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Urine levels of nicotine and its metabolites in young population exposed to second-hand smoke in nightclubs: a pilot study

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The aim of this study was to investigate the extent of second-hand smoke exposure in younger population visiting nightclubs in Croatia by comparing the levels of nicotine and its main metabolites cotinine and trans-3'-hydroxycotinine (3HC) in urine samples taken from 22 participants before and after spending about three hours in a nightclub, stratified by smoking status (smokers and non-smokers). The samples were prepared by liquid-liquid extraction and analysed with gas chromatography-mass spectrometry. The presence of nicotine, cotinine, and 3HC was confirmed in all urine samples. Their median concentrations significantly differed between the two measurements in non-smokers. Our findings show that even a three-hour exposure to second-hand smoke can significantly increase the levels of nicotine and its metabolites in urine, which are indicative of exposure to other, harmful tobacco smoke substances. They also call for raising awareness of the health risks of exposure to second-hand smoke in the general population and among individuals who frequent nightclubs in particular.

KEY WORDS: urine sample; cotinine; nicotine; trans-3'-hydroxycotinine

According to the World Health Organization (WHO) estimates, there are 1.25 billion tobacco users in the world (1). Tobacco is associated with over eight million deaths a year, 1.3 million of which are owed to second-hand smoke (2). Second-hand or environmental tobacco smoke (ETS) is a mixture of smoke exhaled by an active smoker and the smoke released from a burning cigarette (3). Childhood exposure is associated with an increased risk of acute respiratory infections, ear problems, asthma, and sudden infant death syndrome (SIDS) (3). During pregnancy, it significantly increases the risk of congenital malformations and stillbirth (4). In adults, second-hand smoke exposure has been associated with a 20–30 % higher risk of lung cancer in people living with a smoker and a 16–19 % higher risk in people exposed at the workplace (3, 5). It is also associated with a higher risk of cardiovascular diseases and stroke (6, 7).

Nicotine is the main psychoactive ingredient in tobacco and although it is not a direct cause of tobacco-related diseases, it is responsible for the development of addiction to tobacco products (8). Nicotine is metabolised by the liver to primary metabolites, mostly cotinine. Most of cotinine is metabolised to trans-3'-hydroxycotinine (3HC). The final concentration of cotinine in the urine makes 10–15 % of the absorbed dose of nicotine, while 3HC and its glucuronide in urine make 40–50 %, and only 8–10 % of the absorbed nicotine is excreted unchanged (8, 9).

There are several biological markers used to assess nicotine exposure, the most common being nicotine in the urine, hair, saliva, and plasma and cotinine in the urine, saliva, and plasma (10, 11). 3HC is also an important biomarker of exposure, and its levels in urine are 3–4 times higher than those of cotinine (12). Given its specific source, i.e., tobacco, total cotinine is used to estimate exposure to environmental tobacco smoke in non-smokers (13).

Despite the overwhelming evidence of the harmful effects of tobacco smoke, second-hand exposure is still common and almost impossible to avoid in everyday social life. A 2001–2002 study that measured exposure to second-hand smoke at airports, train stations, hospitals, schools, universities, restaurants, and nightclubs across seven European cities (14) found that it was present in most public places, with the highest nicotine concentrations measured in cafes and nightclubs. The amount of tobacco smoke to which people were exposed during a four-hour stay in some of those nightclubs was comparable to the amount to which a person living with a smoker is exposed over an entire month. Considering that younger people most often frequent this type of public places they are the most vulnerable group to this kind of exposure and its adverse effects.

The aim of this pilot study was to establish changes in the levels of nicotine and its metabolites in the urine as biomarkers of

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exposure to tobacco smoke in young 20–30 years old participants who attend nightclubs.

PARTICIPANTS AND METHODS

This study included a random sample 22 participants from Split and Kutina (15 women and seven men), of whom 17 non-smokers and five smokers (all of them women). Their average age was 24 years. Three male non-smoking participants worked as waiters in a nightclub. Between contacts with the researcher, the participants lived their everyday life without interference. Relevant demographic data were collected after the participants gave informed consent to participation. The study was carried out in accordance with the Helsinki Declaration and approved by the Ethics Committee of the University of Split School of Medicine on 17 December 2018 (file class No. 003-08/21-03/0001; record No. 2181-198-03-04-18-0062).

Urine samples were collected from the participants at two time points. The first sample was collected before going to the nightclub (baseline) and the second sample after having spent 190 min in average (between 153 and 222 min) in the nightclub and slept six-hours (post-exposure). A detailed procedure is given in Figure 1. The reason for such a timeline are the half-lives of nicotine and its metabolites. Nicotine half-life is around 2 h, while that of cotinine and 3HC around 16 h (15).

All solvents used for extraction and dissolving were of analytical grade. Dichloromethane, ethyl acetate, chloroform, and sodium tungstate dihydrate were purchased from Merck (Darmstadt, Germany). Reference nicotine, cotinine, and 3HC (in 1 mg/mL of methanol) were purchased from Lipomed AG (Arlesheim, Switzerland). All working standard solutions were prepared by dilution with methanol to concentrations from 100 μg/mL to 10 ng/mL and used to prepare calibration samples.

All urine samples (5–10 mL each) were labelled with the study number, participant code to ensure anonymity, test number, and date and time and sent directly to the laboratory, where they were stored at -20 °C until analysis. At the time of analysis, we inserted

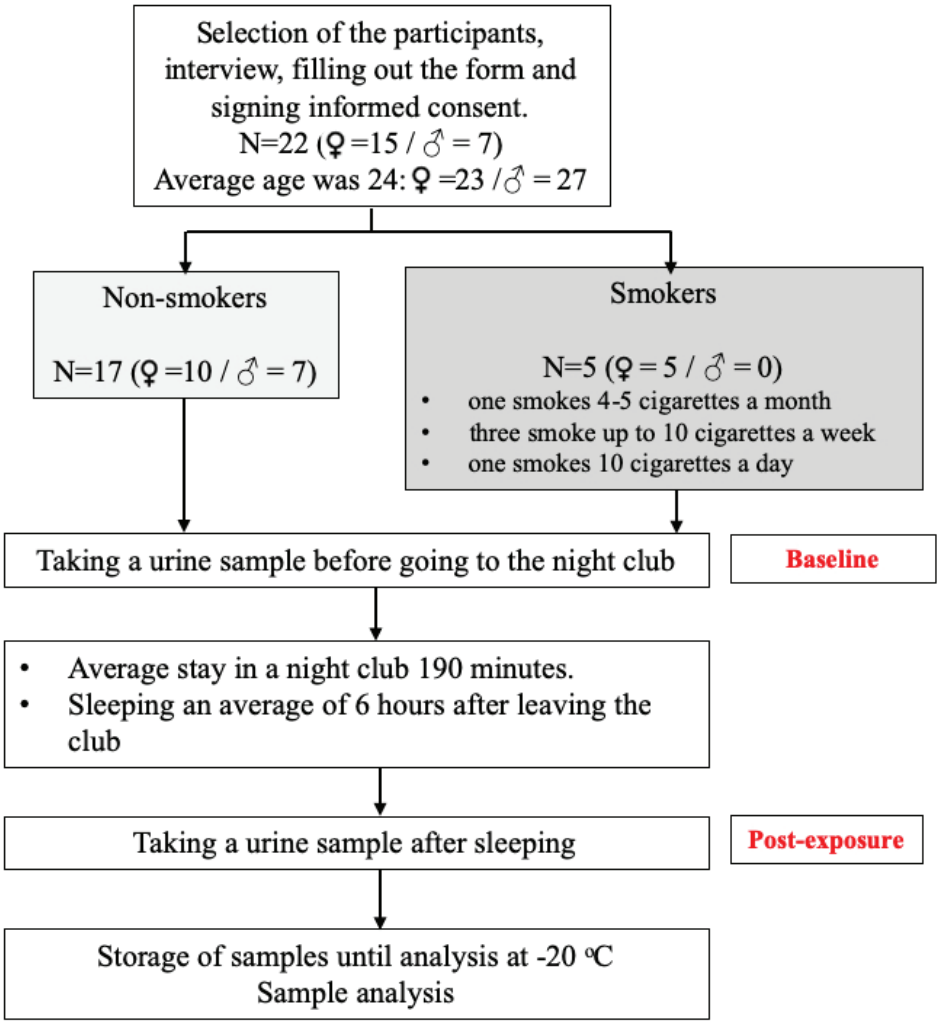

Figure 1 Study design

1.8 g of sodium tungstate dihydrate and 3 mL of dichloromethane/ethyl acetate mixture (*v*/*v*=3:1) to a glass tube with a screw and a cap (16) and added 2 mL of urine, mixed in a rotary mixer at 0.56 *g* for 10 min, and then centrifuged at 1500 *g* for another 10 min. The eluate was evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 30 μL chloroform and transferred to a glass tube for gas chromatography-mass spectrometry (GC-MS) run on a Shimadzu GCMS-QP2010 (Kyoto, Japan). The chromatographic column was RTX–5MS (5 % diphenyl-95 % dimethyl polysiloxane, length 30 m, diameter 0.25 mm, film thickness 0.25 μm). The initial column temperature of 90 °C was held for 3 min, then ramped to 240 °C at 15 °C/min and held for the total run time of 13 min. For carrier gas we used ultra-pure grade helium at the flow rate of about 1.5 mL/min. One microlitre of a sample was injected in splitless mode with an injection temperature of 250 °C. Ion source temperature was 200 °C, and the interface temperature was 280 °C. GC-MS analysis was performed using the selected ion monitoring mode with characteristic ions (m/z: 84, 133 and 162; 98, 176 and 42; 106, 192 and 135; for nicotine, cotinine and 3HC, respectively). Detector voltage was 1.5 kV in absolute mode.

Method validation

The method was validated in the linear range of 0.1–1000 ng/mL with correlation coefficients consistently higher than 0.998. To assess linearity, a calibration curve was calculated by analysing drug and nicotine-free urine samples spiked with standards at the concentrations of 0.1, 1, 10, 100, and 1000 ng/mL. Repeatability was assessed by analysing blank urine samples spiked with two different concentrations of nicotine and cotinine (10 and 333 ng/mL) in six replicates. Mean recovery was ≥95.0 %, with linearity (R2) greater of 0.998 for all analytes. Relative standard deviation for each analyte was <5 %. The limits of detection (LOD) for nicotine, cotinine, and 3HC were 0.2, 0.1, and 0.3 ng/mL, respectively, whereas the limits of quantitation (LOQ) were 0.66, 0.33, and 0.99 ng/mL, respectively.

Statistical analysis

Descriptive statistics and differences between the groups were run on the GraphPad Prism software, version 8.0.0 for Mac (GraphPad Software, San Diego, CA, USA). The normality of distribution was checked with the Kolmogorov-Smirnov test. Due to non-normal distribution of the data, continuous variables are presented with the minimum, maximum, and median (interquartile range, IQR). Medians were compared using the chi-square test and the groups were compared with the Mann-Whitney *U* test. Statistical significance was set to $p<0.05$.

RESULTS AND DISCUSSION

Table 1 shows a rise in nicotine, cotinine, and 3HC concentrations in all participants between the baseline and post-exposure measurements, which was significant in non-smokers. In smokers, only nicotine concentrations rose significantly. In addition, Figure 2 shows that the difference in the rise was significantly higher in smokers than non-smokers for all three compounds. In non-smokers nicotine, cotinine, and 3HC concentrations increased 20.05, 18.70, and 16.18 ng/mL, respectively, while in smokers they increased 177.24, 285.31, and 342.83 ng/mL, respectively.

However, when we calculated the concentration ratios between baseline and post-exposure measurements (obtained by dividing the

Table 1 Nicotine, cotinine, and trans-3'-hydroxycotinine concentrations in urine samples of 22 study participants before (baseline) and after having spent 190 min in a nightclub and slept for six hours in average (post-exposure)

Variable		Baseline concentrations (ng/mL)			Post-exposure concentrations (ng/mL)			Differences between baseline and post-exposure measurements*
		Min	Max	Median (IQR)	Min	Max	Median (IQR)	
Smokers (N=5)	Nicotine	0.66	323.34	16.22 (172.21)	25.20	509.58	364.72 (370.88)	**p<0.05**
	Cotinine	2.61	323.15	54.44 (235.22)	54.82	946.04	373.06 (639.29)	p=0.095
	3HC	5.46	451.51	168.41 (328.16)	<LOD	5933.42	575.15 (3681.79)	p=0.529
Non-smokers (N=17)	Nicotine	0.67	42.25	2.66 (14.26)	6.91	588.27	32.37 (49.29)	**p<0.05**
	Cotinine	<LOD	63.00	35.36 (40.49)	2.62	190.55	65.09 (66.94)	**p<0.05**
	3HC	1.23	77.76	5.46 (16.52)	2.46	148.77	32.95 (68.96)	**p<0.05**

* Mann-Whitney *U* Test

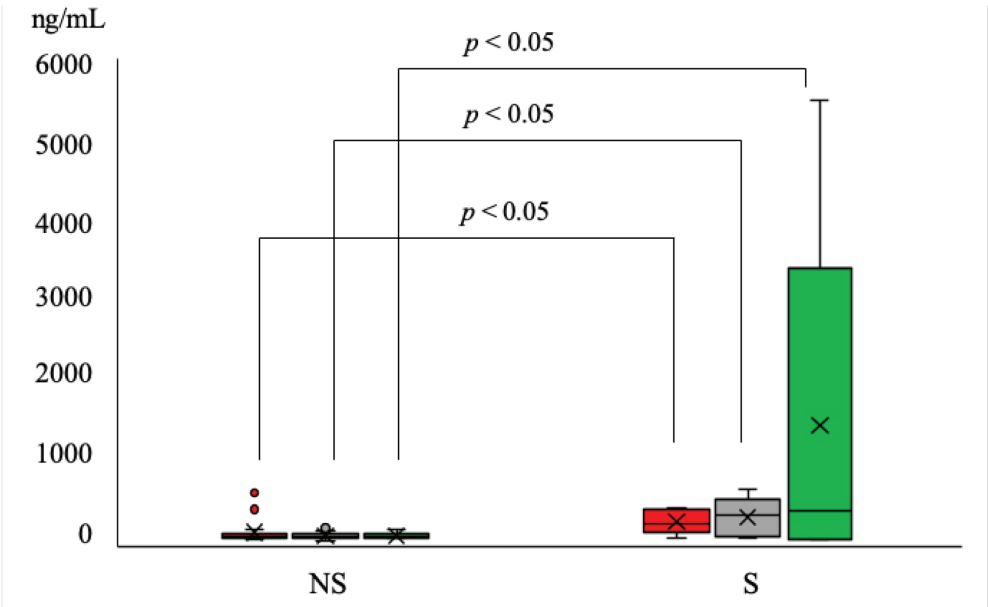

Figure 2 Differences in median nicotine (red column), cotinine (grey column), and 3HC (green column) increase (in ng/L) in urine between non-smokers (NS) and smokers (S)(chi-squared test). Box plots show the median, interquartile range (box), and overall range (whiskers). p<0.05 – statistically significant

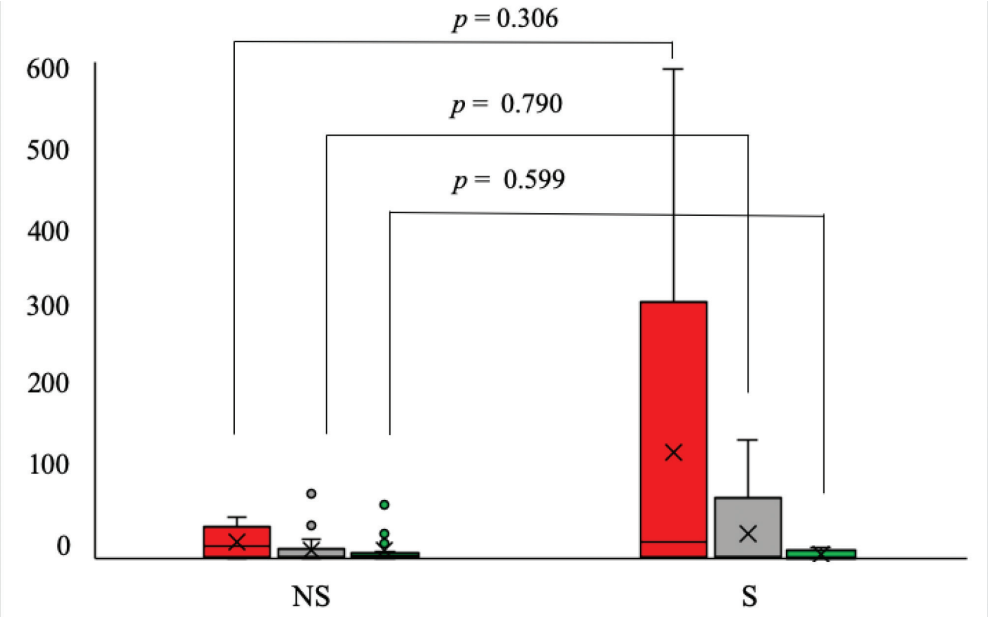

Figure 3 Differences in the ratio between median baseline and post-exposure nicotine (red column), cotinine (grey column), and 3HC (green column) concentrations in urine between non-smokers (NS) and smokers (S) (chi-squared test). The y-axis shows how many times urine concentration increased between baseline and the post-exposure measurement. p<0.05 – statistically significant

post-exposure concentration with the corresponding baseline, there was no statistically significant difference in the rise between non-smokers and smokers, even though the nicotine increase in smokers was 14.74-fold, while in smokers it was 20.85-fold. Cotinine increase in non-smokers was 1.86 and in smokers 2.41-fold, but the increase in 3HC was higher in non-smokers (3.80 vs 2.48-fold in smokers).

Because we could not control the conditions of tobacco smoke exposure in our participants, none had zero concentrations of nicotine and its two metabolites at baseline. Precisely for this reason we determined the increase in their concentrations after exposure at the nightclub. As expected, the increase in all three compounds was higher in smokers, because not only were they exposed to second-hand smoke, but they also actively smoked at the nightclub.

Furthermore, our study included three non-smoking male waiters who, due to the nature of their workplace, were exposed to second-hand smoke several hours longer than the rest of the participants. Because of that, their increase in nicotine, cotinine, and 3HC concentrations is 4.42, 1.62, and 4.04 times higher, respectively than in non-smoking male participants. In a study conducted in nightclubs and bars across 24 cities in North and South America, Eastern Europe, Asia and Africa between 2007 and 2009, hair nicotine concentrations in smoking and non-smoking employees

were higher than before the shift, which confirms that exposure to second-hand smoke is an important occupational health risk (17).

A particularly interesting finding in our study concerns a male non-smoking participant who spent the entire nightclub stay near the ventilation. In his sample, we determined a considerably lower increase in cotinine and 3HC than in other non-smoking participants. Although, in this particular case, ventilation seems to considerably reduce second-hand smoke exposure, it cannot prevent it completely. The American Society of Heating, Refrigerating, and Air-Conditioning Engineers (ASHRAE) believes that ventilation, even the most advanced such as dilution ventilation, cannot prevent tobacco smoke exposure and that the only way is to ban smoking (18).

STUDY LIMITATIONS AND CONCLUSION

Our pilot study and the interpretation of its findings are limited by the small sample (22 participants) and the fact that it could not be run under controlled conditions. Also, in order to determine the real impact of passive smoking by excluding the contributions of active smoking, any further research should have two separate groups of participants, smokers and non-smokers.

Regardless of its limitations, however, this pilot study clearly shows a considerable increase in second-hand tobacco smoke exposure and a higher risk of its adverse effects in non-smokers attending nightclubs. It also calls for further, more comprehensive research. Our next study will include more participants and will pay attention to variations in nicotine metabolism, primarily the genetic variations in the main nicotine-metabolising enzyme CYP2A6.

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Razine nikotina i njegovih metabolita u mokraći mlade populacije izložene pasivnom pušenju u noćnim klubovima: pilot-istraživanje

Cilj ovog istraživanja bio je istražiti opseg izloženosti pasivnom pušenju u mlađoj populaciji koja posjećuje noćne klubove u Hrvatskoj usporedbom razina nikotina i njegovih glavnih metabolita kotinina i trans-3'-hidroksikotinina (3HC) u uzorcima urina uzetim od 22 sudionika prije i nakon trosatnog boravka u noćnom klubu, stratificiranih prema statusu pušenja (pušači i nepušači). Uzorci su pripremljeni za analizu metodom ekstrakcije tekuće-tekuće i analizirani vezanom tehnikom plinske kromatografije sa spektrometrijom masa. Prisutnost nikotina, kotinina i 3HC potvrđena je u svim uzorcima urina. Njihove srednje koncentracije značajno su se razlikovale između dva mjerenja kod nepušača. Naši nalazi pokazuju da čak i trosatna izloženost pasivnom pušenju može značajno povećati razinu nikotina i njegovih metabolita u urinu, što ukazuje na izloženost drugim, štetnim tvarima duhanskog dima. Također pozivaju na podizanje svijesti o zdravstvenim rizicima izloženosti pasivnom pušenju u općoj populaciji, a posebno među pojedincima koji posjećuju noćne klubove.

KLJUČNE RIJEČI: kotinin; nikotin; pasivno pušenje; trans-3-hidroksikotinin; uzorak mokraće