Official Study Title: Investigating the Cardiovascular Toxicity of Exposure to Electronic Hookah Smoking

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Study Protocols for Vaping and Smoking Exposure Sessions:

E-Hookah Vaping Protocol. To determine the acute effect of e-hookah vaping on FMD, blood pressure, heart rate, and FMD were measured before and after (within ≤10 minutes) 30 minutes of e-hookah bowl vaping. Ten minutes after FMD testing (to allow arterial diameter to return to baseline size), endothelium-independent dilatation was assessed by administering sublingual nitroglycerin. Using an e-hookah bowl (Starbuzz Wireless E-head) placed on a traditional waterpipe, subjects were instructed to vape e-hookah fruit-flavored liquid containing 50/50 blend of propylene glycol and vegetable glycerin and 6 mg/mL nicotine, according to the package label (Starbuzz Tobacco, Inc.).

Traditional Charcoal-Heated Hookah Smoking Protocol. A traditional water-pipe was used for hookah smoking protocol. Participants were instructed to smoke the most popular brand of maassel cited by hookah smokers and manufactured in the United States (5%-10% tobacco fermented with molasses, fruit, and glycerin; Starbuzz Tobacco, Inc.) heated with two charcoal briquettes (Coco Nara 100% Natural Coal, Coco Nara).

To mitigate the impact of carryover effects, both exposure sessions were separated by a minimum of a 7-day washout period. Topography were standardized in accordance with hookah smoking puffing parameters observed in natural settings. Thus, for the duration of the 30-min inhalation sessions, all participants were cued verbally to inhale a 3-s puff at 20-s intervals, with vapor remaining in the lungs for approximately 3 s of breath-holding after inhalation. Supervision was carried out to prevent superficial vaping or hyperventilation. Experimental sessions took place in a specifically designed

smoking/vaping area, housed within the University of California, Los Angeles, Clinical and Translational Research Center.

Study Protocol for Flow-Mediated Dilation and Vascular Endothelium-Independent Dilation

Brachial artery flow-mediated dilation (FMD) was performed in strict accordance with guidelines described by Thijssen et al. *European Heart Journal*. 2019. Briefly, after a resting period of 15 minutes, the left arm was abducted at heart level, placed on a foam pad, and the brachial artery (3–7 cm above the antecubital crease) was imaged using a 5-to-12-MHz linear array transducer attached to a high-resolution ultrasound machine (Toshiba, Xario XG 2000). To ensure the location of the same arterial segment after the exposure session, anatomical landmarks were noted and the distance from the antecubital crease was recorded. A rapid-inflation/deflation pneumatic cuff (Hokanson) was placed on the upper forearm for 5 minutes and inflated to suprasystolic pressure (250 mm Hg). Doppler velocity was measured continuously with a fixed insonation angle of 60°, using a stereotaxic instrument to stabilize probe position.

Baseline diameter and velocity were recorded for 45 seconds and resumed 30 seconds before cuff deflation and continuously for 2 minutes after deflation to obtain true peak vasodilatory response. Recordings were triggered and captured at the R-wave of the ECG (end-diastolic diameter) using AccuSync 72 ECG trigger monitor and stored for offline analysis using validated edge-detection software (Brachial Analyzer for Research, Medical Imaging Applications, LLC). FMD measurements were calculated as absolute and percent changes in brachial artery diameter. Time to peak was calculated as the interval from the point of occlusion cuff deflation to the maximum brachial artery diameter. Peak hyperemic shear rate was calculated as (8× time averaged peak velocity)/occlusion diameter, based on a wide-centered sample volume from the first 15

velocity envelopes following cuff release. Because the main stimulus for FMD is an acute increase in hyperemic shear stress, to account for potential differences in peak hyperemic shear rate between conditions, FMD values were also normalized for the magnitude of the hyperemic stimulus (i.e., change in diameter divided by the hyperemic shear rate).

Vascular endothelium-independent dilatation was assessed with sublingual nitroglycerin (0.15 mg) with brachial artery images recorded continuously for 10 minutes. Since hormonal changes can affect FMD, women were studied in a standardized phase of the menstrual cycle (i.e., follicular phase).

Study Protocol for Intravenous Ascorbic Acid Protocol. To determine if oxidative stress plays a mechanistic role in the e-hookah vaping-associated decline in FMD, the acute effects of e-hookah vaping on FMD and exposure biomarkers were assessed before and after intravenous administration of a supra-physiological dose of ascorbic acid (Mylan Institutional Inc.): priming bolus of 0.06 g per kg fat-free mass dissolved in 100 mL of saline infused at 5 mL min−1 for 20 minutes followed by an intravenous infusion of 0.02 g per kg fat-free mass dissolved in 30 mL of saline administered over 60 minutes at 0.5 mL min−1. The maximum dose of ascorbic acid administered did not exceed 5 g. Ten minutes after FMD testing, endothelium-independent dilatation was assessed by administering sublingual nitroglycerin (0.15 mg) with brachial artery images recorded continuously for 10 minutes.

Study Protocol for Central Arterial Stiffness

Carotid-femoral PWV was measured by simultaneous waveform capture using both a thigh-specific cuff and carotid artery applanation tonometry (SphygmoCor XCEL; AtCor Medical). Velocity (*d*sf – *d*sc (m) / time (s)) was calculated by measuring the time difference between the initial upstroke of the recorded waveforms at each site. The linear distance was measured manually from the suprasternal notch to the top of the thigh cuff at the center line of the leg, at the location of the femoral artery (*d*sf), and subtracting the distance from the suprasternal notch to the location of the carotid pulse (*d*sc). The transit time between the carotid and the femoral pulse waves was determined automatically by the SphygmoCor software.

The augmentation index was derived from the contour of the brachial BP waveform. The brachial-artery waveform, calibrated using oscillometric brachial artery BP, was analyzed by the validated brachial-to-aortic SphygmoCor transfer function to generate a central waveform and associated parameters. The augmentation index was calculated as the ratio of augmentation pressure (difference between the second and first systolic peaks of the aortic pressure waveform) and pulse pressure expressed as a percentage.

Study Protocol for Biomarkers of Oxidative Stress and Inflammation

Blood samples were obtained from the antecubital vein, drawn into pre-iced heparinized vacutainers, and placed on ice. Tubes were sent to the University of California, Los Angeles, Clinical Laboratory for inflammatory biomarker analyses, which were performed within 24 h after collection. One tube was centrifuged to separate plasma for antioxidant biomarker analyses, and samples were frozen at –80°C in a cryopreservative solution.

Arylesterase Activity: Activity was determined by the rate of hydrolysis of phenyl acetate to phenol. Briefly, 4 mL plasma was incubated with 3.5 mM phenyl acetate in 9 mM Tris-HCl buffer (pH, 8.0) containing 0.9 mM CaCl2 at RT. The kinetics of phenol formation were determined by recording the absorbance at 270 nm every 15 s for 2 min. The activity was expressed as nanomoles of product formed per minute per milliliter of plasma.

PON-1 Activity: The ability of PON-1, associated with HDL, to hydrolyze paraoxon substrate was determined. The hydrolysis of paraoxon (diethyl-p-nitrophenyl phosphate) to p-nitrophenol by PON-1 was determined by incubating 5 mL of plasma with 1.0 mM paraoxon in 100 mM tris-HCl buffer (pH, 8.5). The kinetics of p-nitrophenol formation was determined by recording absorbance at 405 nm every 15 s for 4 min. The enzyme activity was expressed as micromoles of p-nitrophenol formed per minute for every 1 mL plasma and assayed in triplicates. The intra-assay CV for the assay was 2.60% and the interassay CV was 8.95%.

HDL Antioxidant Capacity: Capacity was determined as the ability of HDL to inhibit LDL-induced oxidation of dihydrodichlorofluorescein into the fluorescent dichlorofluorescein. Capacity was expressed as an HDL oxidative index, determined by the ratio of dichlorofluorescein fluorescence in the presence and absence of HDL and assayed in triplicates. An index of < 1.0 denotes protective antioxidant HDL, whereas an index of > 1.0 indicates pro-oxidant HDL. The within-assay CV was 6.89%. The interassay CV for four separate measurements over a period of 2 months was 7.30%.

Serum hsCRP levels were analyzed using the immunoturbidimetric method using the Siemens Vista Dimension analyzer, which provides a minimum detection level of < 0.2 mg/L; and TNFα was analyzed using quantitative multiplex bead assay.

Biomarkers of Exposure. Plasma nicotine levels were assayed by gas chromatography with nitrogen-phosphorus detection, using 5-methylnicotine and 1-methyl-5-(2-pyridyl)-pyrrolidin-2-one (ortho-cotinine) as internal standards. Expired carbon monoxide (CO) measurements were carried out using a CO meter (Micro Smokerlyzer; Bedfont Scientific Ltd.).