Methods:

Animals. Male Sprague Dawley rats ([H1a: (SD) CVF, n = 6 rats/group; 6-7 weeks of age, 200 – 250 g) were obtained from Hilltop Lab Animals, Inc., Scottdale, PA. All animals were free of viral pathogens, parasites, *mycoplasms*, *Helicobacter* and cilia-associated respiratory (CAR) bacillus. The rats were acclimated to the facilities for 1 week after arrival. All animals were housed in cages ventilated with HEPA-filters under controlled temperature and humidity conditions and a 12h light/12h dark cycle. Food (Teklad 7913) and tap water were provided *ad libitum*. The animal facilities are pathogen-free, environmentally controlled, and accredited by AAALAC International. All procedures were approved by the NIOSH Animal Care and Use Committee and are in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals.

Exposure. After 1 week of acclimation to the facility, animals were assigned to purified air (control) or PC-emissions. More specifically, three desktop 3D-printers (Manufacturer 1, NY) were placed in a stainless-steel chamber. Black polycarbonate filament was fed into each printer and the printers were operated continuously during a 4 h exposure period. Concentrations of measured PC-emissions were 0.6 mg/m^3 (Farcas et al. 2019).

At the beginning of each exposure, animals were placed into the designated animal chamber (air or PC-emissions exposure). Animals were individually housed during the exposure. A complete description of the exposure chamber and system can be found in (Farcas et al. 2020). Emissions from the printing chamber were pumped into the whole-body inhalation chamber. At the end of each exposure, animals were removed from the exposure chamber, placed into their home cages and returned to the colony room until the next exposure.

Analysis of volatile organic compounds by gas chromatography/mass spectroscopy (GC/MS). The collection of samples and the measurement of volatile organic components (VOCs) of PC-emissions is described in detail in (Krajnak, Farcas, McKinney, et al. 2023). The method used for measurement of VOCs by GC/MS can be found in (National Institute for Occupational Safety and Health, 2018). It is unlikely that levels of VOCs generated during 3D printing with PC induced any significant health effects in this study because all levels were well below the permissible established limits (PELs; see Table 1).

BPA and bisphenol A diglycidyl ether were also assessed in the PC-emissions. Samples were collected as described in (Krajnak, Farcas, McKinney, et al. 2023). Particulate was collected over a 4 h period at 1 liter per/min onto glass fiber filters (SKC lot #21600-7E5-274; n = 6 samples) from chambers where the particulate concentration was approximately 0.6 mg/m^3. The filters were then analyzed by BVNA labs (Novi, MI). The average level of bisphenol in the samples was 5.3 ± 0.18 µg. Using a mass particle distribution model to determine BPA deposition in the nose, trachea, alveolae and lung (where the tidal volume is 1.7 ml, the breathing rate is 120 breaths/min and the exposure time is 240 min), the estimated cumulative deposition of BPA in the entire respiratory tract was 36 ng per day 1.

Tissue collection: Animals were exposed to purified air or PC-emissions for 4 h/day, 4 days/week. Groups of animals (6 air control and 6 treated) were euthanized by injection of pentobarbital (100-300 mg/kg i.p.) followed by exsanguination 24 h after 1, 4, 8, 15 or 30d of exposure. Heart tissue was collected and processed for histological and immunohistochemical analyses to look for morphological changes and changes in various protein concentrations, and qRT-PCR to measure changes in transcript expression. Ventral tail arteries were also dissected

and vasoconstriction and vasodilation in response to phenylephrine and acetylcholine were measured in a microvessel system (Scincta, Ontario).

Tissue preparation: Histology and Immunohistochemistry. The heart was dissected from each animal. Each sample was fixed in 10% formalin for 24h, paraffin embedded and sections (5 μm) were cut on a microtome. Five slides were collected from each animal, with each slide having 1-2 tissue sections. One set of sections was stained with Harris hematoxylin and eosin (H&E) for histological analyses (assessment of number of inflammatory cells in cardiac muscle and arterial diameter and muscle thickness). The other sections were stored in boxes until used for immunohistochemical identification of steroid receptors, oxidative stress and vascular remodeling.

Slides used for immunohistochemistry were de-paraffinized by heating slides at 60ºC in an oven and then putting them through 2-20 min rinses with xylene, and a descending series of ethanol rinses to remove the paraffin. Sections were then placed and kept in water to rehydrate the sections prior to immunohistochemistry. Antibody retrieval was performed by putting slides into 0.5 M citrate buffer and microwaving for approximately 5 min (4 x 1 min 20 second intervals at the lowest power to prevent the citrate buffer from boiling over). Slides were then incubated in fresh citrate buffer for an additional 5 min, rinsed briefly with water, and then with 0.1 M phosphate buffered saline (PBS: pH 7.4).

Immunohistochemistry. Immunohistochemistry was performed using a modification of the protocol described in (Stefaniak et al. 2017; Hubbs et al. 2011; Krajnak, Farcas, McKinney, et al. 2023). Briefly, a liquid proof barrier was drawn around tissue sections on each slide to keep all solutions on the tissues. One hundred μl of each solution was pipetted onto the tissue. Endogenous peroxidase activity was reduced by incubating tissues in 0.3 % hydrogen peroxide

in methanol for 20 min then rinsing with 0.1 M PBS (3 x 5 min rinses). Sections were then incubated in primary antibody diluted in normal serum and PBS+ 0.3% Triton-X 100 overnight in humidified chambers at 4ºC. The primary antibodies used were all from Santa Cruz Biotechnology (Dallas, TX) and included mouse-anti estrogen receptor (Er), nitrotyrosine, inducible nitric oxide synthase, (iNOS), endothelial NOS (eNOS), androgen receptor (Ar) and vascular endothelial growth factor (VEGF). Sections were then rinsed 3 x in PBS and incubated in the appropriate, fluorescently labeled secondary antibody (Jackson Immunolabs, Fisher Scientific, Pittsburgh, PA) for 1h at room temperature. All slides were then rinsed in 0.1M PBS, and the process was repeated with another primary antibody. After completion of the secondary antibody step for the second antibody, sections were air dried in the dark, cover slipped with Fluorogold containing DAPI mounting media (Fisher Scientific, Pittsburgh, PA) and stored at 4ºC.

Microscopy. For immunostaining, multiple photomicrographs of arteries were taken at 20x magnification. ImageJ was used to quantify the immuno-stained area. All pictures for a specific antibody were taken at the same intensity so that the area labeled and the intensity of the labeling could be measured using Image J (NIH, Bethesda, MD). To measure labeled area and intensity of labeling in the endothelial cells and vascular smooth muscle (VSM), the inside of the lumen was circled and a measurement of the labeled area was made using Image J. This was followed by drawing another circle along the internal elastic membrane, and the labeled was measured. The area of the lumen was subtracted from the area containing the elastic membrane, and this was used as an estimate of endothelial cell labeling. Similar steps were performed to estimate VSM labeling; measures collected after outlining the elastic membrane and lumen of the artery were subtracted from measures made after outlining the whole artery and this number

was used as the staining of the VSM. An average of the measures from each photo was calculated and this average was used for analyses.

H&E staining was also performed on one set of sections. Arteries were located within the tissue and photomicrographs of arteries were taken at 20 x magnification. The internal and external perimeters of five randomly chosen arteries in the lateral atria and ventricles were chosen for analyses. The ratio of the internal to external diameter was calculated. In addition, four measures of arterial wall muscle thickness were collected from each artery and averaged. The number of inflammatory cells and the number of vacuoles in endothelial cells was also counted in each photomicrograph. The number of vacuoles within endothelial cells has been used as a measure of injury (an increase in vacuoles is equivalent to endothelial cell injury (Curry et al. 2005). The average number of each quantified structure per section was calculated and used for analyses.

Microvessel physiology: Animals were euthanized by i.p. injection of sodium pentobarbital solution (100-300 mg/kg) and exsanguination. Tails were dissected from rats after exsanguination and placed in cold Dulbecco's modified Eagle's medium with glucose (Invitrogen/Gibco; Carlsbad, CA). Ventral tail arteries from the C18-20 region of the tail were dissected, mounted on glass pipettes in a microvessel chamber (Living System; Burlington, VT), and perfused with bi-carbonated HEPES buffer (130 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES, 1.80 mM KH_2PO_4, 0.03 mM EDTA, plus 10 % glucose) warmed to 37°C. Arteries were pressurized to 60 mm Hg and allowed to equilibrate for approximately 1 h. After the hour acclimation period, the chamber buffer was replaced with fresh HEPES buffer and responsiveness of the arteries to phenylephrine (PE)-induced vasoconstriction and acetylcholine (ACh)-induced re-dilation was measured. All chemicals for microvessel exposures were

purchased from Sigma (Indianapolis, IN) unless otherwise noted. To assess the effects of treatment on sensitivity to α_1-adrenoreceptor-mediated vasoconstriction, PE was added to the chamber so that changes in the concentration occurred in half-log increments (-9.0 to -5.5 M) and the internal diameter of the artery was recorded after the arteries stabilized (approximately 5 min between concentrations). After measuring the dose-dependent vasoconstriction that occurred in response to PE, the chamber buffer, was removed and replaced with fresh, oxygenated HEPES buffer. After rinsing in oxygenated HEPES buffer for 15 min, arterial diameter returned to near baseline levels. Because ventral tail arteries usually display little basal tone, endothelial-mediated re-dilation was assessed after arteries were pre-constricted to approximately 50% of their baseline diameters with PE. In pilot work, we demonstrated that re-constricting arteries with PE did not affect subsequent responses to ACh or other vasomodulating factors. To assess the dilatory effects of ACh, the agonist was added cumulatively in half-log increments (-10.0 to -5.0) and changes in the internal diameter of the vessel were measured as described for PE.

Quantitative reverse transcriptase-polymerase chain reaction (qtRT-PCR). qRT-PCR was performed to determine if exposure to PC-emissions resulted in changes in transcript levels in heart tissue (Krajnak et al. 2013). Heart tissue was collected and fixed in 10% formalin for 24 h prior to being paraffin embedded and sectioned. RNA was extracted from eight 5 μm sections using the RNeasy FFPE Kit (Catalogue number 73504: Qiagen, Valencia, CA). First strand cDNA was synthesized from 1 mg of total RNA using a Reverse Transcription System (Invitrogen, Carlsbad, CA). Because only a limited amount of RNA could be isolated from paraffin-embedded sections, transcripts for factors that changed in response to inhalation of other types of particulate or toxic fumes were chosen for analyses (Krajnak, Farcas, Richardson, et al. 2023; Krajnak et al. 2017; Roberts et al. 2014; Hubbs et al. 2011). In the current study, qRT-PCR

for the antioxidant enzyme *catalase (Cat)*, *endothelial nitric oxide synthase (eNOS), endothelin-1a (Et1a), hypoxia-induced factor-1(Hif1)* and *vascular endothelial growth factor (Vegf)* was performed. To determine if the treatment resulted in a change in transcript levels, fold changes from the same day controls were calculated. This was done by calculating the average response for the control group and then subtracting the individual CT values for each sample from the average of the controls.

References.

Farcas, M.T., A.B. Stefaniak, A.K. Knepp, W.K. Mandler, M. Kashon, S.R. Jackson, T.A. Stueckle, J. Sisler, S.A. Friend, C. Qig, J. Matheson, V. Castranova, and Y. Qian. 2019. Acrylonitrile butadiene styrene (ABS) and polycarbonate (PC) filaments three-dimensional (3-D) printer emissions-induced cell toxicity. *Toxicol Lett.* . 317:1-12.

Farcas MT, McKinney W, Qic C, Mandler WK,. Friend SA, Stefaniak A, Jackson M, Winn A, Kashon M, LeBouf RF, Russa KA, Hammond DR, Burns D, Ranpara A, Thomas TA, Matheson J, and Qian Y. 2020. Pulmonary and systemic toxicity in rats following inhalation exposure of 3-D printer emissions from acrylonitrile butadiene styrene (ABS) filament. *Inhal Toxicol.* 32:403-418.

Krajnak, K., M. Farcas, D. Richardson, M.A. Hammer, S. Waugh, W. McKinney, M. Jackson, J. Matheson, T. Thomas, and Y. Qian. 2023. Inhalation of polycarbonate emissions generated during 3D printing processes affects neuroendocrine function in male rats. *J Toxicol Environ Health A* 86:575-596.

Stefaniak, A. B., R. F. LeBouf, M. G. Duling, J. Yi, A. B. Abukabda, C. R. McBride, and T. R. Nurkiewicz. 2017. Inhalation exposure to three-dimensional printer emissions stimulates acute hypertension and microvascular dysfunction. *Toxicol Appl Pharmacol* 335:1-5.

Hubbs, A. F., R. R. Mercer, S. A. Benkovic, J. Harkema, K. Sriram, D. Schwegler-Berry, M. P. Goravanahally, T. R. Nurkiewicz, V. Castranova, and L. M. Sargent. 2011. Nanotoxicology--a pathologist's perspective. *Toxicol Pathol* 39 (2):301-24.

Curry, B.D., S.R. Govindaraju, J.L.W. Bain, L.L. Zhang, J.-G. Yan, H.S. Matloub, and D.A. Riley. 2005. Evidence for frequency-dependent arterial damage in vibrated rat tails. *Anat Rec A Discov Mol Cell Evol Biol* 284:511-521.

Krajnak, K.M., S. Waugh, C. Johnson, G.R. Miller, X. Xu, C. Warren, and R.G. Dong. 2013. The effects of impact vibration in peripheral blood vessles and nerves. *Ind Health* 51 (6).

Roberts, J. R., S. E. Anderson, H. Kan, K. Krajnak, J. A. Thompson, A. Kenyon, W. T. Goldsmith, W. McKinney, D. G. Frazer, M. Jackson, and J. S. : Fedan. 2014. Evaluation of Pulmonary and Systemic Toxicity of Oil Dispersant (COREXIT EC9500A((R))) Following Acute Repeated Inhalation Exposure. . *Environ Health Insights* 8 (Suppl 1):63-74.