Assessment of the Exposure to Aromatic Amines in Users of Various Tobacco/Nicotine Products

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ABSTRACT: Aromatic amines such as ortho-toluidine (o-Tol), 2-aminonaphthalene (2-AN), and 4-aminobiphenyl (4-ABP) are human bladder carcinogens and occur at various workplaces, in ambient air, in food products, as well as in tobacco smoke. In a clinical study comprising a period of 74 h under confinement, we investigated the exposure to these three aromatic amines as well as to 3-aminobiphenyl (3-ABP) by measuring them in urine of habitual users of combustible cigarettes (CCs), electronic cigarettes (ECs), heated tobacco products (HTPs), oral tobacco (OT), and nicotine replacement therapy products (NRTs). Non-users (NU) of any tobacco/nicotine products served as (negative) control group. Smokers (CC) exhibited the highest levels for all four aromatic amines measured, significantly elevated compared to NU and non-CC users. Urinary levels in users of EC, HTP, NRT (mostly nicotine gum), and OT (mostly snus) were not significantly different from those in NU. Users of HTP showed slightly elevated urinary excretion levels of o-Tol, 3-ABP, and 4-ABP compared to some other non-CC groups. Dose markers such as daily consumption, urinary nicotine equivalents (Nequ), and plasma cotinine (CotP) were found to be consistently and significantly correlated with the excretion of aromatic amines for smokers (CC) only. Excretion levels of 3- and 4-ABP in smokers were significantly lower in the urine collected overnight compared to that collected during the day, which is just the opposite of what we observed for other biomarkers in this study. The possible reason for this observation is discussed. In conclusion, in contrast to smoking of CCs, the use of ECs, HTPs, nicotine gum, and oral tobacco was not observed to be associated with significant exposure to the aromatic amines o-Tol, 2-AN, 3-ABP, and 4-ABP. The observed slight increase in o-Tol, 3-ABP, and 4-ABP excretions in HTP users has to be verified in larger studies.

INTRODUCTION

Tobacco use, particularly smoking of combustible cigarettes (CCs), is associated with an increased risk for diseases such as cancer, cardiovascular (CVD), and chronic obstructive pulmonary diseases (COPD).1,2 Cigarette smoking is an established risk factor for bladder cancer with a relative risk of about 5 compared to nonsmokers.3 Aromatic amines have been identified as causally related to the induction of bladder tumors in numerous human and animal studies.4,5 In this biomonitoring study, we focus on the four aromatic amine ortho-toluidine (o-Tol), 2-aminonaphthalene (2-AN), 4-aminobiphenyl (4-ABP), and 3-aminobiphenyl (3-ABP), the first three being classified as human carcinogens (Group 1) and 3-ABP not evaluated by the International Agency for Research on Cancer (IARC).4,5 The chemical structures of these four aromatic amines are shown in Figure 1.

Apart from tobacco smoke, exposure sources for the general population are emissions from cooking oil, hair dyes, and heated food.4,5 Exposure to o-Tol occurs ubiquitously, mostly of unknown origin. The local anesthetic prilocaine is immediately metabolized to o-Tol, leading to high exposure levels in the patients.6

Reported mainstream smoke yield ranges (derived with the ISO smoking regime) of market cigarettes were 8.6−144.3, 1.47−14.06, and 0.30−2.31 ng/cig for o-Tol, 2-AN, and 4-ABP, respectively.7 Yields of 3-ABP in the mainstream smoke of reference cigarettes were found to be in the range of 0.7−1.8 ng/cig.8 Releases per cigarette of these aromatic amines into sidestream smoke are usually 20−40 times higher than into mainstream smoke.9 Increases in indoor air concentrations of 4-ABP and 2-AN due to environmental tobacco smoke (ETS) were reported to be <1 ng/m³, whereas those for o-Tol can reach 10 ng/m³.10,11 In a recent review of the literature,12 the

Received: September 22, 2022
Accepted: October 20, 2022
Published: November 1, 2022
median daily intakes of o-Tol, 2-AN, and 4-ABP by smoking combustible cigarettes (CC) were estimated to amount to 750.8, 121.7 and 25.1 ng/day, respectively. From the concentrations in indoor air, an ETS-related intake in nonsmokers of <10 ng/day (for 4-ABP and 2-AN) and about 100 ng/day for o-Tol can be deduced. Only few data are available for the release of aromatic amines from the next-generation tobacco/nicotine products so that no reliable daily intakes can be estimated.\cite{12} Biomonitoring results of these aromatic amines in the various groups will be discussed below. More recently published biomarker data for users of various types of tobacco/nicotine products\cite{13-16} are also considered in the comparison with our data performed in the Discussion section.

With respect to the metabolism of the four aromatic amines dealt with in this study, primarily two competing pathways are of interest: (i) N-oxidation to the N-hydroxyamine by means of the inducible CYP 1A2 or peroxidases (e.g., the prostaglandin H synthase), in the case of o-Tol CYP 2A6 or 2E1 play the main role; (ii) N-acetylation by means of the N-acetyl transferase 2 (NAT2).\cite{4,17-19} Both the free and the N-hydroxylated aromatic amines can be N-glucuronidated and are transported together with the N-acetylated metabolite from the liver via the bloodstream into the bladder and finally excreted with the urine. O-Acetylation (via NAT1), O-sulfation (via the sulfotransferase SULT1A1), or acidic pH can lead to the release of highly reactive nitrenium ions, which can undergo tautomerization to carbenium ions; both ions are capable of forming DNA adducts. Acidic pH in the bladder may release free and N-hydroxylated aromatic amines; the former can be again oxidized to the N-OH metabolite by an urothelial peroxidase.\cite{18,20} Formation of the N-acetylated amines can be viewed as a detoxification pathway. A minor part of aromatic amines absorbed may also reach the bladder lumen in unchanged (free) form.\cite{21}

As suitable biomarkers of exposure to the four aromatic amines of interest, hemoglobin adducts as well as the free and N-acetylated amines excreted in urine have been established (for an overview, see Turesky and Lemarchand\cite{23}). Hemoglobin adduct formation is preceded by the co-oxidation of oxyhemoglobin and the N-hydroxy-metabolite to met-hemoglobin and the nitroso aryl compound, respectively, the latter of which covalently binds to cysteny-SH groups in hemoglobin.\cite{23-25}

The adduct levels are, therefore, a direct dosimeter for the proximate carcinogen (the N-OH aryl amine) formed from the absorbed parent compounds in the liver. Biomonitoring the exposure to aryl amines in urine assesses the N-acetyl amines (after alkaline hydrolysis only)\cite{19} as well as the N-glucuronidated and free aryl amines in urine, i.e., that part of the dose which is not activated to the proximate carcinogen, which represents the largest part. All of the metabolites mentioned above can be excreted in urine; the applied analytical method using acid hydrolysis assesses the N-glucuronidated and the free form of the aromatic amines.\cite{19,20}

The objective of this study was to compare the exposure to o-Tol, 2-AN, 3-ABP, and 4-ABP by urinary biomarkers in a controlled clinical study over 74 h under confinement in habitual users of combustible cigarettes (CCs), electronic cigarettes (ECs), heated tobacco products (HTPs), oral tobacco (OT, primarily snus), and NRTs (nicotine replacement therapy products, primarily nicotine gum). As a (negative) control group, non-users (NU) of these products were included. In addition, the dose dependence of these biomarkers is studied in all user groups. Results were compared with corresponding published data.

### MATERIALS AND METHODS

**Study Design and Biological Samples Collected for Analysis.** A controlled, single-center, open-label trial was conducted. Five nicotine product user groups, including reportedly exclusive users of CC, EC, HTP, OT, and NRT with a control group of non-users (NU) recruited. The study design and the study population were described in a previous publication.\cite{27} The study protocol has been approved by the authorized ethics committee (Medical Association Hamburg, Germany). The purpose of the study was to identify specific biomarkers or biomarker profiles for the users of respective tobacco/nicotine products by means of targeted and untargeted analytical methods. Briefly, 10 subjects per group, which were self-reported exclusive users of the respective products for at least 6 months, were confined for 74 h to a clinic. Product use (own brands, described in more detail elsewhere\cite{28,29}) was allowed between 8 am and midnight during the subjects’ stay in the clinic.

On each study day, blood samples were collected at 7 am and 5 pm, starting in the evening of Day −1, when the subjects were admitted to the clinic. Throughout the course of the study, all urine voids were collected separately. For each void, the total volume and the time were recorded. For the analytical determinations, urine fractions were pooled, yielding six urine samples for each subject, three collected overnight (comprising 14 h) and three collected during the day (comprising 10 h). The “overnight” urine was collected from about 5 pm to 7 am (including the first morning urine) of the following day. The “during-the-day” urine was collected between 7 am (after the first morning urine) and 5 pm of the same day. A collection scheme for the biological samples has been published previously.\cite{28-30}

**Analytical Methods.** The aromatic amines o-Tol, 2-NA, 3-ABP, and 4-ABP in urine were determined by means of gas chromatography/mass spectrometry (GC-MS) as described previously,\cite{31} with modifications. Briefly, to 5 mL of urine, the internal standards o-Tol-D9, 2-AN-13C6, and 4-ABP-D9 were added. The isotopic purity of labeled internal standards was >99%. The mixture was acidified with 1 mL of hydrochloric acid (37%) and hydrolyzed (1 h, 80 °C). After adjusting the pH to 6.0–6.4 by adding 1.25 mL of 10 N sodium hydroxide solution and 3 mL of 2-(N-morpholino)-ethanesulfonic acid buffer pH 6.0, the mixture was extracted twice with 5 mL of n-
hexane. The combined extracts were dried over sodium sulfate and derivatized with 50 μL of pentafluoropropionic acid anhydride and 25 μL of pyridine (1 h, 80–85 °C). The derivatization mixture was washed with 3 mL of phosphate buffer (pH 8). After addition of 200 μL of toluene as keeper, the organic phase is reduced to 50 μL by means of a SpeedVac centrifuge. GC-MS analysis with negative-ion chemical ionization (NICI) was performed as described in the literature.

The actual device was a DSQ GC-MS instrument (Thermo Scientific, Dreieich, Germany). An Optima 35 MS, 60 m, 0.25 mm ID, 0.25 μm df (Macherey-Nagel, Düren, Germany) was used as an analytical column. The quantifier ions used were m/z 233, 240, 275, 295, 304 for o-Tol, 2-Tol-D9, 2-AN, 3-ABP, 4-ABP, and 4-ABP-D9, respectively. No qualifiers ions were available for the four analytes at the concentration ranges of interest. Limit of detection (LOD)/limit of quantification were: 0.8:10 (o-Tol), 0.6:1.7 (2-AN), 0.5:1.3 (3-ABP), and 0.5:1.5 (4-ABP) ng/L.

Urinary nicotine equivalents (Nequ), comprising the molar sum of nicotine and its 10 major metabolites, namely, cotinine, trans-3′-hydroxycotinine, nicotine-N-glucuronide, cotinine-N-glucuronide, trans-3′-hydroxycotinine-N,O-glucuronide, 4-OH-4-(3-pyridyl)-butanoic acid, nornicotine (NN), norcotinine, nicotine-N′-oxide, and cotinine-N′-oxide, were determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS) according to a published method, with modifications.

Plasma cotinine was determined by an LC-MS/MS method as described earlier.

All methods were fully validated according to FDA guidelines.

**Statistical Evaluation.** Normal distribution tests of Shapiro–Wilk, D’Agostino & Pearson, and Kolmogorov–Smirnov were applied. Since the concentrations of the aromatic amines were mostly not normally distributed, the nonparametric Mann–Whitney U test (comparison of two groups) and Kruskal–Wallis–ANOVA (comparison of multiple groups) was used to determine statistical significances between the groups. The nonparametric Spearman rank test was utilized calculating correlations. p-Values of <0.05 were rated as significant. GraphPad Prism Software (San Diego, CA), Version 9.2.0 was utilized for statistical analysis and generation of graphs.

**RESULTS**

Sixty (60) subjects completed the clinical study, 10 in each of the six groups (NU, CC, EC, HTP, NRT, OT). In all groups, the number of males and females were balanced, except for OT users, for which sex distribution was 9:1 (m/f). Group means for age ranged from 28.1 to 36.1 years and were not statistically different between groups. The reported average daily consumption prior to the study for users of CC, EC, HTP, NRT, and OT was 16.1 cigarettes, 9.75 mL of e-liquid, 15.5 sticks, 8.3 nicotine gums, and 6.9 g (mainly snus), respectively.

The time courses of the urinary excretion of o-toluidine (o-Tol), 2-aminonaphthalene (2-AN), 3-aminobiphenyl (3-ABP), and 4-aminobiphenyl (4-ABP) for the six study groups during the confinement period of the study (74 h) are shown in Figure 2. Smokers (CC) exhibited the highest mean levels for all four aromatic amines at all time points. 3-ABP and 4-ABP excretions in the night and day urine fractions of smokers (CC) showed a characteristic pattern with lower levels in the urine fractions collected overnight compared to those collected during the day. This is notably different from all urinary biomarkers of exposure in smokers (CC), where a reverse pattern (if any) was observed.

Reasons for this phenomenon are discussed below. Excretion levels for the five other groups (non-CC users including NU) were found to be similar with slight elevations in some urine fractions of all four aromatic amines in HTP users (Figure 2). Since the biomarker data for Day 3 of the study can be regarded as those obtained under the most controlled conditions in terms of product use and other environmental
markers were the recorded consumption and amount of amines excreted on Day 3 and three different dose markers for the non-CC groups (Table 1).

The correlations between the amounts of the aromatic amines excreted on Day 3 were used for further evaluations. Descriptive statistics for the four aromatic amines are shown in Table 1. The slightly elevated excretion of aromatic amines were observed (Table 2).

The correlations between the amounts of the aromatic amines excreted on Day 3 and three different dose markers for product use on that study day are shown in Table 2. The dose markers were the recorded consumption and amount of nicotine equivalents (Neq) excreted on Day 3 as well as plasma cotinine concentrations at 5 pm on Day 3. For smokers (CC), all dose–response relationships were found to be highly significant ($p < 0.01$) with Spearman correlation coefficients in the range of 0.86–0.95. For the other user groups, no consistent associations between the dose markers and the excretion of aromatic amines were observed (Table 2).

### Discussion

The presented results are part of a research project on biomarkers of exposure to be analyzed by targeted and untargeted methods in various biological matrices in users of five tobacco/nicotine products. The project aims to distinguish different nicotine user groups by either specific biomarkers or biomarker patterns. In this paper, results for urinary biomarkers of four aromatic amines ($\alpha$-Tol, 2-AN, 3-ABP, 4-ABP) are presented. To date, results from this study for exposure and uptake of nicotine,28 tobacco-specific nitrosamines (TSNA),29 polycyclic aromatic hydrocarbons (PAH),35 benzo[a]pyrene,36 1,2-propanediol glycol, and glycerol36 have been published. Results for 15 mercapturic acids have been submitted for publication. Furthermore, an evaluation of literature data on the intake and uptake of 38 chemicals (including $\alpha$-Tol, 2-AN, and 4-ABP) in the same user/non-user groups as in this investigation has been published.12

The toxicological importance of the aromatic amines studied ($\alpha$-Tol, 2-AN, and 4-ABP are human bladder carcinogens, 3-ABP has not been evaluated by the IARC) is out of question. Based on the release data of the five tobacco/nicotine products investigated, already briefly described in the Introduction section, excretion of $\alpha$-Tol in smokers (CC) on Day 3 was in the range of 91.0–260.7 ng/24 h (Table 1). This is overall in good agreement with a series of other studies.13,37–46 NU of any tobacco/nicotine products in our study showed $\alpha$-Tol excretion rates in the range of 30.4–77.2 mean: 60.2) ng/24 h (Table 1). Agreement with reported $\alpha$-Tol excretions in nonsmokers is acceptable.13,37–46 Urinary $\alpha$-

### Table 1. Descriptive Statistics of Four Aromatic Amines Excreted in the 24 h Urine of Day 3 by User/Non-User Groups

| biomarker | NU | CC | EC | HTP | NRT | OT |
|-----------|----|----|----|-----|-----|----|
| $\alpha$-Tol (ng/24 h) | <LOD, n of N | 0 of 20 | 0 of 20 | 0 of 20 | 0 of 20 | 0 of 20 |
| mean ± SD | 60.2 ± 15.4 | 150.2 ± 61.9 | 43.9 ± 18.6 | 85.8 ± 40.9 | 50.0 ± 24.8 | 65.7 ± 20.6 |
| median (IQR) | 64.2 | 130.7 (104.5–190.1) | 37.2 (32.2–54.6) | 68.3 (52.7–109.8) | 51.1 (30.0–70.9) | 68.6 (53.6–81.9) |
| min–max | 30.4–77.2 | 91.0–260.7 | 24.3–85.9 | 45.8–176.0 | 11.4–89.9 | 22.9–88.9 |
| different from | CC | NU, EC, HTP, NRT, OT | CC, HTP, OT | CC, EC, NRT | CC, HTP | CC, EC |
| 2-AN (ng/24 h) | <LOD, n of N | 8 of 20 | 0 of 20 | 9 of 20 | 7 of 20 | 3 of 20 |
| mean ± SD | 1.4 ± 1.0 | 25.7 ± 14.4 | 1.0 ± 0.8 | 1.7 ± 0.9 | 1.3 ± 0.7 | 1.5 ± 0.9 |
| median (IQR) | 1.1 (0.8–1.6) | 20.1 (12.5–39.1) | 0.8 (0.5–1.1) | 1.8 (0.7–2.2) | 1.2 (0.7–2.0) | 1.2 (0.8–1.9) |
| min–max | 0.4–3.9 | 10.0–48.9 | 0.2–3.2 | 0.6–3.2 | 0.4–2.5 | 0.6–3.7 |
| different from | CC | NU, EC, HTP, NRT, OT | CC | CC | CC | CC |
| 3-ABP (ng/24 h) | <LOD, n of N | 12 of 20 | 0 of 20 | 15 of 20 | 10 of 20 | 12 of 20 |
| mean ± SD | 0.6 ± 0.3 | 6.6 ± 3.6 | 0.4 ± 0.2 | 0.8 ± 0.3 | 0.5 ± 0.2 | 0.6 ± 0.3 |
| median (IQR) | 0.6 (0.4–0.8) | 5.3 (3.7–9.5) | 0.4 (0.2–0.6) | 0.9 (0.5–1.1) | 0.5 (0.3–0.7) | 0.5 (0.4–0.9) |
| min–max | 0.4–1.1 | 2.6–13.7 | 0.2–0.7 | 0.2–1.2 | 0.2–0.9 | 0.2–1.2 |
| different from | CC | NU, EC, HTP, NRT, OT | CC, HTP | CC, EC | CC | CC |
| 4-ABP (ng/24 h) | <LOD, n of N | 0 of 20 | 0 of 20 | 2 of 20 | 1 of 20 | 2 of 20 |
| mean ± SD | 2.3 ± 0.5 | 18.5 ± 9.9 | 1.5 ± 0.6 | 3.2 ± 1.2 | 2.1 ± 1.0 | 2.6 ± 0.9 |
| median (IQR) | 2.3 (1.9–3.2) | 14.7 (9.5–28.2) | 1.4 (1.2–1.7) | 3.2 (2.3–4.3) | 2.0 (1.2–2.8) | 2.5 (2.1–3.2) |
| min–max | 1.5–3.2 | 8.3–16.5 | 0.8–3.0 | 1.0–4.9 | 1.0–3.7 | 1.2–4.3 |
| different from | CC | NU, EC, HTP, NRT, OT | CC, HTP, OT | CC, EC | CC | CC |

**Notes:** $n$ = number of samples; <LOD of all evaluated samples (N) in a group; note that the 24 h urine of Day 3 is the sum of two urine fractions which were analyzed separately; for the group statistics, values <LOD were set to 0.5 × LOD. IQR: interquartile range (25th–75th percentile). Statistically significant differences (ANOVA, Kruskal–Wallis test, $p < 0.05$) to the other groups are indicated.
Table 2. Spearman Correlation Coefficients between Four Aromatic Amines and Three Dose Markers on Day 3 for Users of Five Tobacco/Nicotine Products (Statistically Significant Correlations Coefficients Are Highlighted in Gray)

| biomarker          | EC | NRT | OT |
|--------------------|----|-----|----|
| o-Tol (ng/day)     | 0.94*** | 0.95*** | 0.87*** |
| 2-AN (ng/day)      | 0.92*** | 0.95*** | 0.93*** |
| 3-ABP (ng/day)     | 0.86**  | 0.93*** | 0.86**  |
| 4-ABP (ng/day)     | 0.80*** | 0.92*** | 0.80*** |

Note: Consumption recorded for Day 3: CC: cig/day, EC: mL of e-liquid/day (average of 3 study days), HTP: sticks/day, NRT: pieces of nicotine gum/day (range: 8), 2 subjects used nicotine inhalators, OT: g/day. Nicotine equivalents (Nequ) excreted on Day 3, mg/day.

Possibly, there were issues with the specificity of the analytical methods applied in these studies. Alkaline hydrolysis, used in some studies, can also split the N-acetyl metabolites excreted in urine and thus increases the measurable level of aromatic amines in urine. Other factors were discussed elsewhere. o-Tol released from HTP was reported to be in the range of 0.4–1.3 ng/stick. For the other non-CC products (EC, NRT, OT), no data for the release of o-Tol were available. The urinary o-Tol excretion rates, which we observed for HTP users (Figure 2 and Table 1), suggest that there might be some exposure to this chemical in this group. This, however, has to be verified in larger studies.

On Day 3 of the study, smokers (CC) excreted 8.3–36.5 mg/24 h of 4-ABP, whereas excretion levels for NU were 1.5–3.2 mg/24 h (Table 1). Again, the excretion rates were in good agreement with median levels of 20.9 and 2.4 mg/24 h for smokers and nonsmokers, respectively, obtained in a recent literature evaluation. Only trace amounts of 2-AN (<0.1 ng/unit) were found to be released by EC and HTP, whereas no data for the release of 2-AN were available for NRT and OT. The product release data are compatible with our observation that excretion of 2-AN in the users of non-CC products was not distinguishable from NU (Figure 2 and Table 1).

We found an excretion rate of 3-ABP in the range of 2.6–13.7 ng/24 h on Day 3 in smokers (CC) and 0.4–1.1 mg/24 h in NU (Table 1). In another study, mean excretion of 5.39 and 1.11 mg/24 h for smokers and nonsmokers, respectively, was reported, which is in good agreement with our findings. Release of 3-ABP from CC was reported to amount to 3.5–4.2 ng/cig. In HTP, 3-ABP release was found to be at or below the LOQ of about 0.03 ng/stick. No release data of 3-ABP for the other products were available. Our findings of urinary excretion of 3-ABP (Figure 2 and Table 1) are, therefore, in line with product release data of 3-ABP in the literature.

The unusual excretion pattern of 3- and 4-ABP in smokers (CC) was already mentioned in the Results section (Figure 2). All biomarkers which exhibited day versus night excretion rates in smokers (CC) showed higher levels in the fraction collected overnight compared to that collected during the day. We explained this by the fact that about 15% more cigarettes were consumed in the time period between 3 pm and midnight (which is mainly reflected in the amounts of biomarkers in the urine fraction collected overnight and completed with the first
morning urine) than in the time period between 7 am until 5 pm (when smoking was allowed and is mainly reflected in the urine fractions collected during the day). This is illustrated in Figure 3, which shows the time patterns of 4-ABP in comparison to those of nicotine equivalents and 2-cyanoethyl-mercaturic acid (CEMA, a biomarker for exposure to acrylonitrile). How can this divergence, which is unique for 3- and 4-ABP, be explained?

A possible explanation for this observation might be the fact that bladder content remained for longer in the overnight urine fraction than in the urine fraction collected during the day (because of more urine voids taking place during the day). The longer residence time in the overnight urine fraction increases the probability that the free 4-ABP is reabsorbed through the urothelial wall or N-hydroxylated by the urothelial cells. In addition, the N-glucuronide of 4-ABP might easily release the free form under acidic conditions. Since both the free and the N-glucuronidated forms are particularly assessed by our analytical method, this would lead to a lower level of measurable 4-ABP in the overnight urine fraction, as we observed. These considerations are compatible with a pharmacokinetic model to predict the exposure of the bladder epithelium to urinary N-hydroxarylamine carcinogens as a function of urine pH, voiding interval, and resorption, published 40 years ago. This finding is also in line with a study, in which dogs were dosed with radio-labeled 4-ABP, showing that less frequent urination and, to a lesser extent, acidic pH in the bladder lumen, increased the level of 4-ABP-DNA adducts in the urothelium. To our knowledge, our data provide experimental evidence that these processes also are relevant for humans exposed to aromatic amines. We, however, can provide no explanation, why 2-NA and o-Tol did not show a similar urinary excretion pattern to 3- and 4-aminobiphenyl. If our hypothesis for explaining the unusual time course of 3- and 4-ABP is appropriate, this would imply that infrequent urination (occurring, for example, overnight) leads to an underestimation of the exposure by our method and, more critical, increases the bladder cancer risk since the formation of the procarcinogen (N-hydroxy-4-ABP) can increase. However, as long as no further evidence is available, these considerations have to be classified as speculative.

**CONCLUSIONS**

Our results derived from a clinical study with habitual users of CC, EC, HTP, NRT, and OT show significantly elevated urinary levels for the aromatic amines o-Tol, 2-AN, 3-ABP, and 4-ABP in smokers (CC) compared to all other groups. We observed a slight increase in o-Tol, 3-ABP, and 4-ABP excretion in HTP users compared to the other non-CC groups. This observation requires verification by larger studies. In contrast to other biomarkers determined in this study, excretion levels of 3- and 4-ABP in smokers (CC) were lower in the urine fraction collected overnight compared to the fraction collected during the day. We hypothesize that this is due to less frequent voiding in the former compared to the latter time period. We suggest that a lower frequency of urination can increase the risk of bladder cancer.

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**Author Contributions**

M.S., G.S., and N.P. contributed to conceptualization. K.R. conducted formal analysis. G.S. involved in writing—original draft preparation. M.S., N.P., and K.R. contributed to writing—review and editing. M.S. and N.P. performed supervision. M.S. involved in project administration. M.S. and N.P. contributed to funding acquisition. All authors have read and agreed to the published version of the manuscript.

**Funding**

This study was funded with a grant from the Foundation for a Smoke-Free World (FSFW), a US nonprofit 501(c)(3) private foundation. FSFW had no role in the planning and execution of this study, data analysis, and publication of the results. The Foundation accepts charitable gifts from PMI Global Services Inc. (PMI); under the Foundation’s Bylaws and Pledge...
Agreement with PMI, the Foundation is independent of PMI and the tobacco industry.

Notes
The authors declare no competing financial interest.
The authors declare no conflict of interest. The funders had no role in the design of the study; collection, analyses, or interpretation of data; writing of the manuscript; and the decision to publish the results.

■ ACKNOWLEDGMENTS
The authors thank Filip Sibul for helping organize and monitor the conduction of the clinical study and Marta Latawiec for her technical assistance in performing the analysis of aromatic amines. They also thank Janina Mütze for conducting the analysis of nicotine equivalents in urine and cotinine in plasma.

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