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Article

Possible Relationship between Respiratory Diseases and Urinary Concentrations of Polycyclic Aromatic Hydrocarbon Metabolites

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Abstract: Aim: Exposure to polycyclic aromatic hydrocarbons (PAHs) has been in the past associated with adverse effects on human health among which belong also respiratory diseases. Our study is focused on the evaluation of PAH exposure by measuring the concentrations of their monohydroxylated metabolites (OH-PAHs) in urine and comparing their concentrations with the incidence of respiratory diseases in 2-year-old children from 2 locations in the Czech Republic – a control location Ceske Budejovice and a previously (in 1970s and 1980s) highly contaminated mining district Most. Now, the air pollution and lifestyle in both cities are very similar. However, based on our previous data, we suspect that there might be long-term changes in the Most population**. Methods:** The total number of 248 participants gave samples of their urine which were analyzed for the presence of 11 OH-PAHs. The analysis was carried out by liquid-liquid extraction with ethyl acetate; clean-up employed dispersive solid phase extraction with a sorbent Z-Sep. Separation, identification, and quantification of the target compounds was realised using ultra-high performance liquid chromatography coupled to tandem mass spectrometry. The incidence of respiratory diseases was evaluated according to the questionnaires provided by the pediatricians. **Results and discussion:** The concentrations of measured OH-PAHs were higher in the urine samples collected from 2-year-old children living in Most compared to 2-year-old children from Ceske Budejovice. The same trend was observed also when the urine samples were analyzed when these children were studied as newborns in our previous study. From all of the monitored respiratory diseases, only influenza due to unidentified influenza virus showed a difference between the tested locations: the 2-year-old children living in Most were more frequently diagnosed with this disease. **Conclusion:** We have observed a higher incidence of respiratory diseases as well as a higher concentration of OH-PAHs in urine of the 2-year-old children from Most. Therefore, we hypothesize that the population living in this previously highly contaminated location can carry some long-term health burden.

Keywords: 2-year-old children; respiratory diseases; influenza; OH-PAHs; metabolites; urine

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are non-polar environmental contaminants that are formed during incomplete combustion or pyrolysis of organic matter [1, 2]. Due to their ubiquitous presence in the environment, people can be easily exposed to these compounds mainly through the digestion of contaminated foods or inhalation of polluted air or cigarette smoke [3]. Occupationally exposed individuals (firefighters, asphalt workers, coke-oven workers) can be also significantly exposed to PAHs by transfer through the skin [4, 5].

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PAHs are not considered to be bioaccumulative substances because when they enter the human body, they are relatively quickly metabolized (excretion half-life 2-6 h). It is known that PAHs become more harmful to human health after the metabolic transformation. PAH metabolites (mainly reactive epoxides) can form adducts with DNA which can result in carcinogenesis [6]. A formation of reactive oxygen species is also a very important consequence of PAH exposure. These reactive compounds can cause oxidative stress in the cells and also damage DNA with production of 8-hydroxy-2′-deoxyguanosine (8-OHdG) [7, 8]. The exposure to PAH metabolites in uterus may lead to dysregulation of lung development and result in respiratory symptoms early after birth [9-11]. Jedrychowski et al. (2005) observed the increased risk for barking cough, wheezing, sore throat, and ear infection [12]. The prenatal exposure to immunotoxic PAHs impairs the immune function; subsequently, it may be responsible for the increased susceptibility of newborns and young infants to respiratory infections [12]. The impact of PAH exposure on respiratory diseases is explained by three main physiological mechanisms: systemic oxidative stress, epithelial dysfunction, and immune disturbance [11].

However, not all of the PAH metabolites result in producing DNA adducts. The reactive epoxides can be transformed to hydroxylated metabolites (OH-PAHs) that are in the second phase of the metabolism conjugated with glucuronic acid, sulphates, or glutathione to produce more water-soluble compounds that can be excreted from the body via urine [4, 13]. In such biological material, these metabolites can be then reliably measured.

In our previous study [14], we have observed higher concentrations of urinary OH-PAHs of newborns from Most compared to the second sampling location Ceske Budejovice, but the concentrations of benzo[*a*]pyrene (BaP) in ambient air in both of these locations are similar. Therefore, we hypothesized that the population of highly polluted mining districts at 40-50 years ago can carry some long-term changes (maybe changes in the genetic information) which also affect the metabolism of PAHs [15].

The aim of this study has been to evaluate if there is any relationship between the levels of OH-PAHs in urine and respiratory diseases in 2 cities of the Czech Republic – the control location Ceske Budejovice and the previously highly polluted mining district Most. Also, there had been the aim to try to find the cause of the differences in PAH exposure by investigating the information provided in personal questionnaires.

2. Materials and Methods

2.1. Certified standards and materials

Certified standards of 1-OH-NAP and 2-OH-NAP were bought from Absolute Standards, Inc. (USA); 3-OH-PHEN, 9-OH-PHEN, 1-OH-PYR, and 3-OH-BaP were supplied by Neochema (Germany), and 6-OH-CHRY was purchased from AccuStandard® (USA). Analytes 2-OH-FLUO, 1-OH-PHEN, 2-OH-PHEN, 4-OH-PHEN, and isotopically labelled analogues of target compounds, specifically d_7-1-OH-NAP, d_7-2-OH-NAP, d_9-2-OH-FLUO, d_9-1-OH-PHEN, d_9-2-OH-PHEN, d_9-3-OH-PHEN, d_8-9-OH-PHEN, d_9-1-OH-PYR, and d_{11}-3-OH-BaP were obtained from Toronto Research Chemicals Inc. (Canada). Creatinine was bought from Sigma-Aldrich (USA). All of the supplied standards and their isotopically labelled analogues were of at least 98 %purity. All mixtures were prepared in methanol and stored in the freezer (-20 °C). The Standard Reference Material® 3673 (Organic Contaminants in Non-Smokers´ Urine) (SRM 3673) was delivered by the US National Institute of Standards and Technology (NIST, USA).

2.2. Chemicals, reagents and other materials

Sorbent Supel™ QuE Z-Sep, enzyme β-glucuronidase (type HP-2, glucuronidase activity ≥ 100 000 units/mL, sulfatase activity ≤ 7 500 units/mL), ethyl acetate, picric acid, and polypropylene centrifuge tube filters (nylon, pore size 0.22 μm) were purchased from Sigma-Aldrich (USA). Methanol (HPLC gradient) was obtained from Merck (Germany). Unsterile polyvinylidene fluoride (PVDF) filters (0.22 μm, Ø 33 mm) were bought from Rotilabo® (Germany) and 96-well micro plates were supplied by the Gama Group (Czech Republic).

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2.3. *Sample collection*

The urine samples were collected within the grant of the Czech Health Agency. The urine was sampled in 2 locations of the Czech Republic, Ceske Budejovice and Most, from 2-year-old children. All urine samples were collected as spot samples. The study was approved by the Ethics Committee of the Faculty of Health and Social Science, University of South Bohemia in Ceske Budejovice. Each mother signed the approval to include their child in the study. A total of 248 samples (130 samples from children from Ceske Budejovice and 118 samples from children living in Most) were collected. The urine collection was carried out from February 2019 to December 2020. For each child, there was available a questionnaire provided by the pediatricians which included the information such as disease incidence, dietary habits, length of breastfeeding, child's development (height, weight, head circumference), smoking habits in the family, and a type of household heating - Table 1.

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Table 1. Study demographic overview

Parameter			All (n=248)	Ceske Budejovice (n=130)	Most (n=118)
			Mean ± SD	Mean ± SD	Mean ± SD
Age	Maternal	years	33.6 ± 5.1	34.2 ± 4.7	33.1 ± 5.4
	Child	years	2.7 ± 0.5	2.6 ± 0.6 *)	2.8 ± 0.4 *)
Maternal Education	Elementary	%	5	0	11
	Low Secondary	%	14	11	17
	Upper Secondary	%	35	30	40
	University	%	44	56 +++)	31 +++)
Breast Feeding	Full	months	6.7 ± 5.2	6.2 ± 4.2	7.3 ± 6.1
	Partial	months	12.7 ± 8.0	12.2 ± 7.1	13.2 ± 8.9
Maternal Smoking	Smoking	%	10	6 +)	14 +)
	Smoking Before Pregnancy	%	24	16 ++)	32 ++)
	ATS - Pregnancy	cig/day	0.3 ± 1.1	0.2 ± 1.0	0.4 ± 1.2
	ATS - 1st Year	cig/day	0.6 ± 1.8	0.4 ± 1.5	0.7 ± 2.0
	ATS - 2nd Year	cig/day	1.6 ± 3.6	1.6 ± 3.8	1.7 ± 3.4
	ETS - Pregnancy	cig/day	2.9 ± 5.8	1.6 ± 4.8 *)	4.3 ± 6.6 *)
	ETS - 1st Year	cig/day	3.0 ± 5.9	1.7 ± 4.8 *)	4.4 ± 6.7 *)
	ETS - 2nd Year	cig/day	3.0 ± 5.9	1.8 ± 4.8	4.4 ± 6.8
Heating Type	Remote Heating	%	34	24 +++)	46 +++)
	Gas Heating Out of Flat	%	9	10	8
	Gas Heating In Flat	%	12	14	10
	Coal Heating Out of Flat	%	7	11 +)	3 +)
	Coal Heating In Flat	%	0	1	0
	Electric Heating	%	14	20 ++)	7 ++)
	Fireplace in Flat	%	10	12	8
Length	At Birth	cm	50.0 ± 1.8	49.6 ± 1.8 ***)	50.4 ± 1.8 ***)
	18 Months	cm	82.8 ± 3.4	83.6 ± 3.6 ***)	81.9 ± 2.9 ***)
Weight	At Birth	kg	3.4 ± 0.5	3.4 ± 0.5	3.4 ± 0.5
	18 Months	kg	11.2 ± 1.4	11.4 ± 1.4	11.0 ± 1.3
Head Circumference	At Birth	cm	34.5 ± 1.4	34.6 ± 1.3	34.4 ± 1.5
	18 Months	cm	47.8 ± 1.5	47.9 ± 1.5 *)	47.6 ± 1.6 *)

Results of Mann Whitney U-test compared by region *) p<0.05, **) p<0.01, ***) p<0.001 ,logistic regression by region +) p<0.05, ++) p<0.01, +++) p<0.001

Notes: ETS = Environmental Tobacco Smoke, ATS = Active Tobacco Smoke

The study enrolled children from whom we collected urine already in the time of delivery [14].

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2.4. Description of the methods

2.4.1. Measurement of urinary creatinine

Concentrations of creatinine were used to correct for the different dilution of each spot of urine sample and it was measured using a Jaffe's spectrophotometric method described in more detail in the study by Lankova et al. (2016) [16].

2.4.2. Analytical method for the determination of urinary OH-PAHs

2.4.2.1. Extraction

The samples were extracted using LLE (extraction solvent ethyl acetate) and a clean-up step (Z-Sep sorbent), as described in more detail in our previous paper [16].

2.4.2.2. Analytical instrumentation

The analysis of 11 OH-PAHs was performed using a UHPLC–MS/MS system 1290 Infinity II (Agilent) liquid chromatograph coupled to a QTRAP® 6500+ (SCIEX) mass spectrometer with electrospray ionization in a negative ion mode (ESI-) with a capillary voltage -4,5 kV and desolvation temperature 500 °C (MRM transitions with corresponding MS parameters are listed in **Table S1** in Supplementary data). Analytes were separated on a PFP (pentafluorophenyl) Kinetex column (Phenomenex) with dimensions 100 mm × 2.1 mm × 1.7 μm. The more detailed LC conditions are described in paper by Lankova et al. (2016) [16].

2.4.2.3. Quality assurance/quality control and validation

The validation of the method for the creatinine determination is described in detail in our previous study (Lankova et al., 2016). The validation of 11 OH-PAHs in urine was performed using SRM 3673 in 6 parallels. Solvent calibration was used for the quantification of target analytes. Limits of quantification (LOQs), recovery and repeatability (RSD - relative standard deviation) are summarized in **Table 2**. Monohydroxylated metabolite of CHRY (6-OH-CHRY) and BaP (3-OH-BaP) that were not certified in this material were validated by artificial contamination of a blank urine sample that was previously tested based on the presence of the target analytes. The used concentration levels were 0.1 ng/mL urine for 6-OH-CHRY and 1 ng/mL for 3-OH-BaP. Recovery for 6-OH-CHRY was 95 % with RSD 13% and LOQ 0.01 ng/mL urine and for 3-OH-BaP 97 % with RSD 16 % and LOQ 0.90 ng/mL urine. Each day, approximately 20 urine samples were analyzed and with each batch of samples, one duplicate sample was prepared.

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Table 2: LOQs, recovery and RSD for the target analytes (n=6)

Analyte	LOQ [ng/mL urine]	Certified value [ng/mL urine]		Measured value [ng/mL urine]		Recovery [%]	RSD [%]
		Mean	Uncertainty	Mean	Uncertainty		
2-OH-NAP	0.025	1.35	0.03	1.43	0.09	106	6
1-OH-NAP	0.025	211	34	146	12	70	8
2-OH-FLUO	0.025	0.107	0.007	0.106	0.016	99	15
2-OH-PHEN	0.010	0.0247	0.0043	0.0267	0.0041	108	15
3-OH-PHEN	0.010	0.0276	0.0014	0.0278	0.0030	101	11
1-OH-PHEN	0.010	0.0488	0.0075	0.0514	0.0063	105	12
9-OH-PHEN	0.010	0.0116	0.0009	0.0134	0.0015	115	11
4-OH-PHEN	0.010	0.0104	0.0010	0.0088	0.0005	84	5
1-OH-PYR	0.025	0.0305	0.0018	0.0307	0.0023	101	8

2.4.3. Respiratory diseases

Respiratory diseases were diagnosed during multiple clinic visits, based on the child's clinical symptoms and International Statistical Classification of Diseases 10th Edition (ICD-10): acute nasopharyngitis (J00), acute sinusitis (J01), acute pharyngitis (J02), acute tonsillitis (J03), acute laryngitis and tracheitis (J04), acute upper respiratory infections of multiple and unspecified sites (J06), Influenza due to unidentified influenza virus (J11), pneumonia, unspecified organism (J18), acute bronchitis (J20), other chronic obstructive pulmonary disease (J44), asthma bronchiale (J45).

2.4.4. Statistical evaluation

To compare the differences between the concentrations of OH-PAHs in urine, statistical t-test was performed. ICD codes of diagnoses have been collected from paediatric documentation filtered for apparent duplicities, normalized to shortened 2-digits codes form for better granularity of the tested data and then counted per individual child. Mann-Whitney U test [17] has been used to test the difference between both regions for individual children per individual diagnoses and for total sum of respiratory diseases, same as for demographical population parameters. For estimate of difference between categorical population parameters such as education, has been used logistical regression. For estimation of relation between OH-PAHs and count of diagnoses has been used linear regression calculated on log transformed values

3. Results and discussion

3.1. Analysis of OH-PAHs in urine

The analytes found in all of the measured samples (100 %) were 2-OH-NAP, 2-OH-FLUO, 1-OH-PHEN, 3-OH-PHEN, and 9-OH-PHEN, followed by 2-OH-PHEN (99 %), 1-OH-NAP (98 %), 1-OH-PYR (96 %), and 4-OH-PHEN (88 %) in urine samples obtained from children from Ceske Budejovice. As far as urine samples collected in Most are concerned, the analytes measured in all of the urine samples (100 %) were 2-OH-NAP, 2-OH-FLUO, 1-OH-PHEN, 2-OH-PHEN, 3-OH-PHEN, 9-OH-PHEN and 1-OH-PYR, followed by 4-OH-PHEN (96 %) and 1-OH-NAP (93 %). In terms of concentration, the analyte with the highest concentration in urine samples collected both in Ceske Budejovice and Most was 2-OH-NAP with median concentration 7.6 μg/g creatinine in the urine samples collected in Ceske Budejovice and 12.4 μg/g creatinine in the urine samples obtained in Most. The concentrations of other target compounds were approximately 10 times lower than in Ceske Budejovice (**Figure 1**).

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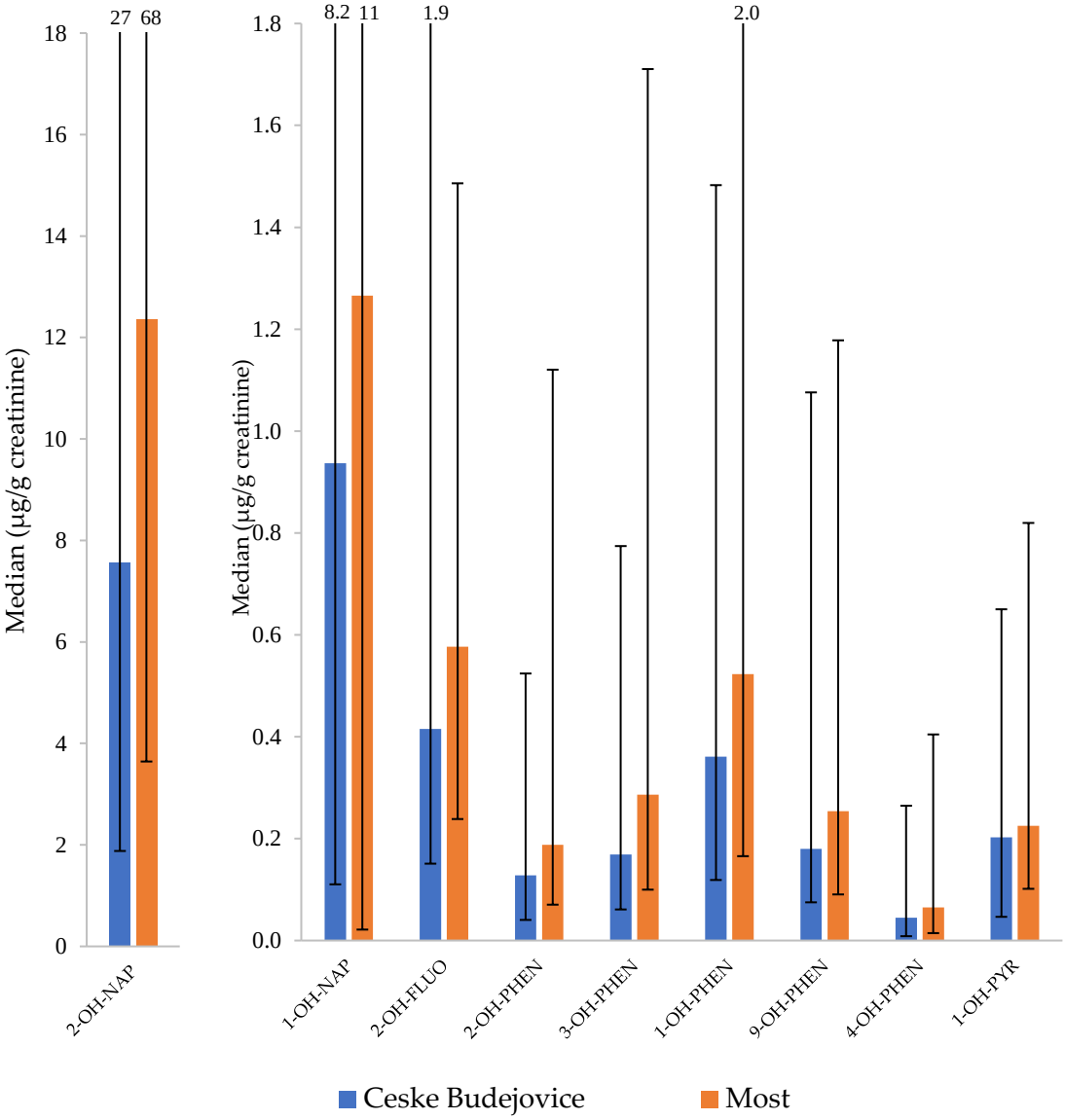
When comparing the concentrations of target analytes in urine between the locations, it can be observed that median concentrations for the OH-PAHs measured in urine are



statistically significantly ($p<0.05$) higher in samples collected in Most. The median concentration of the sum of all measured OH-PAHs in urine (ΣOH-PAHs) was 20.2 µg/g creatinine in samples collected in Most and 11.4 µg/g creatinine in urine samples collected in Ceske Budejovice. The trend of higher median concentration can be seen in all of the target analytes (**Figure 1**).

Figure 1: Median concentrations of target OH-PAHs in urine of 2-year-old children
Note: The error bars represent 5th and 95th percentiles

3.2. *Comparison of urinary OH-PAHs of newborns and 2-year-old children*

The two-year-old children from whom the urine samples in this study were collected, were also sampled within our previous study when these children were newborns [14]. The **Figure 2** shows the comparison of the median concentrations of OH-PAHs in urine when the children were newborns and when they were 2 years old. The same trend of higher levels of OH-PAHs in samples from Most can be observed for both newborns and 2-year-old children. The median concentration of ΣOH-PAHs in urine samples of newborn children from Most was 8.1 µg/g creatinine whereas the median of ΣOH-PAHs in urine samples collected from newborn children from Ceske Budejovice was 3.8 µg/g creatinine.

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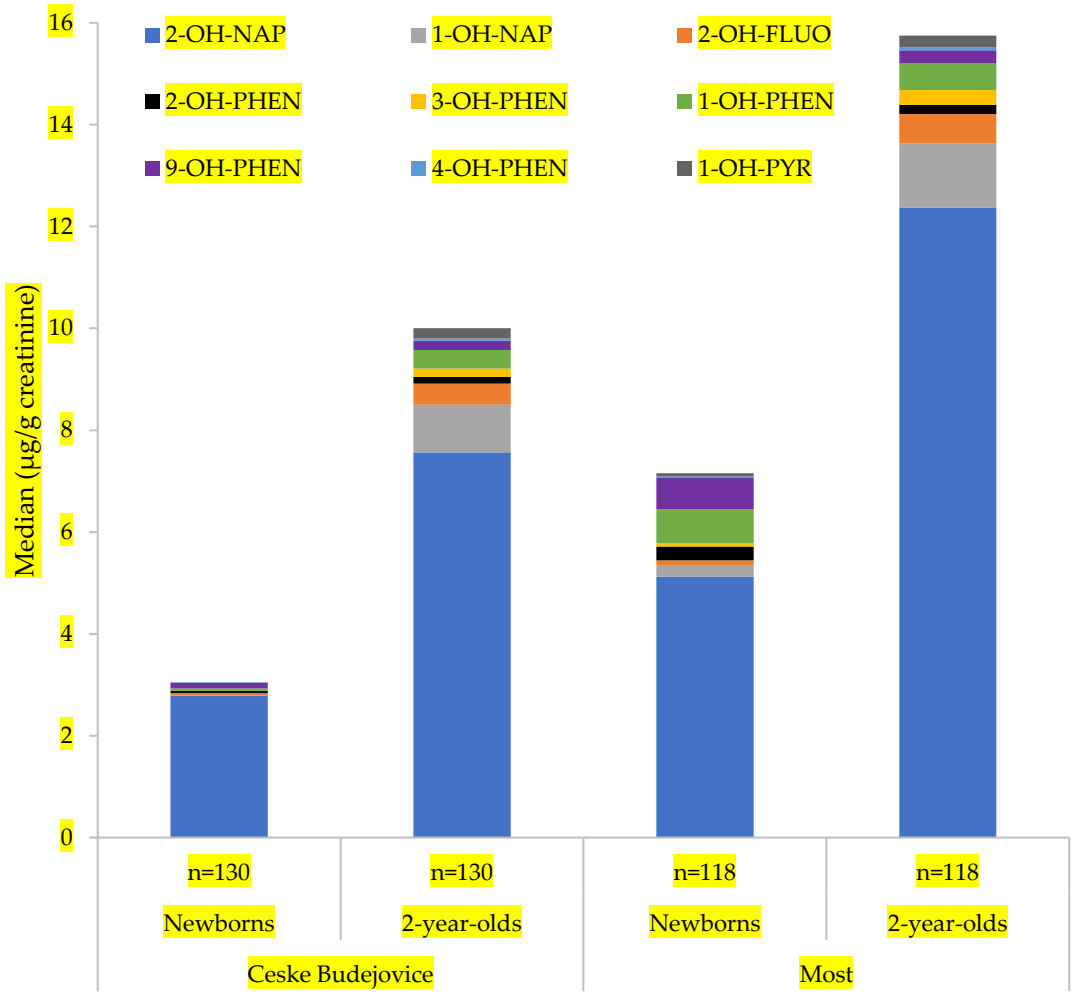

Figure 2: Comparison of the median concentrations of target OH-PAHs in urine of newborns and 2-year-old children

As we already concluded in our previous study [14], such outcome is somewhat surprising due to the fact that the air pollution in both locations is similar. Available questionnaires were carefully studied in order to explain this outcome. Specifically, the impact of breastfeeding, diet, type of household heating, and smoking habits in the family were investigated. However, it was concluded that breastfeeding did not have any impact on the levels of OH-PAHs in urine because both children from Ceske Budejovice and Most were breastfed for similar duration. We saw the same outcome when evaluating the impact of diet and type of household heating as both of these parameters were very similar in Ceske Budejovice as well as in Most. The only slight difference was observed in smoking habits in the family. When investigating smoking habits of the mothers, we concluded that in Most, more mothers claimed that they were smoking before the pregnancy (32 %) compared to the mothers from Ceske Budejovice (16 %). However, after the pregnancy, the number of smoking mothers was lower both in Most (14 %) and Ceske Budejovice (6 %). The mean number of cigarettes smoked during the day by the mothers was comparable between the locations as well as the number of fathers who smoked before and after the pregnancy of their partner.

3.3. Respiratory diseases

When we analyzed the total incidence of respiratory diseases (J00-J99) in our cohorts of 2-year-old children from Most and Ceske Budejovice, they were significantly higher in Most ($p<0.05$), specifically in the diagnosed influenza due to unidentified influenza virus (J11; $p<0.001$) (Table 3). As the concentration of OH-PAHs in urine of newborns from Most was higher at the time of the delivery, we may confirm the hypothesis

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Table 3. Frequency of illnesses

ICD-10	Disease	All (n=248)	Ceske Budejovice (n=130)	Most (n=118)
		Median (Quartile Range)	Median (Quartile Range)	Median (Quartile Range)
J00	Acute nasopharyngitis (common cold)	1 (0 - 2)	0 (0 - 1)	1 (0 - 2)
J01	Acute sinusitis	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)
J02	Acute pharyngitis	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)
J03	Acute tonsillitis	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)
J04	Acute laryngitis and tracheitis	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)
J06	Acute upper respiratory infections of multiple and unspecified sites	0 (0 - 1)	0 (0 - 1)	0 (0 - 1)
J11	Influenza due to unidentified influenza virus	0 (0 - 0)	0 (0 - 0) ***	0 (0 - 1) ***
J18	Pneumonia, unspecified organism	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)
J20	Acute bronchitis	0 (0 - 1)	0 (0 - 1)	0 (0 - 1)
J44	Other chronic obstructive pulmonary disease	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)
J45	Asthma	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)
J00-J99	Respiratory diseases	3 (1 - 4)	2 (1 - 4) *	3 (2 - 5) *

Results of Mann Whitney U-test compared by region *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

presented by Jedrychowski et al. (2015) that in utero exposure to PAH metabolites may result in the dysregulation of lung development and result in respiratory symptoms early after birth [9]. According to another Jedrychowski et al. (2005) hypothesis, prenatal exposure to immunotoxic PAHs may impair the immune function of the fetus and subsequently may be responsible for the increased susceptibility of newborns and young infants to respiratory infections [12]. This premise could explain the higher incidence of influenza in Most compared to Ceske Budejovice.

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Table 4. Impact of OH-PAHs to Respiratory Diseases

PAH	Disease											
	J00	J01	J02	J03	J04	J06	J11	J18	J20	J44	J45	J00-J99
	All (n=248)											
2-OH-NAP	-0.01	0.10	-0.03	0.04	0.08	0.15	0.15	-0.01	-0.01	-0.08	-0.03	0.14
1-OH-NAP	0.01	-0.02	0.03	0.05	-0.19 *)	0.09	-0.01	0.03	-0.05	-0.05	-0.05	0.00
2-OH-FLUO	0.00	-0.10	0.16 *)	0.00	0.05	0.19 *)	-0.02	-0.04	0.02	-0.14	-0.06	0.15
2-OH-PHEN	0.02	-0.15	0.23 **)	-0.03	0.00	0.10	0.04	0.07	0.10	-0.18 *)	-0.13	0.17 *)
3-OH-PHEN	-0.02	-0.06	0.22 **)	-0.03	0.08	0.12	0.05	0.00	0.18 *)	-0.12	-0.06	0.21 **)
1-OH-PHEN	0.01	-0.08	0.26 ***)	-0.07	0.02	0.11	0.02	0.03	0.15 *)	-0.17 *)	-0.09	0.17 *)
9-OH-PHEN	0.03	-0.06	0.17 *)	-0.04	0.02	0.11	0.11	0.03	0.12	-0.08	-0.05	0.19 *)
4-OH-PHEN	0.05	0.05	0.15	-0.03	-0.07	0.10	0.05	0.04	0.17 *)	-0.03	-0.02	0.17 *)
1-OH-PYR	0.02	-0.18 *)	0.21 **)	0.02	-0.04	0.11	0.06	0.05	0.03	-0.13	-0.09	0.15
	Ceske Budejovice (n=130)											
2-OH-NAP	-0.13	0.09	-0.06	0.02	0.05	0.21 *)	0.08	0.09	-0.31 **)	-0.06	-0.02	-0.02
1-OH-NAP	0.12	-0.07	-0.02	-0.04	-0.18	0.02	0.01	0.17	-0.02	-0.06	-0.07	0.03
2-OH-FLUO	0.11	-0.09	0.22 *)	0.07	0.04	0.13	-0.06	0.15	-0.03	-0.14	-0.06	0.18
2-OH-PHEN	0.21	-0.23 *)	0.33 **)	0.05	-0.08	0.02	-0.06	0.10	0.03	-0.21 *)	-0.17	0.18
3-OH-PHEN	0.15	-0.10	0.35 ***)	0.12	0.11	0.04	-0.07	0.05	0.20	-0.12	-0.05	0.28 **)
1-OH-PHEN	0.20	-0.11	0.30 **)	-0.01	0.01	0.04	-0.06	0.03	0.17	-0.19	-0.10	0.2
9-OH-PHEN	0.20	-0.05	0.16	-0.05	-0.01	0.10	0.09	0.08	0.09	-0.07	-0.05	0.23 *)
4-OH-PHEN	0.23 *)	-0.16	0.15	-0.01	-0.06	0.04	-0.06	0.12	0.15	0.00	0.00	0.23 *)
1-OH-PYR	0.17	-0.20	0.26 *)	0.05	-0.06	0.15	0.06	0.09	-0.01	-0.14	-0.10	0.20
	Most (n=118)											
2-OH-NAP	0.05	0.15	-0.02	0.01	0.08	0.09	0.12	-	0.21	-	-	0.21
1-OH-NAP	-0.11	0.05	0.08	0.12	-0.21	0.15	-0.05	-	-0.10	-	-	-0.06
2-OH-FLUO	-0.23 *)	-0.11	0.08	-0.13	0.05	0.28 *)	-0.10	-	0.04	-	-	0.04
2-OH-PHEN	-0.25 *)	-0.02	0.14	-0.13	0.05	0.16	-0.01	-	0.11	-	-	0.09
3-OH-PHEN	-0.28 *)	0.03	0.11	-0.20	0.03	0.19	-0.04	-	0.09	-	-	0.06
1-OH-PHEN	-0.30 **)	-0.02	0.22	-0.16	0.00	0.18	-0.05	-	0.07	-	-	0.06
9-OH-PHEN	-0.25 *)	-0.07	0.18	-0.05	0.05	0.11	0.09	-	0.13	-	-	0.07
4-OH-PHEN	-0.23 *)	-0.06	0.14	-0.08	-0.11	0.16	0.00	-	0.14	-	-	0.04
1-OH-PYR	-0.26 *)	-0.13	0.15	-0.03	-0.03	0.05	0.01	-	0.05	-	-	0.01

Beta coefficient results of regression by PAH-OH - *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

Acute pharyngitis (J02) was increased by 2-0H-FLUO, 2-OH-PHEN, 3-OH-PHEN, 1-OH-PHEN 9-OH-PHEN, and 1-OH-PYR; acute respiratory infections (J06) by2-OH-FLUO; acute bronchitis (J20) by 3-OH-PHE, 1-OH-PHEN, 4-OH-PHEN; respiratory diseases (J00-J99) by 2-OH-PHEN, 3-OH-PHEN, 1-OH-PHEN 9-OH-PHEN, 4-OH-PHEN. When our study is separated by location, in Ceske Budejovice acute pharyngitis (J02) was increased by 2-0H-FLUO, 2-OH-PHEN, 3-OH-PHEN, 1-OH-PHEN, and 1-OH-PYR;

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respiratory diseases (J00-J99) by, 3-OH-PHEN, 9-OH-PHEN, 4-OH-PHEN. In Most was effect observed only for acute respiratory infections (J06) for 2-OH-FLUO.

When we checked the effect of analyzed OH-PAH metabolites, we observed increased incidence of acute pharyngitis, acute respiratory infections, acute bronchitis and all respiratory diseases. When the sample of children is separated to Ceske Budejovice, effect is seen only for acute pharyngitis and all respiratory diseases, in Most only for acute respiratory infections.

Our results are affected by the number of children in each location. We may probably interpret our results as pilot study, indicating possible relationship between OH-PAHs metabolites and respiratory diseases in 2 years old children. 4. Conclusion

As already mentioned, our results seem to be peculiar, as the air pollution in both locations during the prenatal period as well as up to 2 years of age of our cohorts is similar. No significant differences were also observed between the lifestyle of the mothers living in Most during their pregnancy compared to mothers from Ceske Budejovice. The most significant increase from OH-PAH metabolites was observed for 1-OH-NAP. Comparing OH-PAHs in urine between newborns and 2 years old children. OH-PAHs level is approx. twice as high in 2 years old children. As PAHs exposure is immunotoxic, increase exposure may increase also respiratory diseases.

This outcome seems to be in an agreement with our previous idea that differences in the metabolism of PAHs in newborns in Most may be the outcome of the load of air pollution (one of the most polluted districts in Europe in 1970s and 1980s) which already affected their parents. We assume that the population in the air-polluted mining areas in previous years can carry some long-term changes (maybe alterations in the information on genetic level) which can also be connected with the alteration of the metabolism of PAHs [14]. To further confirm this premise, another collection of urine samples in Most is planned.

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Ethical Approval: The study was conducted according to guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Faculty of Health and Social Sciences, University of South Bohemia in Ceske Budejovice, Czech Republic, in June 30, 2017.

Consent to Participate: Informed consent was obtained from the parents of all subjects involved in the study.

Consent to Publish: All authors approved this text.

Conflict of Interest: The authors declare no conflict of interest.

Data availability statement: All data are available in our paper or from the corresponding author on reasonable request.

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