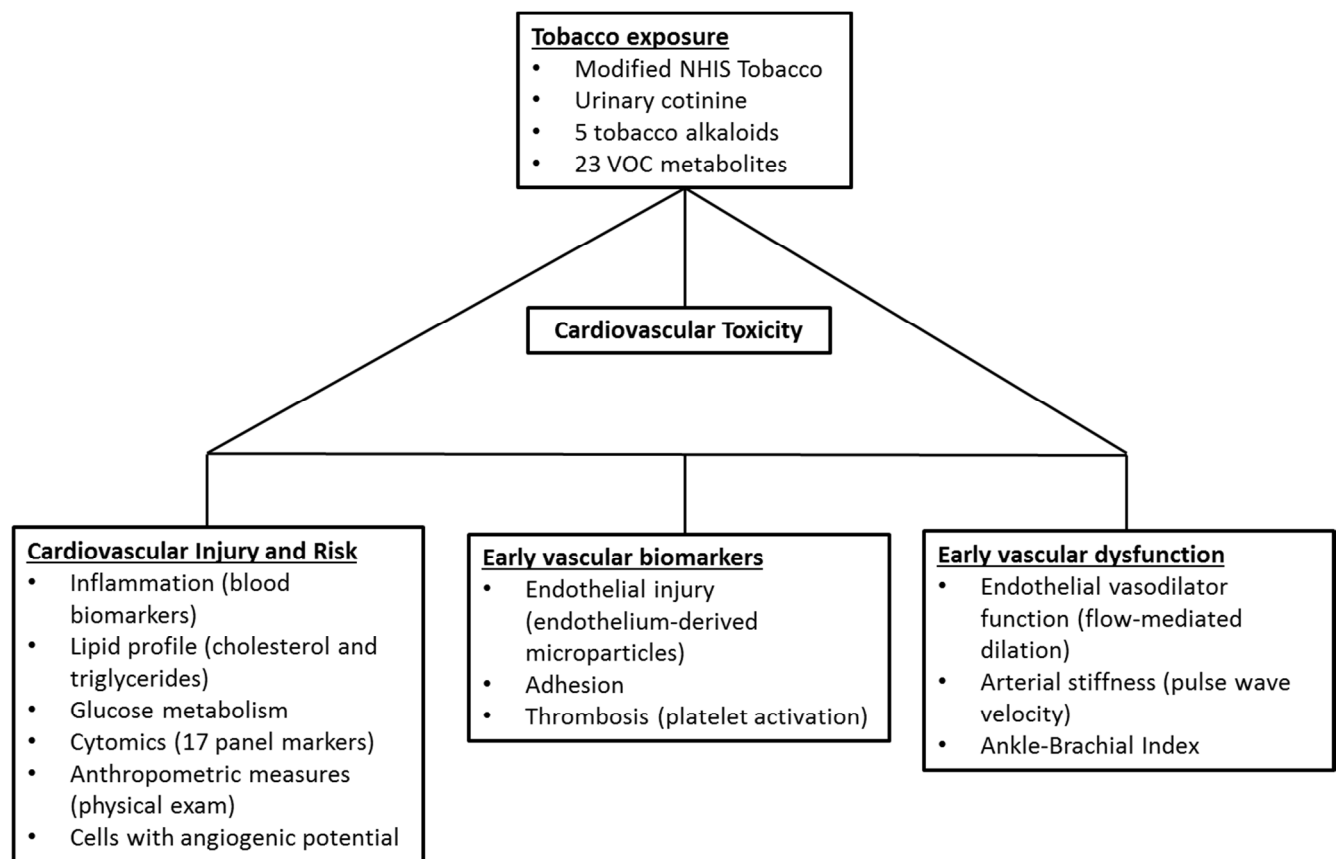
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Figure 1.



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

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

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

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

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

BMJ Open

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

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

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3 1 **Protocol to Assess the Impact of Tobacco-Induced Volatile Organic Compounds**
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5 2 **on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to**
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10 4 Rachel J. Keith, Jessica L. Fetterman, Daniel W. Riggs, Tim O'Toole, Jessica Nystoriak,
11
12 5 Monica Holbrook, Pawel Lorkiewicz, Aruni Bhatnagar, Andrew DeFilippis*, Naomi M.
13
14 6
15 6 Hamburg*

16
17 7 Rachel J. Keith-Division of Cardiovascular Medicine, University of Louisville School of
18
19 8 Medicine 580 S. Preston St. Louisville KY, 40202 rachel.keith@louisville.edu 502-852-
20
21 9 4211

22
23
24 10 Jessica L. Fetterman- Vascular Biology Section, Whitaker Cardiovascular Institute,
25
26 11 Boston University School of Medicine Evans Building, Boston, MA USA

27
28 12 Jasmit Shah- University of Louisville School of Medicine Louisville, KY USA

29
30
31 13 Timothy O-Toole- Division of Cardiovascular Medicine, University of Louisville School of
32
33 14 Medicine Louisville, KY USA

34
35 15 Jessica L. Nystoriak- Division of Cardiovascular Medicine, University of Louisville
36
37 16 School of Medicine Louisville, KY USA

38
39
40 17 Monika Holbrook- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston
41
42 18 University School of Medicine Boston, MA USA

43
44
45 19 Pawel Lorkiewicz- Division of Cardiovascular Medicine, University of Louisville School
46
47 20 of Medicine Louisville, KY USA

48
49 21 Aruni Bhatnagar- Division of Cardiovascular Medicine, University of Louisville School of
50
51 22 Medicine Louisville, KY USA

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

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

28 **Word Count: 2581**

30 **ABSTRACT**

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

41 **Methods and analysis:** We present the design and methodology of the CITU study a
42 cross-section observational tobacco study conducted in Boston MA and Louisville KY
43 starting in 2014. Healthy participants 21 to 45 years of age who use tobacco products,
44 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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3 46 for evaluation of self-reported tobacco exposure, VOC exposure and tobacco-induced
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5 47 injury profiling. Detailed information about participant's demographics, health status and
6
7 48 lifestyle is also collected.

9
10 49 **Ethics and dissemination:** The study protocol was approved institutional review
11
12 50 boards at both participating universities. All study protocols will protect participant
13
14 51 confidentiality. Results from the study will be disseminated via peer-reviewed journals
15
16 52 and presented at scientific conferences.
17
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21 54 **Strengths and limitations**

- 24 55 • Young age to allow for evaluation of early stage disease (e.g. inflammation,
25
26 56 endothelial function) as opposed to end stage clinical consequence (e.g.
27
28 57 myocardial infarction)
- 31 58 • Diverse tobacco product use allows for assessment of a wide range of tobacco-
32
33 59 induced VOC exposure
- 35 60 • All study visits are in English introducing selection bias
- 38 61 • Data will inform regulatory agencies on the cardiovascular health effects of
39
40 62 multiple tobacco products and the contribution of HPHCs
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44
45 64 **Keywords:** Tobacco, smoking, electronic cigarette, vascular injury, cardiovascular risk,
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47 65 cigarettes.
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51 67 **INTRODUCTION**

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3 68 Tobacco product use and smoking are the leading causes of preventable deaths
4
5 69 throughout the world. Of those deaths, one-third are attributed to cardiovascular disease
6
7 70 (CVD)¹. The cardiovascular (CV) effects of tobacco exposure can include
8
9
10 71 atherogenesis, vascular injury, thrombosis, arrhythmias and inflammation² and may be
11
12 72 attributable to the many different harmful and potentially harmful constituents (HPHCs)
13
14 73 present in tobacco products.

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17 74 The HPHCs found in tobacco products include volatile organic compounds
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19 75 (VOCs) of which reactive aldehydes, such as acrolein and crotonaldehyde, are likely the
20
21 76 most significant contributors to CV toxicity³. High levels of aldehydes are present in
22
23 77 cigarette smoke^{4,5} as well as smokeless tobacco (ST)⁶. Risk assessments, using the
24
25 78 prevalence of each individual chemical weighed by its potency, suggest that the non-
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27 79 cancer risk of smoking is dominated by acrolein, which contributes 40-100 times more
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29 80 to risk than any other chemical present in cigarette smoke³.

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33 81 Although HPHCs, including VOC reactive aldehydes, have been suspected to be
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35 82 major contributors to the toxicity of cigarette smoke for over 4 decades, their
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37 83 contribution to CV injury and early CVD risk has not been rigorously evaluated.
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39 84 Experimental studies in animal models suggest that because of low aldehyde-
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41 85 metabolizing capacity, CV tissues are highly sensitive to aldehydes and exposure to low
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43 86 levels of aldehydes can induce CV injury and accelerate CVD⁷⁻¹⁸. The WHO Study
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45 87 Group on Tobacco Product Regulation (TobReg) has marked acrolein, a VOC, along
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47 88 with 8 other cigarette constituents for monitoring and regulation¹⁹ and the U.S.
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49 89 Environmental Protection Agency lists Acrolein as one of most hazardous air
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51 90 pollutants²⁰. Nevertheless, the contribution of tobacco induced VOCs, including acrolein

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3 91 or other aldehydes, toward CV toxicity in humans has not been fully assessed. Greater
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5 92 understanding of how aldehydes affect cardiovascular health and disease will provide
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7 93 new avenues for evaluating the toxicity of cigarette smoke and for assessing the
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9 94 injurious potential of new and emerging tobacco products, such as ENDS, which may
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11 95 also contain VOCs including acrolein²¹⁻²³.
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14 96 The latency period between tobacco exposure and the development of major
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16 97 clinical adverse health effects is long, therefore biomarkers that provide information over
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18 98 a shorter period allow for the identification of harm decades before clinical outcome data
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20 99 is available. Thus, in this paper we present the design and methodology of the
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22 100 Cardiovascular Injury due To Tobacco Use (CITU) study which will evaluate the
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24 101 association of the urinary metabolites of 18 parent VOCs from tobacco exposure with a
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26 102 comprehensive set of CV biomarkers representative of early disease and predictive of
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28 103 future CV events.²⁴
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33 104 **METHODS AND DESIGN**

34 35 105 **Overall design**

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37 106 The CITU study is an investigator-initiated cross-sectional observational study of
38
39 107 around 500 healthy participants 21 to 45 years of age who are never or current tobacco
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41 108 product users in two urban areas at Boston University (BU) and University of Louisville
42
43 109 (UofL) (Boston, MA and Louisville, KY) designed to evaluate CV toxicity due to tobacco
44
45 110 product use, with correlations to VOCs found in the tobacco products (**Figure 1**).
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52 53 113 **Participant Eligibility Criteria**

114 The goal of the study is to examine the impact of tobacco products on healthy
 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).
 117 Participants were self-reported to be healthy therefore we excluded participants if they
 118 had: 1) diagnosis of clinical cardiovascular disease including but not limited to known
 119 heart attack, peripheral artery disease, heart failure or stroke; 2) diagnosis of diabetes
 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,
 122 inflammatory conditions such as lupus or inflammatory bowel disease, HIV/AIDS,
 123 hepatitis, liver disease, anemia, cancer of any type or another medical condition that
 124 might compromise the successful completion of the study; 2) recipients of organ
 125 transplant or renal replacement therapy; 3) individuals that are taking the following
 126 medications: immunosuppressant agents estrogen, testosterone, anti TNF agents,
 127 certain biologics, Procrit, statins, beta-blockers or other cardiovascular medicine; 4)
 128 individuals using nutraceuticals or anabolic steroids beyond the recommended daily
 129 allowance; 5) body weight less than 100 pounds; 6) pregnant women; 7) prisoners and
 130 other vulnerable populations; and 8) active illness or infection. Participants are
 131 rescheduled or considered screen-failures and excluded from the study if symptomatic
 132 of an acute illness, i.e. viral upper respiratory infection, on study date.

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

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

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

137 **Overall Study Procedure**

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

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

151 **Exposure Variables**

152 Tobacco Product Use & Particulate Matter Exposure

153 Comprehensive tobacco product exposure is assessed using a modified version
 154 of the National Health Interview survey on tobacco use²⁵. The survey is modified to
 155 include detailed information on electronic nicotine devices (ENDs) and other new or

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

161 VOC Measurements

162 Standard clean catch urine specimens are obtained from participants. We have
 163 developed a robust Core Lab that utilizes mass spectrometry procedures adopted from
 164 the Centers for Disease Control and Prevention (CDC) protocols, to quantify 23 urinary
 165 metabolites of tobacco smoking related toxins (aldehydes and other VOCs), including
 166 acrolein²⁶ (**Table 2**). The concentration values of analytes are then normalized to
 167 urinary creatinine levels measured using Infinity Creatinine Reagent (Thermo Fisher
 168 Scientific, MA) on a COBAS MIRA-plus analyzer (Roche, NJ).

169 **Table 2 Exposure Variables (Please see end of article)**

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

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

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

170

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

173

174 **Circulating Markers of Cardiovascular Injury**

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

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3 182 overnight to the UofL central laboratory at the completion of each study visit. Samples
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5 183 obtained at UofL are processed to a similar stage, then held overnight prior to analysis
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7 184 to standardize the time to measurement for all samples. The UofL central laboratory, as
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9
10 185 previously reported, will complete fasting and biomarker measurements (**Table 3**), with
11
12 186 the exception of cytomics^{12 29}. For cytomic measurements, mononuclear cells are
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14 187 labeled with the peripheral blood phenotyping panel kit (Fluidigm). Samples are shipped
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17 188 at 4 degree C to Core Lab facilities at the University of Rochester for Mass cytometric
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19 189 analysis.
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190 **Table 3 Blood analysis****Fasting Measurements**

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

Biomarkers

CAC (1-15)¹, Platelet-monocyte aggregates, MP (1-5)¹, PF4, t-PA, TxA2, Factor VII, IL-6, CRP, D-dimer, PAI-1, s-ICAM-1, s-VCAM, s-thrombomodulin, s-TNFR1, MMP-2, MMP-3, MMP-9, cytomics, endothelin, E-selectin and P-selectin

1: Fifteen different CAP subpopulations and 5 subtypes of microparticles were measured by flow cytometry.

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

200 Non-Invasive Vascular Function Testing

201 Smoking, is associated with endothelial damage and vascular dysfunction^{30 31}.
 202 Endothelial cells are exposed to circulating toxins and measures of endothelial function
 203 are reflective of cardiovascular injury³². Thus, we examine the non-invasive endothelial
 204 vasodilator function using flow-mediated vasodilation^{33 34}, arterial stiffness with carotid-
 205 femoral and carotid-radial pulse wave velocity³⁵, and peripheral vascular function with
 206 ankle brachial index. Flow mediated dilation was assessed with a 7.5MHZ ultrasound
 207 probe is used to image the brachial artery while a 10cm blood pressure cuff is attached

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3 208 to the lower arm and a 3 lead ECG is attached to the patient. After baseline images and
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5 209 10 cycles of Doppler images are captured using NIHEM R-wave triggered image
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8 210 capturing software, the blood pressure cuff is inflated to 200mmHg or 50mmHg higher
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10 211 than the systolic pressure. After the 5 minute occlusion, the cuff is released and the
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12 212 NIHEM software records two minutes of imaging. Images were analyzed by a single
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14 213 blinded analyzer using MIA vascular Research Tolls Brachial Analyzer for Research,
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17 214 version 6.8.5. All vascular imagers where trained at BU who have a previously reported
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19 215 reproducibility with intra- and inter-observer correlation coefficients of 0.98 and 0.99 for
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21 216 brachial diameter and 0.78 and 0.92 for FMD.³⁶ Similar equipment and software is used
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24 217 at both sites. All vascular studies are sent to the BU central lab for analysis.

25 26 218 **Anthropometric measures**

27
28 219 Anthropometric measures included height, weight, waist and hip circumference
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31 220 and body fat. All anthropometric measures are completed twice and the average
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33 221 recorded. Standing height measurements are completed on a fixed stadiometer. Weight
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35 222 measurements are completed on a digital scale to the nearest tenth of a pound. Waist
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38 223 circumference is measured at the level of the umbilicus to the nearest tenth of a
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40 224 centimeter. Hip circumference is measured at the maximal protrusion of the gluteal
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42 225 muscle to the nearest tenth of a centimeter. Body fat percentage is calculated by the
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44 226 bioelectrical impedance measured with the Omron fat loss monitor (HBF-306C).

45 46 47 227 **DATA ANALYSIS**

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49 228 We expect that from this study we will be able to identify specific biomarkers of
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51 229 cardiovascular injury due to tobacco use and the relationship of these biomarkers to
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54 230 specific measures of tobacco exposure. For instance, we will identify which biomarkers

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3 231 are affected by tobacco use, and which ones are most sensitive; including their dose-
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5 232 dependence. Additionally we will examine the extent to which biomarkers are
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8 233 associated with exposure to nicotine versus exposure to HPHC of tobacco like
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10 234 aldehydes.

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12 235 All statistical analysis will be performed using SAS version 9.4 software (SAS
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14 236 Institute, Inc., Cary, North Carolina), and a two-sided p-value of <0.05 will be considered
15
16 237 significant for any statistical test. Demographics and other baseline characteristics will
17
18 238 be summarized according to product group. The primary outcomes will be analyzed
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20 239 using multiple regression techniques. Appropriate Interaction variables will be tested for
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22 240 in the regression models and subgroup analyses will be conducted according to the
23
24 241 following factors: significant interactions, sex, age, race, tobacco product group.
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26 242 Multiple imputation method will be used for missing data where appropriate. Sensitivity
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28 243 analysis using different analytic approaches, such as generalized linear models, as well
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30 244 as considering different covariate adjustments, will be used to build concordant results.
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35 245 The dose-dependence of the changes in biomarkers will be determined by
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37 246 analyzing the data obtained from individuals that are exposed to different doses of a
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39 247 single product (e.g. smoking 0, <15, 15-20 and >20 cigarettes per day) and by
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41 248 comparing between tobacco products that have different doses of HPHC constituents.
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43 249 In the US the average cigarettes per day is between 15-20³⁷ and therefore this dose
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45 250 range distribution is reflective of general population exposure. Comparisons of the
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47 251 effects of novel tobacco products and smoking will be informative of the relative toxicity
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49 252 of the two products.
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3 253 We believe that the methods employed in the current project are exquisitely
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5 254 sensitive and responsive to even low dose insults such as ambient air pollution ¹²
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8 255 allowing us to quantify tobacco product-induced changes with high precision. Moreover,
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10 256 levels of acrolein exposure vary between different individuals due to difference in puffing
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12 257 intensity and the time a cigarette is left smoldering. Thus, direct measurements of
13
14 258 acrolein metabolites afford better estimates of acrolein exposure than machine yields.
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16
17 259 We expect to obtain wide variations in acrolein/crotonaldehyde exposure which will
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19 260 enable us to construct a dose-response relationship and identify which injury
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21 261 biomarkers are associated with aldehyde exposure and whether high levels of exposure
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23
24 262 are associated with high levels of injury, despite similar nicotine delivery.
25

26 263 **Sample size**

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28 264 The sample size is justified in terms of the primary dependent measure, FMD,
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30
31 265 given the potential importance of this variable as a direct measure of the impact of
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33 266 tobacco exposure. The main comparisons are between non-tobacco users and tobacco
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35 267 users. Due to one control group, we will conservatively adjust our α (significance level)
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37
38 268 using a Bonferroni correction, and we will set $\alpha=0.01$. Based on preliminary data for
39
40 269 FMD, we have observed mean \pm SD in smoker and nonsmoker groups to be 4.0 ± 1.6
41
42 270 and 6.8 ± 1.0 , respectively. We consider at least 25% (mean FMD=3.0 from 4.0)
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45 271 reduction from smokers to non-smokers is meaningful. Using a two sample, one-sided t
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47 272 test with an α of 0.01 and 80% power ($1-\beta$), assuming a common SD of 1.3, we will
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49 273 need 34 evaluable subjects in each group. We will recruit a total of 120 tobacco using
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51 274 participants per site. This over sampling will allow us to look at multiple endpoints and
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54 275 for associations with VOCs.
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276 ETHICS AND DISSEMINATION

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

280 Participants for the CITU study are being recruited in both Boston, MA and
281 Louisville KY. The two populations show significant differences, therefore recruitment at
282 two sites will ensure a range more reflective of the general population. Although overall
283 racial and ethnic demographics for both cities show a clear majority of Caucasians
284 (70%) and despite smokers typically male, we strive to, and currently are successful in,
285 recruiting a population that was gender balanced and almost evenly split between
286 Caucasian and African Americans. Despite this balanced recruitment, e-cigarette users
287 have been reported as predominantly Caucasian and male³⁸, and thus far our
288 recruitment mirrors these demographics. We expect very few Hispanic/Latino's to
289 participate, due to data suggesting tobacco use, including ENDS, tends to be lower
290 among Hispanic's/Latino's^{38 39}. Thus we have also opted to only recruit English
291 speakers. We have carefully develop our recruitment strategy and exclusion criteria to
292 protect vulnerable populations, which is important since many report a lower
293 socioeconomic status and educational level in smokers in addition to higher rates of
294 reported alcohol and drug use^{40 41}.

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

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3 298 requested by the participant. Participant information is de-identified for analysis and
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5 299 reported in aggregate to protect privacy.
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8 300 Completion of these studies will enable a greater understanding of the biological
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10 301 responses to use of a variety of tobacco products. Specifically, they will help to identify
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12 302 the constituents of these products; and how a panel of exposure and CV injury
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14 303 biomarkers are associated with these different constituents. This data will be available
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16 304 to the FDA and could help guide new policy measures to reduce or eliminate the
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18 305 harmful components of tobacco smoke and other nicotine products. The study is
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20 306 dedicated to the rapid dissemination of their rigorously characterized and well-controlled
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22 307 research findings to the public in the form of peer-reviewed publications. Subsequent to
23
24 308 the initial full-length manuscript publications of the resources generated with funding
25
26 309 from this program, the study will make them available to interested and qualified
27
28 310 investigators upon written request. The study will provide relevant protocols of published
29
30 311 data, upon request (presuming prior publication by the Center members). Participants
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32 312 will be provided a summary of the results as they become available. Finally press
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34 313 releases of relevant findings will inform the general population.
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41 42 315 **LIST OF ABBREVIATIONS**

43
44 316 ABI- Ankle Brachial Index

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46 317 CAC= circulating angiogenic cells

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48 318 CRP= C-reactive protein

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50 319 CVD- Cardiovascular disease

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52 320 ENDS- Electronic nicotine Device (i.e. e-cigarette)
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3 321 FACS- Fluorescence-activated cell sorting
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5 322 FMD- Flow mediated dilation
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7 323 HDL= high density lipoprotein
8
9 324 IL-6= Interleukin 6
10
11 325 MMP- Matrix metalloproteinase
12
13 326 MP= micoparticles
14
15 327 PAI=- Plasminogen activator
16
17 328 PF4= Platelet factor 4
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19 329 PWV- Pulse wave velocity
20
21 330 SAA= serum amyloid A
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23 331 s-ICAM- soluble intercellular adhesion protein inhibitor
24
25 332 s-VCAM= soluble vascular adhesion protein
26
27 333 TNFR1= Tumor necrosis factor receptor 1
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29 334 t-PA= tissue plasminogen activator
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31 335 TxA2=Thromboxane A
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33 336 VOC- Volatile organic compound
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35 337 W:H- ratio: Waist to hip ratio
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44 339 **AUTHORS CONTRIBUTIONS**

46 340 Rachel Keith- Study design, study recruitment, study visits, statistical analysis and
47
48 341 manuscript preparation. Jessica Fetterman- study recruitment, study visits, manuscript
49
50 342 preparation and editing. Dan Riggs- statistical analysis, manuscript preparation and
51
52 343 editing. Tim O'Toole- Biomarker measurements, manuscript preparation and editing.
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3 344 Jessica Nystoriak- study recruitment and study visits. Monica Holbrook- study
4
5 345 recruitment and study visits. Pawel Lorkiewicz- VOC measurements and manuscript
6
7 346 preparation. Aruni Bhatnagar- Study design, study funding and manuscript editing.
8
9
10 347 Andrew DeFilippis- Human subject assessment planning, manuscript preparation and
11
12 348 editing. Naomi M. Hamburg- Study design, study funding, vascular core, manuscript
13
14 349 preparation and editing.

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17
18
19 351 None declared

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22
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24
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29
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31
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33
34 359 represent the official views of the NIH or the Food and Drug Administration.

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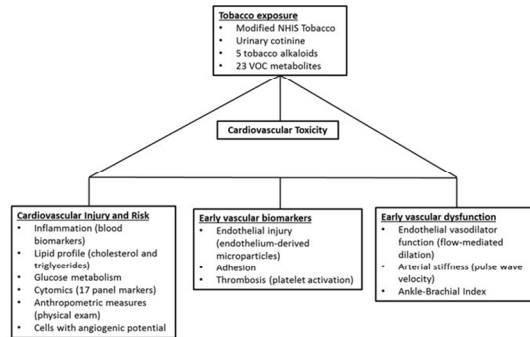
21 22 483 **Figure 1. Cardiovascular Injury due to Tobacco Use**

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24 484 CITU is designed to assess how tobacco related VOC exposure contributes to
25 485 cardiovascular risk factors. Our exposure measurements include a panel of 23
26 486 urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular
27 487 phenotyping includes measures of injury, risk, vascular biomarkers and early
28 488 vascular dysfunction. Tobacco use included use of traditional cigarettes,
29 489 smokeless tobacco, waterpipe tobacco (hookah), electronic nicotine devices
30 490 (ENDS), little cigars, cigarillos, pipes, cigars or any other form of tobacco that is
31 491 available. Enrollment began in July 2014 and is ongoing.

34 492 **Figure 2. Study Visit Design**

35
36 493 Study flow chart for interested participants from screening through study completion.
37 494 Potential participants are pre-screened for eligibility prior to enrollment. Potential
38 495 participants are asked to fast from tobacco for a minimum of 6 hours prior to the
39 496 study visit. On the day of the visit the study lasts approximately 90 minute.

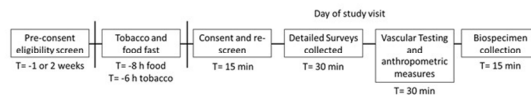
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CITU is designed to assess how tobacco related VOC exposure contributes to cardiovascular risk factors. Our exposure measurements include a panel of 23 urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular phenotyping includes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

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

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

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

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

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

BMJ Open

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2017-019850.R2
Article Type:	Protocol
Date Submitted by the Author:	14-Feb-2018
Complete List of Authors:	Keith, Rachel; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Fetterman, Jessica; Boston Medical Center, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center Riggs, Daniel; American Heart Association- Tobacco Regulation and Addiction Center; University of Louisville, Medicine O'Toole, Timothy; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Nystoriak, Jessica; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Holbrook, Monika; Boston Medical Center, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center Lorkiewicz, Pawel; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Bhatnagar, Aruni; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center DeFilippis, Andrew; University of Louisville, Medicine; American Heart Association- Tobacco Regulation and Addiction Center Hamburg, Naomi ; Boston University, Vascular Biology Section, Whitaker Cardiovascular Institute; American Heart Association- Tobacco Regulation and Addiction Center
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Public health
Keywords:	smoking, tobacco, electronic cigarette, cardiovascular risk, vascular injury, cigarettes

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3 1 **Protocol to Assess the Impact of Tobacco-Induced Volatile Organic Compounds**
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5 2 **on Cardiovascular Risk in a Cross-Sectional Cohort: Cardiovascular Injury Due to**
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7 **Tobacco Study**
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10 4 Rachel J. Keith, Jessica L. Fetterman, Daniel W. Riggs, Tim O'Toole, Jessica Nystoriak,
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12 5 Monica Holbrook, Pawel Lorkiewicz, Aruni Bhatnagar, Andrew DeFilippis*, Naomi M.
13
14 6
15 6 Hamburg*

16
17 7 Rachel J. Keith-Division of Cardiovascular Medicine, University of Louisville School of
18
19 8 Medicine 580 S. Preston St. Louisville KY, 40202 rachel.keith@louisville.edu 502-852-
20
21 9 4211

22
23
24 10 Jessica L. Fetterman- Vascular Biology Section, Whitaker Cardiovascular Institute,
25
26 11 Boston University School of Medicine Evans Building, Boston, MA USA

27
28 12 Daniel W. Riggs- University of Louisville School of Medicine Louisville, KY USA

29
30
31 13 Timothy O-Toole- Division of Cardiovascular Medicine, University of Louisville School of
32
33 14 Medicine Louisville, KY USA

34
35 15 Jessica L. Nystoriak- Division of Cardiovascular Medicine, University of Louisville
36
37 16 School of Medicine Louisville, KY USA

38
39
40 17 Monika Holbrook- Vascular Biology Section, Whitaker Cardiovascular Institute, Boston
41
42 18 University School of Medicine Boston, MA USA

43
44 19 Pawel Lorkiewicz- Division of Cardiovascular Medicine, University of Louisville School
45
46 20 of Medicine Louisville, KY USA

47
48
49 21 Aruni Bhatnagar- Division of Cardiovascular Medicine, University of Louisville School of
50
51 22 Medicine Louisville, KY USA

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

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

28 **Word Count: 2581**

30 **ABSTRACT**

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

41 **Methods and analysis:** We present the design and methodology of the CITU study a
42 cross-section observational tobacco study conducted in Boston MA and Louisville KY
43 starting in 2014. Healthy participants 21 to 45 years of age who use tobacco products,
44 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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3 46 for evaluation of self-reported tobacco exposure, VOC exposure and tobacco-induced
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5 47 injury profiling. Detailed information about participant's demographics, health status and
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7 48 lifestyle is also collected.
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10 49 **Ethics and dissemination:** The study protocol was approved institutional review
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12 50 boards at both participating universities. All study protocols will protect participant
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14 51 confidentiality. Results from the study will be disseminated via peer-reviewed journals
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16 52 and presented at scientific conferences.
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22 54 **Strengths and limitations**

- 23
24 55 • Young age to allow for evaluation of early stage disease (e.g. inflammation,
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26 56 endothelial function) as opposed to end stage clinical consequence (e.g.
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28 57 myocardial infarction)
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30 58 • Diverse tobacco product use allows for assessment of a wide range of tobacco-
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32 59 induced VOC exposure
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34 60 • All study visits are in English introducing selection bias
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36 61 • Data will inform regulatory agencies on the cardiovascular health effects of
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38 62 multiple tobacco products and the contribution of HPHCs
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45 64 **Keywords:** Tobacco, smoking, electronic cigarette, vascular injury, cardiovascular risk,
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47 65 cigarettes.
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52 67 **INTRODUCTION**

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3 68 Tobacco product use and smoking are the leading causes of preventable deaths
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5 69 throughout the world. Of those deaths, one-third are attributed to cardiovascular disease
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7 70 (CVD)¹. The cardiovascular (CV) effects of tobacco exposure can include
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10 71 atherogenesis, vascular injury, thrombosis, arrhythmias and inflammation² and may be
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12 72 attributable to the many different harmful and potentially harmful constituents (HPHCs)
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15 73 present in tobacco products.

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17 74 The HPHCs found in tobacco products include volatile organic compounds
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19 75 (VOCs) of which reactive aldehydes, such as acrolein and crotonaldehyde, are likely the
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21 76 most significant contributors to CV toxicity³. High levels of aldehydes are present in
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23 77 cigarette smoke^{4,5} as well as smokeless tobacco (ST)⁶. Risk assessments, using the
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25 78 prevalence of each individual chemical weighed by its potency, suggest that the non-
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27 79 cancer risk of smoking is dominated by acrolein, which contributes 40-100 times more
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29 80 to risk than any other chemical present in cigarette smoke³.

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33 81 Although HPHCs, including VOC reactive aldehydes, have been suspected to be
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35 82 major contributors to the toxicity of cigarette smoke for over 4 decades, their
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37 83 contribution to CV injury and early CVD risk has not been rigorously evaluated.
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39 84 Experimental studies in animal models suggest that because of low aldehyde-
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41 85 metabolizing capacity, CV tissues are highly sensitive to aldehydes and exposure to low
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43 86 levels of aldehydes can induce CV injury and accelerate CVD⁷⁻¹⁸. The WHO Study
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45 87 Group on Tobacco Product Regulation (TobReg) has marked acrolein, a VOC, along
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47 88 with 8 other cigarette constituents for monitoring and regulation¹⁹ and the U.S.
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49 89 Environmental Protection Agency lists Acrolein as one of most hazardous air
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51 90 pollutants²⁰. Nevertheless, the contribution of tobacco induced VOCs, including acrolein

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3 91 or other aldehydes, toward CV toxicity in humans has not been fully assessed. Greater
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5 92 understanding of how aldehydes affect cardiovascular health and disease will provide
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7 93 new avenues for evaluating the toxicity of cigarette smoke and for assessing the
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9 94 injurious potential of new and emerging tobacco products, such as ENDS, which may
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11 95 also contain VOCs including acrolein²¹⁻²³.
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14 96 The latency period between tobacco exposure and the development of major
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16 97 clinical adverse health effects is long, therefore biomarkers that provide information over
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18 98 a shorter period allow for the identification of harm decades before clinical outcome data
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20 99 is available. Thus, in this paper we present the design and methodology of the
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22 100 Cardiovascular Injury due To Tobacco Use (CITU) study which will evaluate the
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24 101 association of the urinary metabolites of 18 parent VOCs from tobacco exposure with a
25
26 102 comprehensive set of CV biomarkers representative of early disease and predictive of
27
28 103 future CV events.²⁴
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33 104 **METHODS AND DESIGN**

35 105 **Overall design**

37 106 The CITU study is an investigator-initiated cross-sectional observational study of
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39 107 around 500 healthy participants 21 to 45 years of age who are never or current tobacco
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41 108 product users in two urban areas at Boston University (BU) and University of Louisville
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43 109 (UofL) (Boston, MA and Louisville, KY) designed to evaluate CV toxicity due to tobacco
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45 110 product use, with correlations to VOCs found in the tobacco products (**Figure 1**).
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54 113 **Participant Eligibility Criteria**

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3 114 The goal of the study is to examine the impact of tobacco products on healthy
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5 115 young adults who could be classified as a current tobacco product users (Defined in
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7 116 table 1), or never-users (does not have lifetime use of any tobacco product).
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10 117 Participants were self-reported to be healthy therefore we excluded participants if they
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12 118 had: 1) diagnosis of clinical cardiovascular disease including but not limited to known
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14 119 heart attack, peripheral artery disease, heart failure or stroke; 2) diagnosis of diabetes
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16 120 (HbA1c >7.0 or treatment for diabetes), hypertension (systolic blood pressure >140 mm
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18 121 Hg or diastolic blood pressure >90 mm Hg), hypothyroidism or hyperthyroidism,
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20 122 inflammatory conditions such as lupus or inflammatory bowel disease, HIV/AIDS,
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22 123 hepatitis, liver disease, anemia, cancer of any type or another medical condition that
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24 124 might compromise the successful completion of the study; 2) recipients of organ
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26 125 transplant or renal replacement therapy; 3) individuals that are taking the following
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28 126 medications: immunosuppressant agents estrogen, testosterone, anti TNF agents,
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30 127 certain biologics, Procrit, statins, beta-blockers or other cardiovascular medicine; 4)
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32 128 individuals using nutraceuticals or anabolic steroids beyond the recommended daily
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34 129 allowance; 5) body weight less than 100 pounds; 6) pregnant women; 7) prisoners and
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36 130 other vulnerable populations; and 8) active illness or infection. Participants are
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38 131 rescheduled or considered screen-failures and excluded from the study if symptomatic
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40 132 of an acute illness, i.e. viral upper respiratory infection, on study date.
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47 **Table 1. Tobacco product use classifications**

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

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

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

137 **Overall Study Procedure**

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

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

151 **Exposure Variables**

152 Tobacco Product Use & Particulate Matter Exposure

153 Comprehensive tobacco product exposure is assessed using a modified version
 154 of the National Health Interview survey on tobacco use²⁵. The survey is modified to
 155 include detailed information on electronic nicotine devices (ENDs) and other new or

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3 156 emerging tobacco products. Residential addresses are collected for assessment of
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5 157 ambient airborne particulate matter (PM_{2.5}) exposure and future correction of overall
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7 158 exposure. PM_{2.5} data from the day of the study visit, and 3 and 5 days prior to the study
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9
10 159 is collected from publicly available data associated with EPA monitoring stations. Other
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12 160 exposure variables, including occupation, are collected through interview.

14 161 VOC Measurements

16 162 Humans are exposed to VOCs from a variety of sources including indoor and
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18 163 outdoor environments as well as diet. The most significant sources of ambient exposure
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20 164 ambient are air pollution, car exhaust, household products, personal hygiene products,
21
22 165 and solvents^{26 27}. Although concurrent exposures from multiple sources could confound
23
24 166 attribution to smoking, the levels of urinary metabolites of these VOCs in smokers far
25
26 167 exceeds those measured in non-smokers exposed to typical sources of VOCs²⁸.

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28
29 168 Standard clean catch urine specimens are obtained from participants. Though
30
31 169 only a single urine time point is collected, previous studies show spot urine
32
33 170 measurements correlate well with 24-hour urine collections²⁹. Many VOC metabolites
34
35 171 have relatively short half-lives that range from 2 - 25.2h,^{30 31} but given the constant
36
37 172 pattern of tobacco product use by most users, spot collection reflects recurrent use.
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39 173 Moreover, even though some VOC metabolites, such as HPMA, are known vary with
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41 174 time of day,²⁹ synchronizing the study visits and requiring a tobacco fast is likely to
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43 175 minimize diurnal variations in metabolism. Our past work has shown that spot-urine
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45 176 collected at the same time of day reliably reflects daily VOC exposure and is correlated
46
47 177 to CVD risk³².

178 We have developed a robust Core Lab that utilizes mass spectrometry
 179 procedures adopted from the Centers for Disease Control and Prevention (CDC)
 180 protocols, to quantify 23 urinary metabolites of tobacco smoking related toxins
 181 (aldehydes and other VOCs), including acrolein³³ (**Table 2**). The concentration values of
 182 analytes are then normalized to urinary creatinine levels measured using Infinity
 183 Creatinine Reagent (Thermo Fisher Scientific, MA) on a COBAS MIRA-plus analyzer
 184 (Roche, NJ).

185 **Table 2 Exposure Variables (Please see end of article)**

<i>Parent compound</i>	<i>VOC metabolite</i>	<i>Common abbr.</i>
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-carbamoyl-ethyl)-L-cysteine	AAMA
	N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	GAMA
Acrylonitrile	N-Acetyl-S-(2-cyanoethyl)-L-cysteine	CYMA
Acrylonitrile, vinyl chloride, ethylene oxide	N-Acetyl-S-(2-hydroxyethyl)-L-cysteine	HEMA
Anabasine	Anabasine (free)	ANB
Anatabine	Anatabine (free)	ANTB
Benzene	N-Acetyl-S-(phenyl)-L-cysteine	PMA
	trans, trans-Muconic acid	MU

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

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

186

187 *Urine is analyzed for 23 metabolites of 18 parent VOCs and tobacco alkaloids by UPLC-*

188 *MS/MS. Analytes are listed as parent, metabolite and their common abbreviation.*

189

190 **Circulating Markers of Cardiovascular Injury**

191 To assess tobacco product-induced cardiovascular toxicity, we examine
 192 endothelial function, inflammatory mediators, biomarkers, and thrombosis. CV risk is
 193 defined through measurements 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
 197 UofL pathology core. BU biologic samples have minimal processing and are shipped
 198 overnight to the UofL central laboratory at the completion of each study visit. Samples
 199 obtained at UofL are processed to a similar stage, then held overnight prior to analysis
 200 to standardize the time to measurement for all samples. The UofL central laboratory, as
 201 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 peripheral blood phenotyping panel kit (Fluidigm). Samples are shipped

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

206 **Table 3 Blood analysis**

Fasting Measurements

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

Biomarkers

CAC (1-15)¹, Platelet-monocyte aggregates, MP (1-5)¹, PF4, t-PA, TxA2, Factor VII, IL-6, CRP, D-dimer, PAI-1, s-ICAM-1, s-VCAM, s-thrombomodulin, s-TNFR1, MMP-2, MMP-3, MMP-9, cytomics, endothelin, E-selectin and P-selectin

1: Fifteen different CAP subpopulations and 5 subtypes of microparticles were measured by flow cytometry.

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

216 **Non-Invasive Vascular Function Testing**

217 Smoking, is associated with endothelial damage and vascular dysfunction^{37 38}.
218 Endothelial cells are exposed to circulating toxins and measures of endothelial function
219 are reflective of cardiovascular injury³⁹. Thus, we examine the non-invasive endothelial
220 vasodilator function using flow-mediated vasodilation^{40 41}, arterial stiffness with carotid-
221 femoral and carotid-radial pulse wave velocity⁴², and peripheral vascular function with

222 ankle brachial index. Flow mediated dilation was assessed with a 7.5MHZ ultrasound
223 probe is used to image the brachial artery while a 10cm blood pressure cuff is attached
224 to the lower arm and a 3 lead ECG is attached to the patient. After baseline images and
225 10 cycles of Doppler images are captured using NIHEM R-wave triggered image
226 capturing software, the blood pressure cuff is inflated to 200mmHg or 50mmHg higher
227 than the systolic pressure. After the 5 minute occlusion, the cuff is released and the
228 NIHEM software records two minutes of imaging. Images were analyzed by a single
229 blinded analyzer using MIA vascular Research Tolls Brachial Analyzer for Research,
230 version 6.8.5. All vascular imagers where trained at BU who have a previously reported
231 reproducibility with intra- and inter-observer correlation coefficients of 0.98 and 0.99 for
232 brachial diameter and 0.78 and 0.92 for FMD.⁴³ Similar equipment and software is used
233 at both sites. All vascular studies are sent to the BU central lab for analysis.

234 **Anthropometric measures**

235 Anthropometric measures included height, weight, waist and hip circumference
236 and body fat. All anthropometric measures are completed twice and the average
237 recorded. Standing height measurements are completed on a fixed stadiometer. Weight
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
242 bioelectrical impedance measured with the Omron fat loss monitor (HBF-306C).

243 **DATA ANALYSIS**

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2
3 244 We expect that from this study we will be able to identify specific biomarkers of
4
5 245 cardiovascular injury due to tobacco use and the relationship of these biomarkers to
6
7 246 specific measures of tobacco exposure. For instance, we will identify which biomarkers
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9
10 247 are affected by tobacco use, and which ones are most sensitive; including their dose-
11
12 248 dependence. Additionally we will examine the extent to which biomarkers are
13
14 249 associated with exposure to nicotine versus exposure to HPHC of tobacco like
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16
17 250 aldehydes.

19 251 **Sample size**

21 252 The sample size is justified in terms of the primary dependent measure, FMD,
22
23 253 given the potential importance of this variable as a direct measure of the impact of
24
25 254 tobacco exposure. The main comparisons are between non-tobacco users and tobacco
26
27 255 users. Due to one control group, we will conservatively adjust our α (significance level)
28
29 256 using a Bonferroni correction, and we will set $\alpha=0.01$. Based on preliminary data for
30
31 257 FMD, we have observed mean \pm SD in smoker and nonsmoker groups to be 4.0 ± 1.6
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33 258 and 6.8 ± 1.0 , respectively. We consider at least 25% (mean FMD=3.0 from 4.0)
34
35 259 reduction from smokers to non-smokers is meaningful. Using a two sample, one-sided t
36
37 260 test with an α of 0.01 and 80% power ($1-\beta$), assuming a common SD of 1.3, we will
38
39 261 need 34 evaluable subjects in each group. We will recruit a total of 120 tobacco using
40
41 262 participants per site. This over sampling will allow us to look at multiple endpoints and
42
43 263 for associations with VOCs.

49 264 **Analysis Plan**

51 265 All statistical analysis will be performed using SAS version 9.4 software (SAS
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53 266 Institute, Inc., Cary, North Carolina), and a two-sided p-value of <0.05 will be considered
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3 267 significant for any statistical test. Demographics and other baseline characteristics will
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5 268 be summarized according to product group. Differences in VOC's between product
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7 269 groups will be tested using ANOVA for normally distributed data or Kruskal-Wallis test
8
9 270 for non-normal data. The association between primary outcomes of vascular function
10
11 271 as well as circulating markers of cardiovascular injury with individual VOC levels will be
12
13 272 analyzed using multiple regression models, adjusting for appropriate confounders.
14
15 273 Additionally, because we have multiple VOC's, which are highly correlated, we will use
16
17 274 methods such as LASSO to identify the VOC's that are most associated with the
18
19 275 outcomes of interest. Multipollutant approaches, such as principal component analysis
20
21 276 (PCA), will be used to test whether overall VOC exposure is associated with the health
22
23 277 outcomes. Interaction variables will be tested for in the regression models and
24
25 278 subgroup analyses will be conducted according to the following factors: significant
26
27 279 interactions, sex, age, race, tobacco product group. Multiple imputation method will be
28
29 280 used for missing data where appropriate. Sensitivity analysis using different analytic
30
31 281 approaches, such as generalized linear models, as well as considering different
32
33 282 covariate adjustments, will be used to build concordant results.
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40 283 The dose-dependence of the changes in biomarkers will be determined by
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42 284 analyzing the data obtained from individuals that are exposed to different doses of a
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44 285 single product (e.g. smoking 0, <10, 10-20 and >20 cigarettes per day) and by
45
46 286 comparing between tobacco products that have different doses of HPHC constituents.
47
48 287 In the US the average cigarettes per day is between 10-20⁴⁴ and therefore this dose
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50 288 range distribution is reflective of general population exposure. Comparisons of the
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3 289 effects of novel tobacco products and smoking will be informative of the relative toxicity
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5 290 of the two products.
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7
8 291 We believe that the methods employed in the current project are exquisitely
9
10 292 sensitive and responsive to even low dose insults such as ambient air pollution ¹²
11
12 293 allowing us to quantify tobacco product-induced changes with high precision. Moreover,
13
14 294 levels of acrolein exposure vary between different individuals due to difference in puffing
15
16 295 intensity and the time a cigarette is left smoldering. Thus, direct measurements of
17
18 296 acrolein metabolites afford better estimates of acrolein exposure than machine yields.
19
20 297 We expect to obtain wide variations in acrolein/crotonaldehyde exposure which will
21
22 298 enable us to construct a dose-response relationship and identify which injury
23
24 299 biomarkers are associated with aldehyde exposure and whether high levels of exposure
25
26 300 are associated with high levels of injury, despite similar nicotine delivery.
27
28
29

30 301 **ETHICS AND DISSEMINATION**

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32
33 302 The CITU study was approved at each institution by their institutional review
34
35 303 board (BU #H-32613 and UofL #13.0590) and all participants provide written consent.
36
37 304 No study related procedures will be completed until after participant consent.
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40 305 Participants for the CITU study are being recruited in both Boston, MA and
41
42 306 Louisville KY. The two populations show significant differences, therefore recruitment at
43
44 307 two sites will ensure a range more reflective of the general population. Although overall
45
46 308 racial and ethnic demographics for both cities show a clear majority of Caucasians
47
48 309 (70%) and despite smokers typically male, we strive to, and currently are successful in,
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50 310 recruiting a population that was gender balanced and almost evenly split between
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52 311 Caucasian and African Americans. Despite this balanced recruitment, e-cigarette users
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3 312 have been reported as predominantly Caucasian and male⁴⁵, and thus far our
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5 313 recruitment mirrors these demographics. We expect very few Hispanic/Latino's to
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7 314 participate, due to data suggesting tobacco use, including ENDS, tends to be lower
8
9 315 among Hispanic's/Latino's^{45 46}. Thus we have also opted to only recruit English
10
11 316 speakers. We have carefully develop our recruitment strategy and exclusion criteria to
12
13 317 protect vulnerable populations, which is important since many report a lower
14
15 318 socioeconomic status and educational level in smokers in addition to higher rates of
16
17 319 reported alcohol and drug use^{47 48}.

20
21 320 Our study is an observational study where participants have already assumed
22
23 321 the risk of using tobacco. Study procedures pose minimal risk. Given the known harms
24
25 322 associated with smoking, we will provide information on tobacco treatment when
26
27 323 requested by the participant. Participant information is de-identified for analysis and
28
29 324 reported in aggregate to protect privacy.

30
31 325 Completion of these studies will enable a greater understanding of the biological
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33 326 responses to use of a variety of tobacco products. Specifically, they will help to identify
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35 327 the constituents of these products; and how a panel of exposure and CV injury
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37 328 biomarkers are associated with these different constituents. This data will be available
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39 329 to the FDA and could help guide new policy measures to reduce or eliminate the
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41 330 harmful components of tobacco smoke and other nicotine products. The study is
42
43 331 dedicated to the rapid dissemination of their rigorously characterized and well-controlled
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45 332 research findings to the public in the form of peer-reviewed publications. Subsequent to
46
47 333 the initial full-length manuscript publications of the resources generated with funding
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49 334 from this program, the study will make them available to interested and qualified
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3 335 investigators upon written request. The study will provide relevant protocols of published
4
5 336 data, upon request (presuming prior publication by the Center members). Participants
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7 337 will be provided a summary of the results as they become available. Finally press
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9 338 releases of relevant findings will inform the general population.
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340 **LIST OF ABBREVIATIONS**

341 ABI- Ankle Brachial Index

342 CAC= circulating angiogenic cells

343 CRP= C-reactive protein

344 CVD- Cardiovascular disease

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

346 FACS- Fluorescence-activated cell sorting

347 FMD- Flow mediated dilation

348 HDL= high density lipoprotein

349 IL-6= Interleukin 6

350 MMP- Matrix metalloproteinase

351 MP= micoparticles

352 PAI=- Plasminogen activator

353 PF4= Platelet factor 4

354 PWV- Pulse wave velocity

355 SAA= serum amyloid A

356 s-ICAM- soluble intercellular adhesion protein inhibitor

357 s-VCAM= soluble vascular adhesion protein

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2
3 358 TNFR1= Tumor necrosis factor receptor 1
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5 359 t-PA= tissue plasminogen activator
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7
8 360 TxA2=Thromboxane A
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10 361 VOC- Volatile organic compound
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12 362 W:H- ratio: Waist to hip ratio
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14
15 363

16 17 364 **AUTHORS CONTRIBUTIONS** 18

19 365 Rachel Keith- Study design, study recruitment, study visits, statistical analysis and
20

21 366 manuscript preparation. Jessica Fetterman- study recruitment, study visits, manuscript
22

23 367 preparation and editing. Dan Riggs- statistical analysis, manuscript preparation and
24

25 368 editing. Tim O'Toole- Biomarker measurements, manuscript preparation and editing.
26
27

28 369 Jessica Nystoriak- study recruitment and study visits. Monica Holbrook- study
29

30 370 recruitment and study visits. Pawel Lorkiewicz- VOC measurements and manuscript
31

32 371 preparation. Aruni Bhatnagar- Study design, study funding and manuscript editing.
33
34

35 372 Andrew DeFilippis- Human subject assessment planning, manuscript preparation and
36

37 373 editing. Naomi M. Hamburg- Study design, study funding, vascular core, manuscript
38

39 374 preparation and editing.
40
41

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46

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7
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9
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11

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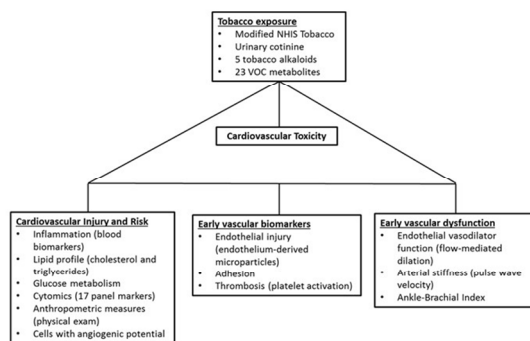
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18 531 **Figure 1. Cardiovascular Injury due to Tobacco Use**

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21 532 CITU is designed to assess how tobacco related VOC exposure contributes to
22 533 cardiovascular risk factors. Our exposure measurements include a panel of 23
23 534 urinary metabolites of 18 parent VOCs and tobacco use patterns. Cardiovascular
24 535 phenotyping includes measures of injury, risk, vascular biomarkers and early
25 536 vascular dysfunction. Tobacco use included use of traditional cigarettes,
26 537 smokeless tobacco, waterpipe tobacco (hookah), electronic nicotine devices
27 538 (ENDS), little cigars, cigarillos, pipes, cigars or any other form of tobacco that is
28 539 available. Enrollment began in July 2014 and is ongoing.

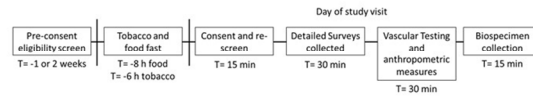
30 540 **Figure 2. Study Visit Design**

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32 541 Study flow chart for interested participants from screening through study completion.
33 542 Potential participants are pre-screened for eligibility prior to enrollment. Potential
34 543 participants are asked to fast from tobacco for a minimum of 6 hours prior to the
35 544 study visit. On the day of the visit the study lasts approximately 90 minute.



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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 minutes.

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

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

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

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

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