Biomarkers of carcinogenic compounds of tobacco smoke constituents in the urine of delivering women

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Abstract
In order to monitor the exposure of smokers to two most important cancerogens – polycyclic aromatic hydrocarbons (PAH) and nitrosamines – it is possible to apply biomarkers. For PAH, it is 1-hydroxypyrene (1-HP), and for nitrosamines, 4-(methylamino)-4-(3-pyridyl)-1-butanol (NNAL). The aim of the study was to evaluate the exposure of delivering women to PAH and tobacco specific nitrosamines, using biomarkers for these compounds. Seventy delivering women took part in the study, i.e. 38 who did not smoke and who were not-exposed to ETS and 39 smokers. A urine sample was taken from each woman to determine cotinine and 1-HP by means of the HPLC and NNAL by LC/MS/MS. Questionnaire surveys were carried out among patients. The first part of the questionnaire survey concerned the socio-economic status, the second one – exposure to tobacco smoke. The smoking women were characterised by lower age, education level and income per person. More than sixty percent of smoking women are in a relationship with smoking partners which was an additional source of exposure to tobacco smoke. The exposure to tobacco smoke was monitored owing to the measurement of cotinine. In the urine of non-smoking women, the level of this biomarker was below the limit of detection, but in the case of smokers, the concentration was 228.2 ng/mg of creatinine, which, confirmed tobacco smoking. The concentration of 1-HP for smoking women amounted to 0.46 ng/mg of creatinine and was statistically higher than in the case of non-smokers (0.21 ng/mg of creatinine). A weak correlation between the concentration of 1-HP and cotinine in the urine of smoking women was observed. The concentration of NNAL in the urine of smokers amounted to 72.6 pg/mg of creatinine. In the urine of non-smokers, in three cases, measurable concentrations were found, which can suggest the exposure of these women to tobacco smoke. No correlations between the concentration of cotinine and NNAL or between NNAL and 1-HP were demonstrated. The results of the study indicate that tobacco smoking is real source of carcinogenic compounds. For pregnant women and foetuses, however, cotinine is a valuable biomarker for evaluation of smoking or exposure to ETS. Its application for the purpose of measuring the exposure to PAH and nitrosamines present in tobacco smoke is insignificant. In order to evaluate the exposure to these compounds, specific markers such as 1-HP and NNAL have to be applied.

Key words: tobacco specific nitrosamines, polycyclic aromatic hydrocarbons, pregnancy

Introduction
Tobacco smoke contains about 4200 chemical compounds and at least a few hundreds of substances which have not been identified so far. Carcinogens belonging to many chemical groups such as polycyclic aromatic hydrocarbons, N-nitrosamines specific for tobacco (NAST), aromatic amines, aldehydes and non-organic compounds constitute a numerous group. During tobacco smoking, the polycyclic aromatic hydrocarbons, present in the main and side stream of tobacco smoke are produced in the process of pyrosynthesis. Among over 60 polycyclic aromatic hydrocarbons identified in tobacco smoke, eleven have a carcinogenic action that has been proved in experiments on animals. In the case of smokers and persons exposed to environmental tobacco smoke (ETS), these compounds participate in the creation of pulmonary carcinoma, lymphatic carcinoma and oral cavity carcinoma [1].

The tobacco-specific N-nitrosamines contained in tobacco smoke are produced as a consequence of N-nitrosation of 2,3-diamines during tobacco drying and burning [2]. Among the seven N-nitrosamines identified in tobacco, initiators of the neoplastic process, four constitute protooncogenes requiring a metabolic activation by hydroxylation in order to achieve the carcinogenic activity [3]. N'-nitrosonornicotine (NNN) and 4-(methylamino)-4-(3-pyriyl)-1-butanol (NNK) are of greatest significance in this process. These nitrosamines are an etiological factor of the appearance of oral cavity neoplasm and pancreas neoplasm [4].

Tobacco smoking is responsible for diseases leading to the premature mortality or disability. It is the main cause of damages of the respiratory tract (pulmonary carcinoma and chronic obstructive lung disease) and cardiovascular diseases (coronary heart disease, arterial hypertension and apoplexy).

A group which should be particularly protected from exposure to tobacco smoke (active and passive smoking) are pregnant women. The increased risk of premature detachment from the placenta, more frequent occurrence of the placenta praevia, increased frequency of irregular bleedings during pregnancy, as well as the enhanced risk of premature rupture of the membranes are only some disorders concerning pregnancy. Tobacco smoking among pregnant women is one of the most frequent factors of the intranate risk related to limiting the foetal growth.

The placenta does not constitute a barrier protecting the foetus from the harmful impact of toxic components of

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tobacco smoke, including polycyclic aromatic hydrocarbons and N-nitrosoamines.

The most credible method of evaluation of the exposure to tobacco smoke and its components is the determination of biomarkers. The adducts of polycyclic aromatic hydrocarbons with proteins and DNA are used as biomarkers of exposure to polycyclic aromatic hydrocarbons contained in the tobacco smoke. Most frequently, these are combinations of benzo(a)pyrene with the DNA leucocytes and polycyclic aromatic carbons with blood albumins. The drawback of this indicator is its long durability, which is not favourable when evaluating active and passive smoking (the level of adducts is maintained at a measurable level for about 14 months) [5-7].

The most frequently applied biomarker of exposure to these compounds is 1-hydroxypyrene, which is determined in urine [5-9]. It is produced as a result of metabolic transformations of pyrene, which constitutes about 15-30% of all polycyclic aromatic hydrocarbons occurring in tobacco smoke. The advantage of 1-hydroxypyrene as a biomarker of exposure to polycyclic aromatic hydrocarbons is the correlation between its concentration and the level of benzo(a)pyrene, the basic carcinogenic compound from this group [10]. 1-hydroxypyrene indicates a degree of exposure to polycyclic aromatic hydrocarbons irrespective of the absorption path; mainly these are lungs, skin, and the alimentary system. Some authors propose to apply 1-hydroxypyrene glucuronide as a biomarker instead of free 1-hydroxypyrene due to its greater content in urine. The concentration of the conjugated compound in comparison to the free one is twice as high [5, 11]. The basic drawback of this biomarker is its little specificity. Its concentration in urine depends on the exposure from all sources (environmental pollution, smoked and grilled food), and not only from tobacco smoke.

The metabolite of 4-(methylnitrosoamino)-1-(3-pyridyl)-1-buthanol (NNAL) produced during the biotransformation of NNK may be used as a biomarker of exposure to this group of carcinogens. The few studies carried out so far indicate an increased secretion of NNAL among people exposed to tobacco smoke (the level of adducts is maintained at a measurable level for about 14 months) [12-15]. Apart from this, Hehst et al. demonstrated the existence of correlation between the concentration of cotinine and NNAL in urine in the case of these people [16]. The undeniable advantage of NNAL as a biomarker of (active and passive) exposure to N-nitrosamines contained in tobacco smoke is its almost total specificity. On the other hand, very low concentration of this compound in urine is a drawback, as it causes considerable analytic difficulties and a significant cost of this type of research.

The aim of the conducted research is to evaluate the exposure of delivering women, who smoke tobacco, to carcinogens from the group of polycyclic aromatic hydrocarbons and tobacco specific N-nitrosoamines.

Material and method

77 pregnant women, who were admitted to the Maternity Department of the Gynaecological-Obstetric Clinical Hospital of the Medical University in Warsaw and Medical University in Poznań, took part in the research conducted in years 2005 and 2006.

The research protocol was approved by Bio-ethical Committees of both Universities. The research was carried out in conformance with the recommendations of the Helsinki Declaration from 1964, 1975, 1983, 1989 and 1996, and participation in it was voluntary. The patients were notified about the aim of the research.

In conformance with the declarations of the patients regarding (active or passive) exposure to tobacco smoke during pregnancy and the determined main nicotine metabolite – cotinine, the tested women were divided into two groups. The first group was formed by patients who smoked tobacco during pregnancy – 39 persons. The non-exposed smoking women and women passively exposed to smoke during pregnancy were assigned to the control group – 38 persons.

A morning sample of urine collected from a woman in the course of the day from the time of admission to the hospital constituted the material for toxicological research (determinations of biomarkers). Until the time of analysis, the urine was kept at the temperature of –18°C.

The cotinine in the urine was marked earlier by means of the method of high performance liquid chromatography with spectrophotometric detection, with the use of norephedrine as an internal pattern, after the prior extraction by means of the liquid-liquid method.

The prepared method was lineal with regard to concentrations from 5 to 1000 ng/ml. The limit of detection (LOD), which was indicated as the value of the ratio of the signal to noise S/N = 3 amounted to 5 ng/ml, whereas the limit of quantification (the lowest concentration on the calibration curve) amounted to 10 ng/ml [8].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cotinine [ng/mg creatinine]</th>
<th>1-Hydroxypyrene [ng/mg creatinine]</th>
<th>NNAL [pg/mg creatinine]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>Smokers</td>
<td>Nonsmokers</td>
</tr>
<tr>
<td>Mean</td>
<td>0</td>
<td>228.2</td>
<td>0.095</td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>293.5</td>
<td>0.141</td>
</tr>
<tr>
<td>Range</td>
<td>0-13.10.2</td>
<td>0-1.560</td>
<td>0-1.273</td>
</tr>
</tbody>
</table>

* three positive results of NNAL determination
1-hydroxypyrene after the enzymatic hydrolysis (total concentration) was determined by means of the method of high performance liquid chromatography with spectrophotometric detection, after solid phase extraction (SPE). The prepared method was linear with regard to the concentrations ranging from 0.1 to 200 ng/ml.

The limit of detection, which was indicated as the value of the ratio of signal to noise S/N = 3 amounted to 0.05 ng/ml, and the limit of quantification (the lowest concentration on the calibration curve) amounted to 0.1 ng/ml.

NNAL in urine was determined by means of the method of the high performance liquid chromatography coupled with mass spectrometry – tandem (LC/MS/MS), with the use of \( d_3 \)-NNAL as the internal pattern, after a prior hydrolysis and solid phase extraction. The prepared method was linear with regard to the concentrations ranging from 10 to 1000 pg/ml. The indicated limit of detection (LOD) assuming the value of the ratio: signal to noise S/N = 2 amounted to 5 pg/ml. The concentration of the lowest pattern on the calibration curve (10 pg/ml) was the assumed limit of quantification (LOQ) \([13]\).

The concentration of cotinine, 1-hydroxypyrene and NNAL in the urine was calculated into creatinine, which was determined by means of the spectrophotometric method.

**Statistical analysis of results**

Information from the questionnaire, after a prior encoding, were collected in the computer data base. The results of questionnaire surveys and toxicological surveys were subjected to a statistical analysis. The differences in the distributions of the tested variables were evaluated by means of the analysis of ANOVA variance and T-student test. The mean values were compared applying the variance analysis.

**Results**

The research included a group of pregnant women delivering at the Gynecological-Obstetrics Clinical Hospital of the Medical University in Warsaw. In conformance with the assumptions of the paper, the patients were divided into two groups on the basis of their declarations: women smoking tobacco during pregnancy (39 women) and women who were neither actively nor passively exposed to tobacco smoke during pregnancy (38 women). The declarations of patients were verified by means of the cotinine determination in the urine samples. The presence of cotinine in urine, in spite of the declaration about non-smoking, excluded such a patient from the research. During the tests, in order to standardise the results, the concentration of cotinine was calculated into mg of creatinine due to the fact that the elimination of this compound from urine depends on the efficient functioning of kidneys, blood flow through the kidneys and pH of urine.

On the basis of the questionnaire surveys, it was stated that women smoking throughout the period of pregnancy, smoked 11 to 15 cigarettes a day and more than 16.

Due to the assumed criteria of selection of patients for the tests (declaration on smoking and determination of cotinine), the concentration of cotinine within the group of non-smoking women was below the limit of detection. On the other hand, the concentration of cotinine in the urine of women smoking tobacco amounted to zero in 7 cases, and its mean concentration amounted to 228.2 ± 263.5 ng/mg of creatinine.

The maximal concentrations of cotinine in the group of smoking women amounted to 1310.2 ng/mg of creatinine.

Within the group of non-smoking women, the concentration of 1-hydroxypyrene ranged from 0 to 0.71 ng/mg of creatinine, and the mean value amounted to 0.095 ± 0.141 ng/mg of creatinine (Table 1). The concentration of this biomarker of exposure to polycyclic aromatic hydrocarbons in urine of smoking women was statistically higher in urine of the non-smoking women and amounted to 0.462 ± 0.358 ng/mg of creatinine (range from 0 to 1.27 ng/mg of creatinine). In 7 urine samples from smoking women and 11 urine samples from non-smoking women, the concentration of 1-hydroxypyrene was below the limit of detection. A weak correlation was observed between the concentration of cotinine and 1-hydroxypyrene in urine of the smoking women (Fig. 1).

The mean concentration of 4-(methylnitrosamino)-1-(3-pyridyl)-1-buthanol in urine of the smoking women amounted to 72.6 ± 70.4 pg/mg of creatinine. In the case of five patients smoking tobacco, the presence of NNAL in their urine was not confirmed. On the other hand, in the case of three patients declaring non-smoking, the level of NNAL was 34.2; 54.3; 87.4 pg/mg of creatinine.

The obtained results did not indicate the existence of correlation either between the concentration of NNAL and cotinine or NNAL and 1-hydroxypyrene in urine of the smoking women (Fig. 1).

**Discussion**

The epidemiological research demonstrate that the common knowledge on the subject of harmful impact of tobacco smoking is not directly correlated with the number of smo-
A significant increase in the number of smoking women was noticed in the 90's. In Poland, at the end of the 90's, about 25-30% of pregnant women smoked tobacco [17]. The greatest number of women smoked in the first trimester, and the number of smokers was smaller and smaller in the course of pregnancy and so about 12% declaring tobacco smoking would be admitted to hospitals to deliver babies [18].

The carcinogenic impact of tobacco smoke components (pulmonary carcinoma, oesophagus carcinoma, larynx carcinoma, oral cavity carcinoma, pancreas carcinoma, carcinoma of urinary bladder and others) was demonstrated in many experimental research and confirmed in epidemiological research [19]. The exposure to polycyclic aromatic carbons contained in tobacco smoke and their detrimental action was proved quite a long time ago [20], whereas the evaluation of exposure to tobacco specific N-nitrosamines has not been conducted for too long due to analytical difficulties related to the determination of compounds in the biological material [21].

In the present research, the concentration of cotinine – a biomarker of exposure to tobacco smoke – amounted to $228.2 \pm 263.5 \text{ ng/ml of creatinine}$ in the case of 39 women smoking tobacco during pregnancy. The obtained values of cotinine concentration below the limit of detection in the case of seven women prove that the time that must have passed by from the moment of smoking of the last cigarette to the moment of collection of a urine sample for tests – arrival at the Clinic was long. In all probability, the women quit smoking before the delivery in spite of the fact that they declared smoking of 6-10 cigarettes per day during pregnancy. Such cases are also described by other authors. The biological period of half-life of cotinine is long and amounts to 10-27 hours [23]. The low concentration of cotinine in the urine of smoking pregnant women is accounted for by some authors, who claim that it is a result of faster metabolism of this compound in this group of patients [24]. With reference to most tests that have been carried out, it follows that the values of cotinine concentrations among pregnant women are lower than the ones assumed for the total number of smokers (above 3000 $\mu$g/g of creatinine) [25, 26].

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Within the group of women who declared non-smoking and lack of exposure to tobacco smoke due to the accepted research protocol, no cotinine was observed in the urine. The lack of cotinine in the urine of most women declaring non-smoking was also observed in other research [8, 18, 23, 27-29].

In accordance with the aim of the paper, the level of 1-hydroxypyrene as a biomarker of exposure to polycyclic aromatic hydrocarbons was determined in urine of the smoking pregnant women and the women who did not smoke tobacco. The mean concentration of 1-hydroxypyrene in urine of patients smoking tobacco during pregnancy amounted to $0.462 \pm 0.358 \text{ ng/mg of creatinine}$, whereas in the case of women who did not smoke it amounted to $0.095 \pm 0.141 \text{ ng/mg of creatinine}$. These differences were statistically crucial.
The pathological impact of polycyclic aromatic hydrocarbons on the functioning of a placenta is considered to be an important factor, which is responsible for the effects of the impact of this group of compounds contained in tobacco on the foetus [30,32]. These compounds are well-known enzymatic inducers, and numerous studies proved that an increase in the activity of microsomal enzymes and metabolism of xenobiotics in the placenta (which is related to it) are an important factor with regard to the impact of xenobiotics on the foetal development. The toxic metabolites of polycyclic aromatic hydrocarbons produced in the organism of a mother and a foetus as a result of tobacco smoking, may impact the embryo, foetus and many functions of the placenta. An increase in the activity of enzymes metabolising polycyclic aromatic hydrocarbons in the first trimester of pregnancy, as a response to tobacco smoking, was statistically important already in the 8th week of pregnancy [33].

The determination of 1-hydroxypyrene as a biomarker of exposure to polycyclic aromatic hydrocarbons started from using it in the evaluation of exposure to these compounds in industrial conditions [34,35], and later on was applied in the evaluation of exposure to polycyclic aromatic hydrocarbons through food [36], atmospheric air, work environment [34,37,39] and in therapeutic actions [40].

The conducted tests confirmed the results of earlier experiments [39,41,42] regarding the usefulness of this biomarker in the evaluation of the increased exposure of tobacco smokers to polycyclic aromatic hydrocarbons. The concentrations of 1-hydroxypyrene in urine of smoking women were much higher than non-smoking women living in similar conditions.

The mean concentration of NNAL in urine of the tobacco smoking women, observed in the tests that were carried out, amounted to 72.6 ± 70.4 pg/mg of creatinine. The scope of changes of the level of this metabolite ranged from 0 pg/ml to 300.1 pg/ml.

Within the group of patients who did not smoke and who were not exposed to tobacco smoke, the presence of NNAL in urine was observed in three cases in spite of the fact that the urine was free from cotinine. The statistical analysis of obtained results demonstrated a tendency to the increase in the concentration of NNAL as the level of cotinine grew, but it was not statistically significant. This lack of correlation can be explained by differences in the speed of elimination of tested compounds. The biological period of half-life of NNAL amounts to about 3-4 days, whereas the half-life of cotinine lasts only 17 hours, hence the metabolites of 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone after quitting smoking remain longer in the organism than metabolites of nicotine [43].

The results of research carried out by Lackmann et al. prove that tobacco specific N-nitrosamines permeate through the placental barrier and may have an impact on the foetus [14].

The tobacco specific N-nitrosamines are an etiological factor that plays a crucial role in the development of the cervical carcinoma [44]. Prokopczyk et al. demonstrated that the mean concentration of NNK in the mucus collected from the uterine cervix of smoking women amounted to 46.9 ± 32.5 ng/g [15].

4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone observed in the present research, in urine of tobacco smoking pregnant women, for which the placental barrier does not exist, creates a risk of carcinoma development in adult life, however the epidemiological research proved that the exposure to tobacco smoke through the placenta and occurrence of tumour in the age of childhood or in the later period of life should be interpreted as the effect of the combined action of tobacco smoke components [45,46].

References


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