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EFFECT OF MATERNAL TOBACCO SMOKING OR EXPOSURE TO SECOND-HAND SMOKE ON THE LEVELS OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANOL (NNAL) IN URINE OF MOTHER AND THE FIRST URINE OF NEWBORN

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Tobacco smoking during pregnancy is associated with a variety of negative consequences not only for the mother, but also for the developing fetus. Many studies have shown that carcinogens contained in tobacco smoke permeate across the placenta, and are found in fetus. The aim of the study was to determine the prenatal exposure to tobacco-specific carcinogenic N-nitrosamines on the basis of measurements of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in urine of smoking and second-hand smoke (SHS) exposed women and in the first urine of their newborns. A questionnaire documenting demographics and socio-economical data, smoking habits and exposure to SHS was completed by 121 delivering women near or at term. Maternal concentrations of cotinine and NNAL were measured in urine of the mother and the first urine of her newborn infant by liquid chromatography tandem mass spectrometry (LC/MS/MS). The mean concentration of cotinine was 439.2 ng/mg creatinine and NNAL concentration in urine of smoking women was 74.0 pg/mg creatinine, and for her newborn 78.6 pg/mg creatinine. Among mothers exposed to SHS, cotinine and NNAL mean concentration were 23.1 ng/mg creatinine, and 26.4 pg/mg creatinine. In newborns of SHS exposed mothers during pregnancy the mean concentration of NNAL was 34.1 pg/mg creatinine, respectively. Active tobacco smoking as well as passive exposure to smoking during pregnancy is an important source of tobacco specific N-nitrosamines to the fetuses as evidenced by increased concentrations of this carcinogen. Determination of NNAL in maternal urine samples can be a useful biomarker of prenatal exposure of newborn to carcinogenic nitrosamines.

Key words: *4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), biomarkers, cotinine, newborn, pregnancy, second-hand smoke*

INTRODUCTION

In many studies it has been demonstrated that approximately 40 compounds contained in tobacco smoke have a carcinogenic properties (1). Epidemiological data have shown an increase of cancer risk in smokers and persons exposed to second-hand smoke (SHS) (2, 3). To evaluate active smoking and passive exposure to tobacco smoke known as second-hand smoke, variable biomarkers have been used (4). Cotinine is the most popular and specific marker of tobacco smoking and SHS exposure (5, 6). Although this biomarker is appropriate for evaluation of general exposure to tobacco smoke, it does not reflect the exposure to tobacco smoke carcinogens. In case of NNAL it may result from differences in pharmacokinetic parameters between cotinine and NNAL. Biological half-life of NNAL is 3-4 days, NNAL-Gluc 40-45 days and cotinine merely 17 hours, thus after smoking cessation the metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanone are eliminated slower in relation to the fast elimination of cotinine. N'-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and five other N-nitrosamines in the tobacco smoke originate from the nicotine present in tobacco. It is commonly accepted that NNK plays a major role in the development of lung cancer in smokers (7, 8). Current literature recommends 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the metabolite of NKK as a biomarker for evaluation of exposure to tobacco-specific carcinogens (9). In some studies NNAL and its glucuronides are measured, however, for estimation of NNK uptake the total concentration of NNAL (NNAL and NNAL-glucuronides) is the critical parameter (10).

Smoking during pregnancy is associated with a variety of negative consequences *e.g.* ectopic pregnancy, low birth weight and length, spontaneous abortion, placenta previa, and abruptio placenta (11-13). In Poland 20 to 29.9% women continue to smoke during pregnancy (11). Although there is general awareness of harmful effects of tobacco smoking on mothers' and fetus' health only 12% of pregnant women stop smoking during pregnancy and as many of (38%) of Polish men smoke tobacco daily (14, 15). After pregnancy is confirmed many women reduce the number of cigarettes smoked daily; however, their exposure to SHS may not be diminished either in their workplaces or homes.

Previous studies have shown that carcinogens permeate across placenta and are found in fetus. In the fetus benzo[a]pyrene-DNA adducts have been detected (16) and in the newborns' urine and metabolites of NNK have been found (17).

The aim of the study was to determine the prenatal exposure to tobacco-specific carcinogenic N-nitrosamines based on the measurements of NNAL in the urine of smoking women as well as women exposed to SHS in order to find relationship between maternal and fetal exposure to toxic carcinogens.

MATERIALS AND METHODS

Study population

The study population consisted of 121 women and their newborns born at the Maternity Ward of the Department of Obstetrics and Gynaecology of the University of Medical Sciences in Warsaw. The protocol was approved by the Bioethics Commission of the University of Medical Sciences in Warsaw, and medical procedures in this study were carried out in concordance with ethical standards of Helsinki Declaration (1975 with revision of 2000), GCP and ICH guidelines. Participation in the study was voluntary and informed consent was obtained from all mothers enrolled into the study. To the study was selected women with singleton pregnancy and correctly developing uterine foetus. Based on the responses to the questionnaire verified by cotinine levels in urine, mothers were divided into three groups: smoking (62), women exposed to SHS (28), and non-smoking and non-SHS exposed women (31). The questionnaire consisted of eight questions related to demographics, socio-economical data, smoking habit (tobacco smoking during pregnancy, number of cigarettes smoked, place of exposure to SHS) and occupational exposure to carcinogenic compounds. Urine samples from women (100 ml) were collected soon after admission to the hospital, and the first newborn urine was collected in sterile adhesive plastic bags. Urine samples were frozen and stored at -20°C until analyzed.

Determination of cotinine and creatinine

After liquid-liquid extraction (dichloromethane: isopropanol 9:1, pH 9), cotinine was determined by previously developed and validated high performance liquid chromatography method with norephedrine as an internal standard. The method was linear from 5-1000 ng/ml, limit of detection (LOD) and limit of quantification (LOQ) were 5 ng/ml and 10 ng/ml, respectively (18). The concentration of creatinine was determined by means of spectrophotometry (wavelength 520-560 nm, Synchron Cx Clinical System analyzer, Beckman Instruments Inc.) with the use of Bio Merieux reagents.

Determination of the total (NNAL and NNAL-glucuronides) level of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

These analyses were carried out using a modified method described previously (19). 100 µl of internal standard, deuterated NNAL (d_3NNAL) (10 pg/µl), was added to 5 ml of urine. The pH was adjusted to 6.8, then 10,000 units of β-glucuronidase (Type IX-A *Escherichia coli* - Sigma) was added. The solution was incubated at 37°C for 20 hours with continuous shaking (250 rpm). After this time the mixture was extracted on Water Oasis MCX columns (Supelco). Analytes were eluted with the mixture of methanol and ammonia (95:5, v/v). The extracts were dried at 40°C under a stream of nitrogen. Before analysis, the samples were redissolved in 150 µl of mobile phase.

The analysis of NNAL was performed by liquid chromatography (LC Waters 2695) coupled with tandem mass spectrometry (Quattro Micro) operating in the multiple reaction monitoring (MRM) mode. The separation was achieved on LiChroCART 125x3 Purospher RP-18e column (Merck, Darmstadt, Germany). For the analysis, gradient elution was applied using a mixture of phase A (water with 100 µl/100 ml of formic acid) and B (acetonitrile with 100 µl/100 ml of formic acid). The following gradient was set: start - 15 min: a linear decrease from 95% to 50% solvent A, 15 min - end: a linear increase to 95% solvent A. A constant flow rate of 0.4 ml/min was applied. Data acquisition time was 21 min. The column was heated to 35°C during the analysis.

An electrospray operated in the positive ion mode (ES+) with multiple reaction monitoring function. Desolvation gas (nitrogen) flow was 600 l/min and the temperature set to 300°C. The collision-induced dissociation transmission m/z 210 to 180 for NNAL and m/z 213 to 183 for d3NNAL was monitored. The collision energy was 40 eV and the pressure in the collision chamber was 0.0003 mbar. Other parameters were as follows: capillary voltage: 3000V, cone potential: 40 V and source temperature 99°C. The limit of detection was 5 pg/ml, and the limit of determination was 10 pg/ml. The calibration curve was linear between 5 to 1000 mg/ml.

Statistical analysis

The results were assessed by the analysis of variance and by chi-square analysis. The homogeneity of variance was verified with the Levene test. Correlations between the respective variables were indicated on the basis of the Spearman coefficient. In addition, the Fisher-Freeman-Hamilton and the Mann-Whitney tests were used in the calculations to determine significant differences between maternal groups according to tobacco exposure, as well as, their infant's.

RESULTS

Characteristics of the studied groups

Based on the response to the questionnaire (preliminary grouping) and concentration of cotinine in maternal urine (final grouping) (below 5 ng/mg of creatinine - non smokers; 5-50 ng/mg of creatinine - exposure to SHS; above 50 ng/mg of creatinine - smokers) women were allocated in the groups of non-smokers (31 women, NS), exposure to SHS (28 women, PS) and smokers (62 women, S). The limits of cotinine concentration in the urine were established on the basis of previous study (5, 20). Newborns of non-smoking women were assigned to N-NS group, of women exposed to environmental smoke to N-PS group, and of smoking women to N-S group.

There was no significant difference in ages of the women in each group, with the majority (65.45%) between 25 and 34 years. The non-smoking women were better educated than smoking (university education - 58.1% - nonsmokers and 35.1% - smokers). Pregnancy duration in completed weeks

Table 1. Characteristic of studied groups.

Parameter	Studied group		
	Nonsmokers	Exposure to SHS	Smokers
No. women in group	31	28	62
Age [year]	27.8±3.8	26.6±4.6	24.1±2.1
No. of cigarettes in pregnancy [%] 1-5 6-10 11-15 >15	NA	NA	 30.7 40.3 25.8 3.2
Partners' smoking [%]	0	57.1	83.9
Exposure to SHS [h] <3 >3	0	 60.7 39.3	 24.2 75.8
Pregnancy duration [week]	35.2±4.1	34.5±4.4	35.3±4.5
Birth body weight [g]	2686±1132	2529±1087	2452±1056
Birth body length [cm]	49.90±7.79	49.32±7.57	48.25±7.07

NA - no applicable

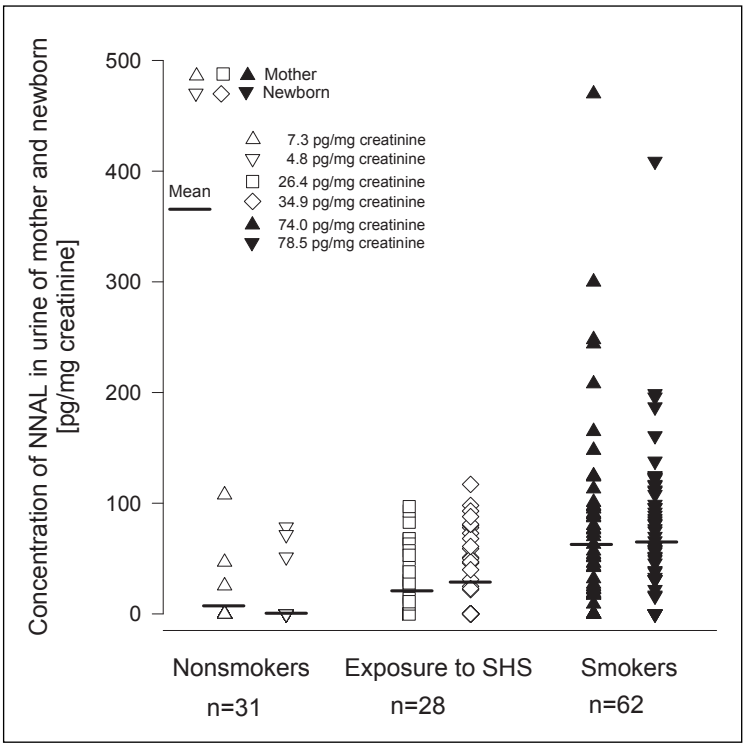

Fig. 1. Concentration of NNAL in urine of women and their newborn (all value and mean).

was not different among the groups of mothers in this study (*Table 1*).

The lowest birth weight and body length occurred among infant of smoking women but these differences were not statistically significant (Kruskal-Wallis test) (*Table 1*).

Maternal urine cotinine levels

Concentration of cotinine - a major metabolite of nicotine was determined in the mothers' urine. According to the eligibility criteria, all of the urine samples of non-smoking and not exposed to tobacco smoke women (n=31) had zero-concentration of cotinine. Among tobacco smoking women (n=62) the mean concentration of cotinine was 439.2 ng/mg creatinine (the minimal value was 4.5 ng/mg creatinine, the maximal value was 6368.6 ng/mg creatinine). In 18 women (29.0% of all smoking) the concentration of cotinine in urine was zero, as they had not smoked for several days prior to hospital admission. Among women exposed to SHS the mean concentration of cotinine in urine was 23.1 ng/mg creatinine (the minimal concentration was

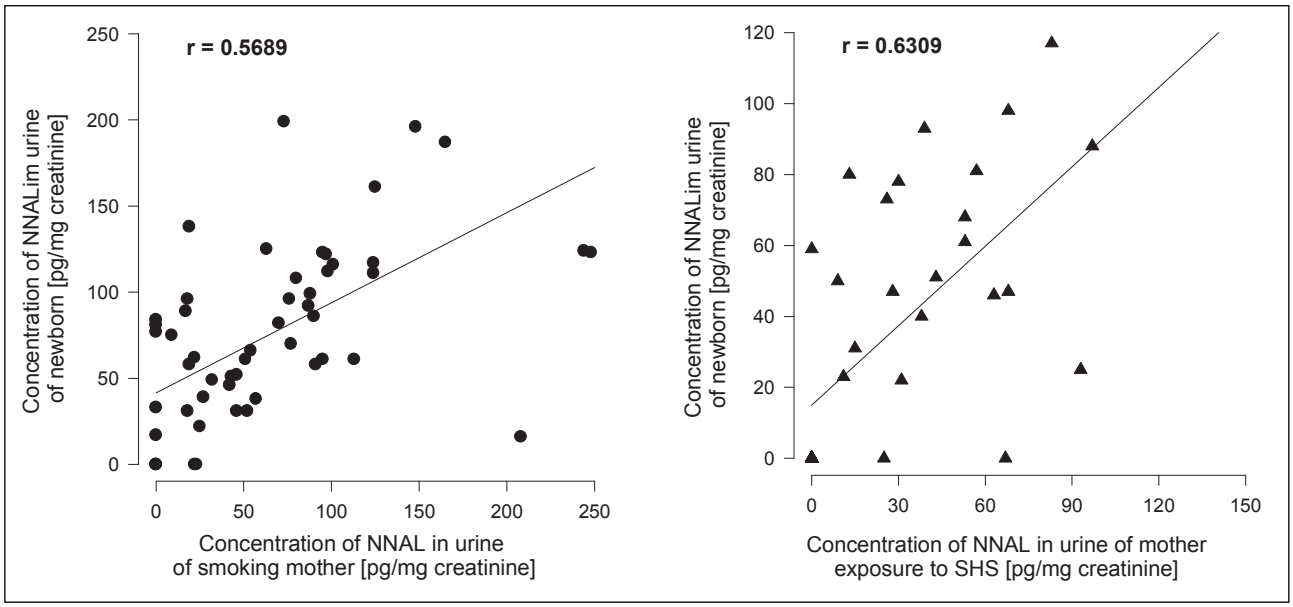

Fig. 2. Correlation between concentration of NNAL o urine of women and newborn. Left - smoking women, right - women exposure to ETS.

7.6 ng/mg creatinine, the maximal concentration was 44.7 ng/mg creatinine). In 19 women (67.9% of all exposed to SHS) zero-cotinine concentration was found.

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in urine of women and newborns

Among women who were smoking during pregnancy in 87.10% of examined samples the concentration of NNAL reached values higher than zero. In the group of mothers who were passively exposed the number of urine samples meeting this criterion amounted to 57.14%. In urine of three non-smoking women NNAL was detected, it can be explained by misclassification of these patients caused by false declaration in the questionnaire and lack of cotinine in urine (longer biological half-life of NNAL then cotinine).

The mean concentrations of NNAL in groups of smoking and exposed women amounted to 74.0±1.9 pg/mg creatinine and 26.4±29.8 pg/mg creatinine, respectively (*Fig. 1*).

Variations in concentration values between the groups of non-smokers and the group of both SHS smoked exposed and active smokers were statistically significant, as well as differences between the group of smokers and exposed only to SHS ($p<0.05$).

The relationship between cotinine and NNAL concentrations in mothers' urine within the entire study group and within individual subgroups (non-smokers, those exposed to SHS and active smokers) did not demonstrate a statistical significance; however, in case of smoking and SHS exposed women an increase of cotinine concentration with an upward trend of NNAL concentration was demonstrated.

Among newborns whose mothers smoked during pregnancy 91.94% of urine samples showed a measurable concentration of NNAL, while in the group of newborns of mothers exposed only to SHS 53.57% had measurable amounts of NNAL. As in the case of their mothers, infant's concentration of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol was above the level of detection. The mean concentration of NNAL in newborns of women who were smoking during pregnancy was 78.6±63.4 pg/mg creatinine, whereas in the group of newborns of mothers exposed to SHS it was 34.1±37.7 pg/mg creatinine (*Fig. 1*). Variations in concentration of 44.5 pg/mg creatinine (mean value) between the group of non-smoking mothers' babies and the group of babies of passive and active smokers were statistically significant, as well as between the groups of smokers' babies and babies of mothers exposed to SHS ($p<0.05$). The correlation between concentrations of NNAL in newborns' urine and concentrations of cotinine in women's urine was not statistically significant. In the group of mothers who smoked during pregnancy, their newborn's had a statistically significant increased correlation between NNAL concentrations their urine compared to their mothers' urine (Spearman rank correlation coefficient was $R=0.5689$ ($p<0.05$) (*Fig. 2*).

Among SHS exposed, there was also a significant correlation between NNAL concentrations in mothers' and newborns' urine ($R=0.6309$, $p<0.05$) (*Fig. 2*).

DISCUSSION

One of the potentially adverse effects of smoking during pregnancy is fetal exposure to carcinogens. Tobacco smoke is a complex mixture of varied types of carcinogens, co-carcinogens, and promoters. The main carcinogenic compounds are polycyclic aromatic hydrocarbons, N-nitrosamines, and aromatic amines. Relatively recently scientists have called particular attention to tobacco-specific N-nitrosamines. Exposure to carcinogenic tobacco-specific N-nitrosamines of fetuses of mothers who were smoking during pregnancy or passively inhaling tobacco smoke was assessed in the studies on the basis of questionnaire and biological markers of exposure - cotinine and N-nitrosamines. The analyzed parameters were determined in mothers' urine (N-nitrosamines and cotinine) and in newborns' first urine (N-nitrosamines).

Cotinine in urine of studied mothers

In the study we used the assumption of Florek *et al*. (21) that urine concentrations of up to 5 ng/mg creatinine suggests no exposure, concentration levels from 5 to 50 ng/mg creatinine

indicate exposure to SHS, and values above 50 ng/mg creatinine confirm active smoking. For the purpose of normalization of the results, the concentration of cotinine was calculated per milligram creatinine, because elimination of cotinine with urine is dependant on kidney function, renal blood flow, and pH of urine. Within the group of smoking women the mean creatinine concentration was 439.2 ng/mg creatinine, and 23.1 ng/mg creatinine in urine of smokers and exposed to SHS, respectively. The zero concentrations of the investigated metabolite were not taken into account while calculating the mean cotinine concentration in the group of smoking women.

NNAL in urine of mothers and their newborns

4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanol and its glucuronide (NNAL-Gluc) are exceptionally useful biomarkers, because they are derived from the carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanone, which is uniquely specific for tobacco products (100-200 ng/cigarette) (22).

In order to assess the potential risk of fetal and neonatal exposure to toxic constituents of tobacco smoke, we determined the concentration of NNAL in urine of mothers and their newborns. The analysis of results demonstrated that the mean concentration of NNAL in urine of smoking women was 74.0 pg/mg creatinine, and 26.4 pg/mg creatinine in urine of mothers passively exposed to tobacco smoke. In non-smoking and tobacco non- exposed women, in only a few cases was the concentration of NNAL above the low limit of determination of the method.

Cotinine and NNAL relationship

A tendency for NNAL concentrations to rise with increasing concentrations of cotinine in urine was shown in the group of smoking and SHS exposed women. Joseph *et al*. (9) described significant correlation between the number of cigarettes smoked per day and total NNAL. A close association between the total NNAL and total creatinine was also demonstrated. The concentration of total NNAL ranged 0-23.9 pmol/mg creatinine. According to the authors, the levels of biomarkers such as carbon monoxide, NNAL and cotinine rise with an increasing number of cigarettes smoked per day, however, the correlation is not linear and reduced number of smoked cigarettes from 50 to 30 per day may result in no significant reduction of exposure to carcinogens (9).

Hecht *et al*. (1) documented metabolites of NNK remain in human body for a long time. One week after smoking cessation, NNAL and NNAL-Gluc were still detected in urine at the level of 34.5% of initial concentration, while the concentration of cotinine, the major metabolite of nicotine, was 11% of the initial concentration. Anderson *et al*. (23) demonstrated, that in the group of women exposed to SHS that the concentrations of NNAL and NNAL-Gluc in urine were 5.6% of the concentration in urine of their partners who smoke, highlights the relationship between the numbers of cigarettes smoked per day and the risk of developing lung cancer.

Exposure of newborns to NNAL

In order to assess the fetal exposure to carcinogenic tobacco-specific N-nitrosamines, we measured the concentration of 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanol in the first urine of newborns. NNAL was determined in 91.94% newborns whose mothers smoked during pregnancy. In smoking mothers, NNAL was detected in 87.10% of cases. In urine of newborns whose mothers were exposed to SHS, NNAL values were above 0 in 53.57% cases, while in their mothers NNAL was measured in 57.14%. The mean NNAL concentration in newborns whose mothers smoked during pregnancy was 78.6 pg/mg creatinine, and in newborns of mothers passively exposed to tobacco smoke during pregnancy the mean concentration of NNAL was 34.1 pg/mg creatinine. Lackmann *et al*. (24) determined two metabolites of NNK: NNAL and NNAL-Gluc in urine of newborns. They confirmed the presence of NNAL-Gluc in 71% of urine samples obtained from newborns whose mothers smoked during pregnancy and NNAL in 4 of 31 samples. Based on our and Lackmann's results we concluded that the metabolites of tobacco-specific N-nitrosamines cross the placental barrier and appear in amniotic fluid and the fetus. In an experiment with Syrian hamsters the administration of NNK to pregnant females resulted in development of tumors in multiple tissues of their offspring, including respiratory tract, pancreas, and adrenal glands. Animal research shows that NNK and/or NNAL cross across placenta to the fetus (Koppang 1992). Both NNK and its main metabolite 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanol are eliminated slowly from the amniotic fluid, with levels still detectable up to 6 h after NNK treatment (25).

On the basis of animal research, two theories explaining the presence of NNAL and NNAL-Gluc in urine of newborns have been postulated. The first theory assumes that the fetus is able to metabolize NNK to NNAL, which is further glucuronidated to NNAL-Gluc. Human data confirm that CYP2A13 is the functional enzyme that catalyzes NNK alpha-hydroxylation in human fetal nasal mucosa (26). The second possible explanation for the presence of NNAL in newborns first urine is metabolism of NNK in the placenta. The results of Collazo and Sultatos (27) confirmed that human placental cytochrome P450 is likely involved in the metabolism of NNK to NNAL and the responsible enzymes are NADPH and NADH-dependent carbonyl reductase(s). Another hypothesis indicates the role of mother's metabolism of NNK to NNAL and NNAL-Gluc, and NNK metabolites which can reach fetal circulation through the placenta (28).

In these studies no statistically significant correlation between NNAL concentrations in mothers' and newborns' urine and concentration of cotinine in mothers' urine was found, which is consistent with previous studies of Hecht *et al*. (29). On the other hand, correlation between NNAL concentration in mothers' urine and the concentration of NNAL in their newborns' urine, both in groups of smoking and SHS women was statistically significant.

Hecht *et al*. determined NNAL and NNAL-Gluc in urine samples collected from 144 babies whose parents smoked tobacco in the home. They determined cotinine and its glucuronides. Total NNAL was detected in 46.5% of babies. The mean value of total NNAL in 144 babies was 0.083 pmol/ml, while the average concentration of total cotinine was 0.133 nmol/ml. This study confirmed for the first time the exposure of babies to carcinogens present in environmental tobacco smoke (30). Also Carmella *et al*. (31, 32) focused on tobacco smoke derived carcinogens. They demonstrated that average rate of NNAL and NNAL-Gluc elimination with urine ranges 0.23-1.0 and 0.57-6.5 μg/24 hours, respectively. The majority of previous studies concerning NNK metabolites were based on urine testing.

However, we also collected blood plasma from 16 smokers and 5 non-smokers and found that the mean concentration value of total NNAL was 42±22 fmol/ml plasma. In 16 tobacco smokers it ranged from 1.7 to 88 fmol/ml plasma. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol was not detected in the plasma of five non-smokers (<8 fmol/ml) (33). Thus studies of many laboratories confirm the high risk following exposure to tobacco-specific N-nitrosamines - carcinogenic compounds found in tobacco smoke.

In this study we demonstrate that NNAL, which is present in urine of pregnant women who smoke tobacco and which crosses the placenta poses a threat of cancer in the developing fetus and baby in future life. Children of women smoking during pregnancy are exposed to toxic constituents of tobacco smoke that cross the placental barrier. Numerous authors suggest that the toxic components of tobacco smoke can freely migrate across the placenta at least from 12th week of pregnancy. Exposure to tobacco smoke *in utero* results in dysfunctions of the respiratory tract in the prenatal and neonatal period.

Epidemiological studies have proved that placental exposure to tobacco smoke and prevalence of different kinds of cancer in children or older individuals may be a cumulative effect of smoke constituents. Cigarette smoke was recently shown to stimulate the expression and secretion of several cytokines including IL-8, IL-6, MPC-1, MIP-1β and IL-10 (34). In additional, when cigarette smoke encounters saliva, there is a massive increase in reactive nitrogen species (RNS), which in turn cause protein alterations in the form of carbonyl formation and decreased enzyme (e.g. salivary α-amylase) activities (35). Recently Öberg and coworkers (36) have reported the burden of disease from SHS exposure in terms of estimated deaths and disability-adjusted life-years throughout the world. Poland was placed in Europe B category which demonstrated that in 2004, 56% of children and 54% of women were exposed to SHS daily. In addition to the burden of significantly increased lower respiratory infections, asthma, and acute otitis media among children, that there was a 1.97 odds ratio for adult onset asthma, a 1.22 odds ration of lung cancer, and 1.27 odds ratio of ischemic heart disease among those exposed to second hand smoke. This translates into 106 deaths from asthma, 1306 deaths from adult onset asthma, and 751 adult deaths from lung cancer with nearly 30,000 deaths from ischemic heart disease in adults (36).

CONCLUSION

Our study suggests that the placenta is not a barrier for tobacco-specific N-nitrosamines and the concentration of NNAL in urine of newborns is approximately three times higher than in urine of their mothers, and furthers the concept of fetal origin of adult, as well as, childhood diseases. Although NNAL concentrations both in women's and newborns' urine are much higher in case of active smoking, SHS exposure must be considered high-risk exposure to the fetus to tobacco smoke carcinogens. Further, the lack of correlation between concentrations of cotinine and NNAL indicates that cotinine, which is a standard biomarker of tobacco smoke exposure, cannot be used as an assessment of carcinogenic nitrosamine exposure to either mother or their fetuses. However, the statistically significant correlation between concentrations of NNAL in urine of newborns and their mothers permits the measurement of this biomarker in urine of the mother for evaluation of prenatal exposure to carcinogenic nitrosamines for both maternal, as well as fetal risk.

Conflict of interests: None declared.

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