Supporting information for:

Nitrosamine and nicotine exposure after switching from filtered to unfiltered cigarette smoking—a cross-over clinical trial

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Urine collection

Subjects collected their urine in a specimen cup. Samples were a minimum of 30 mL, and all urine at a time was collected (urine was not “midstream” only). The urine samples were stored at -20 °C upon collection and until analysis.

Personnel performing the extraction and instrumental analysis were blinded as to the exposure status of participants. To prevent contamination, all sample containers and laboratory tools (pipet tips, syringes, and syringe filters) were rinsed with a solvent prior to use. Laboratory personnel wore disposable caps and laboratory coats when processing samples. All solvents were LC/MS grade.

Quantification of urinary NNAL

Sample preparation. The extraction method followed the molecularly imprinted polymer (MIP) procedure described in Xia et al., (2005)[1] and Hongwei et al. (2012)[2], with minor changes, and used a commercially available NNAL MIP column. One mL of room temperature urine was spiked with 1 ng of the NNAL-d_3 (Toronto Research Chemicals) internal standard, followed by 5 mL of a 1 M phosphate buffer solution containing 10,000 units of β-glucuronidase type IX-A from *E. coli* (Sigma-Aldrich). The β-glucuronidase solution was prepared on the same day and the pH of the urine after addition was approximately 6. Samples were incubated for 24 h at 37 °C in the dark with gentle rocking. The MIP column (SupelMIP SPE - NNAL, bed mass 25 mg, Sigma-Aldrich) was conditioned with 1 mL dichloromethane, 1 mL methanol, and 1 mL water. The sample was passed through a 0.45 µm PTFE syringe filter (Agilent) and loaded onto the MIP column. The loaded column was washed with 1 mL water, 1 mL heptane, and 1 mL hexane, and vacuum dried for 2 min to remove residual solvent. NNAL elution was performed with 3 mL of 9:1 dicholoromethane:methanol. The sample was evaporated to dryness, reconstituted in 1 mL of 98% water, 2% acetonitrile, and transferred to a glass autosampler vial.

Instrumental analysis. NNAL was quantified using an Agilent 1260 liquid chromatography (LC) system coupled to an Agilent 6470 triple quadrupole mass spectrometry (MS) system operated in positive electrospray ionization (ESI+) mode. The injection volume was 2 µL. The chromatographic separation was performed using a Zorbax Eclipse Plus C_{18} column (2.1×100 mm, particle size 1.8 µm, Agilent), with a solvent system of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The LC flow rate was 0.3 mL/min, and the gradient program was 98% A and 2% B for 30 s, then to 5% A and 95% B over 4.5 min, followed by a return to 98% A and 2% B over 1 min, and equilibration 4 min. The retention time of NNAL was approximately 1 min. The multiple-reaction-monitoring (MRM) transitions were 210.2 → 93.2 (quantitative) 210.2 → 180.2 (qualitative), and 210.2 → 149.1 (qualitative) for NNAL; and 213.0 → 93.1 (quantitative), 213.0 → 183.0 (qualitative), and 213.0 → 149.0 (qualitative) for NNAL-d_3. The calibration curve consisted of 8 standard solutions ranging from 0.004 to 20 ng/mL of

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NNAL, each with 1 ng/mL of NNAL-d_3 and was run with every instrumental batch. Linear calibration curves were generated using the analyte/internal standard peak area ratios and concentration ratios.

Quality control. Acceptance criteria were 80% to 120% for the accuracy of injection standards run with each batch. Precision was assessed as the percent coefficient of variation of the measured injection standard values across all instrumental batches and was < 15%. The limit of quantification (LOQ) for NNAL was 0.004 ng/mL urine. LOQ is defined as the lowest concentration that could be consistently measured without bias[3], which we also set as the concentration of the standard solution with the lowest value in the calibration curve. Laboratory blanks consisted of the phosphate buffer processed through the entire sample preparation and instrumental analysis procedure except for the addition of β-glucuronidase (due to the cost) and were included with every sample preparation batch. NNAL was not detected in the laboratory blank samples.

Quantification of urinary cotinine

The sample preparation and instrumental analysis of cotinine was described previously in Quintana et al. (2019)[4].

Quantification of urinary creatinine

Sample preparation. The sample preparation and instrumental analysis of creatinine closely followed the procedures described in Fraselle et al. (2015)[5] and Ou et al. (2015)[6]. The urine sample was vortexed for 1 min and mixed on a rotating platform for 10 min. Fifty µL of urine was removed and diluted stepwise 10,000 times with 95% water and 5% acetonitrile. The sample was then spiked with 0.1 µg creatinine-d_3 (Sigma-Aldrich). The final volume of this solution was 1 mL, and it was passed through a 0.45 µm PTFE syringe filter (Agilent) into a glass autosampler vial.

Instrumental analysis. Creatinine was quantified using an Agilent 1200 liquid chromatography (LC) system coupled to an Agilent 6460 triple quadrupole mass spectrometry (MS) system operated in positive electrospray ionization (ESI+) mode. The injection volume was 2 µL. The chromatographic separation was performed using a Zorbax Eclipse Plus C_{18} column (2.1×100 mm, particle size 1.8 µm, Agilent), with a solvent system of (A) water with 5 mM ammonium acetate and (B) methanol. The LC flow rate was 0.3 mL/min, and the gradient program was 95% A and 5% B for 2 min, then to 50% A and 50% B over 1 min, followed by a return to 95% A and 5% B over 30 s, and equilibration for 4.5 min. The retention time of creatinine was approximately 1.2 min. The multiple-reaction-monitoring (MRM) transitions were 114.3 → 44.2 (quantitative), 114.3 → 86.2 (qualitative), and 114.3 → 43.2 (qualitative) for creatinine; and 117.3 → 47.2(quantitative), 117.3 → 89.2 (qualitative), and 117.3 → 46.2 (qualitative) for creatinine-d_3. The calibration curve consisted of 6 standard solutions ranging from 0.01 to 0.5 µg/mL of creatinine, each with 0.1 µg/mL of creatinine-d_3 and was run with every instrumental batch.

Quality control. The limit of quantification (LOQ) for creatinine was 0.01 µg/mL urine. Acceptance criteria were 85% to 115% for the accuracy of injection standards run with each batch. Precision was assessed as the percent coefficient of variation of the measured injection standard values across all instrumental batches and was < 15%. The limit of quantification (LOQ) for creatinine was 0.01 µg/mL urine. Creatinine was not detected in the laboratory blank

samples run with every sample preparation batch. The original urine creatinine concentration was determined by multiplying the quantitative result of the analyzed solution by 10,000.

References

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