"Supplementary Materials"

Vaping dose, device type, and e-liquid flavor are determinants of DNA damage in electronic cigarette users. *Stella Tommasi, Hannah Blumenfeld, and Ahmad Besaratinia*

Supplemental Materials include:

- Supplementary Methods (text)
- Supplementary References

"Supplementary Methods"

Subject recruitment and enrollment

The study was advertised in online forums, including Craigslist, Reddit, and myUSC (http://my.usc.edu), and on social media (Twitter, Instagram, and Facebook) [1]. Also, flyers and leaflets were used to advertise the study in local colleges, universities, and vape shops. Furthermore, an online survey was developed, validated, and subsequently employed to solicit and query potential participants. Individuals who appeared to have met the study criteria were contacted by phone to complete a screening questionnaire. Based on the information obtained during the phone screen, those who were deemed potentially eligible, were scheduled for an in-person visit to our laboratory. During the visit, an expanded version of the phone screen was administered to reconfirm eligibility and afterward, a written informed consent was obtained from all participants (*see*, below).

Personal interview

Upon reconfirmation of eligibility and informed consent, all participants were interviewed in person to provide detailed information on demographics, socio-economic status, use frequency and patterns of e-cigs, cigarettes, or other tobacco products, dietary habits (*e.g.*, grilled/roasted/broiled food consumption), lifestyle, use of recreational or illicit drugs, alcohol, and prescription- or over-the-counter medicine, specifically vitamins or multivitamins, occupational and residential history, and family history of disease.

Inclusion and exclusion criteria

Health indicators for exclusion from the study consisted of respiratory diseases (*e.g.*, asthma or chronic obstructive pulmonary disease), immune system disorders, diabetes, kidney diseases, body mass index < 18 kg/m^2 or > 40 kg/m^2, local or systemic inflammation or infection, or any medical disorder/medication that could affect subject's safety or study results. Any unstable or significant medical condition in the past 12 months, including but not limited to symptomatic heart conditions, stroke, severe angina, and hypertension was ground for exclusion. Being pregnant or having a baby in the past 12 months was also exclusionary. Other exclusion criteria included uncontrolled mental illness or substance abuse or inpatient treatment for those conditions in the past 12 months, use of recreational or illicit drugs (*e.g.*, marijuana or heroin) in the past six months, and use of any medication known to induce/inhibit CYP450 2A6 enzyme. Physical examination and health assessment of all participants were performed by highly trained staff during the personal visits and interviews.

Sampling and processing of oral epithelial cells

All subjects were required to refrain from eating, smoking, or vaping, at least, 1 h prior to visiting our laboratory. Before sampling, subjects were asked to vigorously rinse their mouths with water to remove saliva, residual food particles, and mucosal debris. An Ultra Soft Oral-B brush (SENSI.SOFT™; Cincinnati, OH) was placed in the subject's mouth, and sufficient pressure was applied to contact the surface of the inside of his/her cheeks. Rotatory motion along the face and edge of the brush was used to gently scrape the entire surface of the inside of the cheek, while avoiding bleeding. The proximal, central, and distal regions of the inside of each cheek were brushed 15 times each. Once brushing of a region was completed, the brush was swirled in a tube pre-filled with 35 ml ice-cold sterile phosphate buffer saline (PBS) to dislodge the cells from the

bristles. Cycles of brushing and washing the cells from the bristles were repeated until all regions from both cheeks were sampled. The two tubes containing the harvested cells from opposite cheeks were centrifuged at 800× *g* for 5 min at 4 °C. Pelleted cells from each tube were re-suspended in PBS, pooled into a single tube, and re-centrifuged as above. The collected cell pellet was snap frozen and kept at −80 °C until further analysis. We have confirmed that this protocol provides, on average, several million cells, the vast majority of which being intermediate and suprabasal oral epithelial cells [1]. To rule out significant contamination by other cell types, we have performed differential cell count on the collected cells and verified the overwhelming presence of oral epithelial cells in all samples. To avoid any potential bias, specimen collection and processing of samples from different groups were done in variable orders, not in batches.

Sampling and processing of peripheral blood

Peripheral blood (30 ml) was drawn from the study subjects by venipuncture. Plasma was collected by centrifugation, aliquoted into multiple microtubes (Eppendorf, Inc., San Diego, CA), snap frozen, and preserved at −80 °C until further analysis.

Quantification of DNA damage by LA-QPCR

LA-QPCR quantification of DNA damage was performed as described in ref. [2], with few modifications. Briefly, genomic DNA was isolated from snap frozen oral epithelial cells, diluted 1:10 with 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and quantified fluorimetrically using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher, Waltham, MA) per the manufacturer's instructions. Following PicoGreen quantification, equal amounts of genomic DNA (20 ng) were used, in duplicate, for long PCR amplification in 1x reaction buffer containing 2.5 U

LongAmp *Taq* DNA polymerase (New England BioLabs, Ipswich, MA), 2% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO), and 300 μM dNTPs (MilliporeSigma, Burlington, MA). The following primers (at 0.2 μM final concentration) were added to the reaction mix to amplify a 12.2 kb region of the DNA polymerase beta *(POLB)* gene: forward primer, 5'-CCTGGAGTAGGAACAAAAATTGCTG and reverse primer, 5'-CATGTCACCACTGGACTCTGCAC. Preliminary assays were carried out to determine the optimal DNA concentration and number of cycles needed to ensure the linearity of the PCR amplification. PCR products were run on agarose gel to verify the size of the long amplicons and assure that no spurious products were generated. Final PCR conditions were set as follows: 94 °C for 2 min; 94 °C for 30 sec, 58 °C for 30 sec, 65 °C for 10 min (x 30 cycles); 65 °C for 10 min; 4 °C. For background adjustment, blanks (samples containing no DNA template) were also included in the assay. After amplification, PCR products and blanks (5 μl/sample) were quantified fluorimetrically by Quant-iT PicoGreen, using dilutions of λ HindIII-cut DNA (ThermoFisher, Waltham, MA) to generate a standard curve [2].

Following the PicoGreen assay, fluorescence values expressed as relative fluorescence units (RFU) were compiled for each sample on an excel spreadsheet for data analysis. Fluorescence readings of duplicate samples from vapers, smokers and non-users were averaged, and blank values (averaged no-DNA samples) were subtracted. To minimize technical noise, fluorescence values from independent PicoGreen plates were normalized using the standards' fluorescence readings. Adjusted RFU values (blank-corrected and standard-calibrated) were then used to calculate the "relative amplification", by dividing each sample's fluorescence by the average fluorescence of all non-users' samples used as the reference. Finally, the resulting values were converted to relative lesion frequencies by applying the Poisson distribution formula:

lesions/amplicon = –ln A_e/A_c, where A_e represents the amplification of each experimental sample from vapers, smokers, and non-users, and A_c is the amplification of the non-users' samples (average) [2]. Lesion frequencies were normalized to number of lesions/10 kb [2-4], and lesion frequencies below the detection level were set to 0.015/10 kb, which is one half of the detection limit of the assay. For validation purpose, we have also interrogated an additional gene target, hypoxanthine phosphoribosyltransferase 1 (*HPRT*), by LA-QPCR using the same protocol as described above. The primer set to amplify a 10.4 kb fragment encompassing exons 2–5 of the *HPRT* gene includes: forward primer, 5′-TGG GAT TAC ACG TGT GAA CCA ACC and reverse primer, 5′-GCT CTA CCC TCT CCT CTA CCG TCC.

Quantification of plasma cotinine by ELISA

Plasma cotinine was measured by a solid phase competitive enzyme-linked immunosorbent assay (ELISA) kit according to the instructions of the manufacturer (Abnova Corp., Walnut, CA). Briefly, aliquots of standard controls and samples of plasma from the study subjects (in triplicate) were loaded (10 µl each) onto a 96-microwell plate pre-coated with a polyclonal antibody raised against cotinine. After adding a cotinine horseradish peroxidase enzyme (100 µl per well), the microplate was incubated for one hour at room temperature in the dark. Unbound cotinine and cotinine enzyme-conjugate were washed off by rinsing the wells six times with distilled water (300 µl each wash). A chromogenic substrate (3,3',5,5'-Tetramethylbenzidine) was added (100 µl per well), and the plate was incubated for 30 minutes at room temperature. The reaction was terminated by adding a stop solution (100 µl per well), and absorbance was read at 450 nm using a SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices, LLC., San Jose, CA). Results are expressed as nanograms (ng) of cotinine measured per milliliter of plasma [5].

Quantification of exhaled CO and COHb by breath monitor

Exhaled CO levels and %COHb were measured using the Bedfont Micro^{+TM} Smokerlyzer® according to the manufacturer's instructions (Bedfont Scientific Ltd., Harrietsham, UK). Briefly, study subjects were instructed to inhale and hold their breath for 15 seconds. Following the completion of the 15 second countdown, subjects blew slowly into the device mouthpiece aiming to empty their lungs completely. The CO levels (ppm) and equivalent %COHb were recorded by the device and displayed on the touchscreen monitor.

Prioritization of endpoints for measurement

Of importance for the present study, we should stress the challenges of research in healthy volunteers with matching characteristics (*i.e.*, age, gender, and race) and strictly defined exposure, whose source materials (*e.g.*, tissues, cells, DNA/RNA) are often limited for molecular analysis. The limited source materials in these studies inevitably leads to prioritization of endpoints for quantification (*e.g.*, selection of target gene). With the same token, prioritization will be required to detect the selected endpoint(s) in specific tissues, cells, or cellular compartments (*e.g.*, nucleolus *vs*. mitochondria). For example, while the significance and importance of damage to the nuclear genome in the pathophysiology of disease is well-established, the role of mitochondrial DNA damage in disease development is beginning to be fully appreciated [6,7]. Given the limited source materials for molecular analysis in population-based studies [8], nuclear DNA has been extensively used as a preferred choice for direct measurement of DNA damage [9]. The focus of the present study was on nuclear DNA damage because the source materials for this study had to be shared

with our ongoing genomic sequencing project, which aims to detect mutations in the nuclear genome. Other distinctions between nuclear and mitochondrial genomes are highlight below:

(1) The mitochondrial genome consists of 16,569 DNA base pairs, whereas the nuclear genome is made of 3.3 billion DNA base pairs;

(2) The mitochondrial genome contains 37 genes with few non-coding DNA sequences, whereas the nuclear genome consist of 20,000-25,000 genes, including protein-coding genes, mitochondrial genes, and thousands of non-coding genes (*e.g.*, microRNAs and long non-coding RNAs) with known regulatory functions;

(3) Mitochondrial DNA is encoded for the genetic information required by mitochondria whereas nuclear DNA is encoded for the genetic information required by the entire cell;

(4) whereas one mitochondrion contains dozens of copies of its mitochondrial genome, each cell contains numerous mitochondria (*i.e.*, hundreds to thousands). Thus, a given cell can contain several thousand copies of its mitochondrial genome, but only one copy of its nuclear genome. This leads to heterogeneous population of mitochondrial DNA within the same cell, and even within the same mitochondrion;

(5) Unlike nuclear genome, the mitochondrial genome is not enveloped and packaged into chromatin. Given the absence of many of the protective protein structures and a relatively less efficient DNA repair machinery, the mitochondrial genome has a much higher mutation rate (~100-fold higher) than the nuclear genome; and

(6) the mitochondrial mode of inheritance is strictly maternal, whereas nuclear genomes are inherited equally from both parents (reviewed in refs. [6,7,10]).

Altogether, while there is a growing recognition of the importance of mitochondria in health *vs*. disease state [7], measuring mitochondrial DNA damage was beyond the scope of the

present study and outside its prioritization scheme. Notwithstanding, the rising appreciation for elucidating the role of mitochondrial DNA damage in pathobiology warrants further investigation whereby mitochondrial DNA damage can be evaluated in vapers *vs*. smokers.

"Supplementary References"

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