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Factors affecting the 27K DNA methylation pattern in asthmatic and healthy children from locations with various environments

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ABSTRACT

Gene expression levels are significantly regulated by DNA methylation. Differences in gene expression profiles in the populations from various locations with different environmental conditions were repeatedly observed.

In this study we compare the methylation profiles in 200 blood samples of children (aged 7–15 years) with and without bronchial asthma from two regions in the Czech Republic with different levels of air pollution (a highly polluted Ostrava region and a control Prachatice region). Samples were collected in March 2010 when the mean concentrations of benzo[a]pyrene (B[a]P) measured by stationary monitoring were $10.1 \pm 2.4 \text{ ng/m}^3$ in Ostrava Bartovice (5.6 times higher than in the control region). Significantly higher concentrations of other pollutants (benzene, NO_2 , respirable air particles and metals) were also found in Ostrava. We applied the Infinium Methylation Assay, using the Human Methylation 27K BeadChip with 27,578 CpG loci for identification of the DNA methylation pattern in studied groups.

Results demonstrate a significant impact of different environmental conditions on the DNA methylation patterns of children from the two regions. We found 9916 CpG sites with significantly different methylation (beta value) between children from Ostrava vs. Prachatice from which 58 CpG sites had differences >10%. The methylation of all these 58 CpG sites was lower in children from polluted Ostrava, which indicates a higher gene expression in comparison with the control Prachatice region. We did not find a difference in DNA methylation patterns between children with and without bronchial asthma in individual locations, but patterns in both asthmatics and healthy children differed between Ostrava and Prachatice. Further, we show differences in DNA methylation pattern depending on gender and urinary cotinine levels. Other factors including length of gestation, birth weight and length of full breastfeeding are suggested as possible factors that can impact the DNA methylation pattern in future life.

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Abbreviations: 5-mC, 5-methyl-cytosine; ACSL3, CoA synthetase long-chain family member 3 gene; ADRB2, beta-2 adrenergic receptor; AHRR, aryl hydrocarbon receptor repressor; B[a]P, benzo[a]pyrene; BCD plates, plates with bisulfite-converted DNA samples; BH *q*-value, Benjamini–Hochberg *q*-value; CYP1A1, cytochrome P450 1A1; GFI1, growth factor independent 1 transcription repressor; IFN γ , interferon γ ; iNOS, inducible nitric oxide synthase; LBW, low birth weight; LINE-1, long interspersed nucleotide element-1; MAGE-1, melanoma-associated antigen 1; PAHs, polycyclic aromatic hydrocarbons; PCA, principal component analysis; PCBs, polychlorinated biphenyls; PLS, partial least square; PM, particulate matter; POPs, persistent organic pollutants; Sat2, pericentromeric satellite 2; ST *q*-value, Storey–Tibshirani *q*-value; SINE, short interspersed nucleotide elements; TCDD, 2,3,7,8-tetrachlorodibenzo-dioxin; VLBW, very low birth weight.

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1. Introduction

Methylation of cytosine ring at position 5 in CpG sites of DNA leading to formation of 5-methyl-cytosine (5-mC) is an important event in epigenetic changes of cells linked to the control of gene function [1]. Studies on nuclear DNA methylation changes in white blood cells are rapidly emerging, and thus, methylation profiles can serve as a useful biomarker. Molecular epidemiologic studies have reported associations between global methylation and several different cancers as well as selected factors including age, gender, race, various environmental exposures or life style factors [2].

The level of DNA methylation and changes of methylation profile can be identified by various methods [3,4], some of which provide quantitative information about global DNA methylation, while others allow qualitative data about gene-specific DNA methylation. Global DNA methylation is most commonly quantified by

analyses of highly repetitive sequences like long interspersed nucleotide elements (LINE, e.g. LINE-1), short interspersed nucleotide elements (SINE, e.g. Alu), and pericentromeric satellites (Sat2). For gene-specific methylation, e.g., array methodologies, including the Illumina Infinium Human Methylation 27K or 450K BeadChips, may be used. The results from these chips provide information on methylation status at each specific CpG site [5]. Generally, obtained data can vary by assay types according to their focus on various CpG sites in the genome [6,7]. Moreover the type of tissue, even different blood cell types can affect global methylation profile [8], which underlines the functional significance of methylation [9].

Age is an important factor which affects various biomarkers including global DNA methylation during life. Some methylation modifications that regulate gene expression are inheritable, but generally DNA methylation is removed during zygote formation and re-established through successive cell divisions during development of the different tissues. Later, this modification can be inherited from cell to cell [10]. According to many studies, global DNA methylation is inversely associated with age. Differences are visible also between genders (global DNA methylation is higher in males than in females [2]), and ethnic differences are also apparent [11].

This inverse association between age and global DNA methylation has also been observed in a study focusing on associations between methylation and child environmental tobacco smoke exposure, birth size, postnatal weight and height, and adult alcohol intake and smoking. Genomic DNA methylation in the repeatedly studied cohort of women born and living in New York City, showed inverse association between prenatal tobacco smoke exposure and Sat2 methylation in these women, and a non-significant association with LINE-1 or Alu methylation [7]. Specific loci that showed significant differences between smokers and non-smokers were recently identified by Infinium Illumina Methylation 27K arrays [12]. A set of genes, *AHRR* (aryl hydrocarbon receptor repressor), *CYP1A1* (cytochrome P450 1A1), and *GFI1* (growth factor independent 1 transcription repressor) with methylation differences present at birth in children whose mothers smoked during pregnancy were each identified by Infinium Illumina Methylation 450K arrays [13].

Other recent studies reviewed the association between level of DNA methylation and exposure to environmental pollutants [2,14,15]. Several studies suggest that exposure to metals, like arsenic, cadmium, chromium, nickel or lead can also affect the epigenome [16]. Rusiecki et al. [17] found an inverse correlation between global DNA methylation of Alu, but not of LINE-1 repeated elements, and plasma persistent organic pollutants (POPs) in a group of Greenlandic Inuits. A study focused on the changes in DNA methylation patterns in subjects exposed to low doses of benzene showed an association with decreased methylation of LINE-1 and Alu sequences, and with hypermethylation of *p15* and hypomethylation of melanoma-associated antigen 1 (*MAGE-1*) genes [18]. Long-term exposure to PM₁₀ (particulate matter with aerodynamic diameters <10 µm) was negatively associated with methylation in both Alu and LINE-1 repeated elements and demethylation within the promoter of inducible nitric oxide synthase gene (*iNOS*), leading to its increased expression [19]. In a recent study, *iNOS* methylation was also decreased after acute exposure to particulate matter less than 2.5 µm in diameter (PM_{2.5}) and black carbon [20]. Results of another study that followed non-smoking women during pregnancy suggests that prenatal air PAH exposure (median 2.47 ng/m³ by personal monitoring) is associated with lower global methylation in umbilical cord blood cells and confirms that global methylation levels are positively associated with the presence of detectable DNA adducts in cord blood [21].

All environmental exposures that occur during preconception or in utero, in early and in adult life, may result in epigenetic deregulation and development of various disorders [22]. Although cancer is the most frequently studied disease in this context, several recent studies focused also on the connection between prenatal exposure to various chemicals and asthma. A recent study suggests acyl-CoA synthetase long-chain family member 3 (*ACSL3*) as a candidate biomarker of PAH exposure and a putative predictor of PAH-associated childhood asthma [23]. Another study suggests methylation in beta-2 adrenergic receptor (*ADRB2* 5'-UTR) as a biomarker of asthma severity and risk for NO₂ exposure [24]. Baccarelli et al. reported increased lower promoter methylation in *IL-6* and *iNOS* genes in nasal cell DNA samples of asthmatic children that fractionally exhaled nitric oxide [25] and Torrone et al. suggest to use buccal cell DNA as a non-invasive method for investigation of methylation in the two asthma genes (interferon γ (*IFN γ*) and *iNOS*) [26].

Gene expression profiles in groups of children and adults living in different regions in the Czech Republic were analyzed [27–30]. Because the level of gene expression is also regulated by DNA methylation, we hypothesized that the level of DNA methylation would vary with significantly different types of air pollution. In this study we verified this statement using Human Methylation 27K BeadChips for 27,578 CpG loci by Illumina and compared the methylation profile in groups of asthmatic and healthy children from a highly polluted region (Ostrava) and a control region (Prachatice). Furthermore, we analyzed the association between bronchial asthma as well as other factors (gender, smoking, length of gestation, birth weight and length of full breastfeeding) and the DNA methylation profile in the studied groups.

2. Materials and methods

2.1. Study population

The study group was recruited from a set of 400 children (200 with bronchial asthma + 200 healthy controls) in which the gene expression levels [28,30], the frequency of micronuclei (MN), markers of oxidative damage to DNA, proteins and lipids and impact of individual susceptibility were previously determined [31]. Our study subset includes 100 children (50 asthmatics and 50 controls) from the highly polluted Ostrava region and 100 children (50 asthmatics and 50 controls) from the Prachatice control region. The average age of all 200 participants was 11.6 ± 2.2 years [median (range): 12.0 (7.3 – 15.4) years]. The study group included 111 boys and 89 girls. Blood and urine samples were collected for the laboratory analyses in this study.

Personal medical history (e.g. birth weight, length of gestation, full breastfeeding, body weight and height, disease, allergy) and life-style (e.g. smoking of parents) were obtained from all participants from questionnaires. The parents of the children signed an informed consent form and could cancel their children's participation at any time during the study, according to the Helsinki II declaration. The ethical committee of the Institute of Experimental Medicine AS CR in Prague approved the study.

2.2. Description of localities

All children in the highly polluted region were recruited from the Radvanice/Bartovice district in the city of Ostrava. Ostrava is the third largest city in the Czech Republic with approximately 300,000 inhabitants. The city is located in the second largest urban agglomeration after Prague, and is located close to the Polish border. The high concentration of heavy industry has a negative impact on the air quality and human health in this region. The Radvanice/Bartovice district belongs to the most polluted places, where the mean concentrations of benzo[a]pyrene (B[a]P) during the study (March 2010) was 10.1 ng/m³ (data obtained from stationary monitoring). By comparison, concentrations of B[a]P in Ceske Budejovice, the monitoring station nearest to the Prachatice control region, were 1.8 ng/m³.

The control Prachatice region (28 children from Prachatice, 45 children from Vimperk, 13 children from Netolice and 14 children from Vlachovo Brezi): Prachatice is a town in the South Bohemian region in the Czech Republic with only 11,500 inhabitants. Vimperk, Netolice and Vlachovo Brezi are small towns/villages near Prachatice with 7800; 2700 and 1700 inhabitants, respectively. All these towns are surrounded by rural areas and local heating may be considered as the main source of air pollution.

2.3. Monitoring of air pollution

The concentrations of all studied pollutants (B[a]P, benzene, NO₂, PM_{2.5}, PM₁₀ and metals: arsenic, cadmium, nickel a lead) for March 2010 were obtained from the tabular overviews of the Czech Hydrometeorological Institute [32]. Air pollution in Ostrava was measured by the Health Institute Ostrava in Ostrava Bartovice. The data for the control region were obtained from the nearest monitoring station in Ceske Budejovice (a regional city located 35 km east from Prachatic) where the measurement was performed by the Czech Hydrometeorological Institute.

2.4. DNA extraction and bisulfite treatment

Genomic DNA was extracted by the salting out procedure [33] adapted by Schumczzerova et al. [34] from whole venous blood collected into EDTA tubes. Genomic DNA (gDNA, 500 ng) was treated overnight with sodium bisulfite using Zymo EZ DNA Methylation Kit for the conversion of unmethylated cytosines to uraciles while methylated cytosines remained unchanged. Plates with bisulfite-converted DNA samples (BCD plates) were stored at –20 °C until use.

2.5. The Infinium Methylation Assay and QA/QC

Bisulfite-converted DNA was processed according to the manufacturer's protocol (Infinium Assay for Methylation Manual Workflow) including the enzymatic fragmentation, precipitation, resuspension and overnight hybridization followed by washing and BeadChip staining. Infinium Human Methylation 27K BeadChips were used in this study (Illumina, catalog number WG-311-2201), each chip for 12 samples. The chips allow interrogating 27,578 CpG sites in 14,495 genes per sample at single-nucleotide resolution. The methylation status at each CpG site was estimated by measuring the intensity of the pair of methylated and unmethylated probes. Quality Assurance/Quality Control was performed by replication of one randomly chosen experiment (5366044015.L) which was repeated as experiment 5366044020.L located on another chip. The concordance between the two experiments was very high, (Pearson's correlation coefficient $r = 0.998$, $p < 0.001$; coefficient of determination $R^2 = 0.996$). This guarantees highly reproducible data and allows both replication of analysis of one sample and comparison of the results between chips. The coefficient of variation (CV) for these two experiments was less than 10% for 56% of CpG sites. The CV exceeded 50% only for 0.4% (=113) CpG sites.

2.6. Cotinine assay

Urinary cotinine levels were analyzed by radioimmunoassay [35]. Children with cotinine levels greater than 450 ng/mg of creatinine were considered active smokers. Earlier, it was reported that some of study children were smokers [31]. Partial least square analysis (PLS) with cotinine as a covariate shows that children with very high cotinine levels (625.9 – 3345.1 ng cotinine/mg creatinine) were outstanding. Therefore, the 5 smokers were excluded from most of the analyses.

2.7. Statistical analyses

The basic statistic analyses of the levels of air pollutants in localities and groups by age, gender, smoking, length of gestation, birth weight and length of fully breastfeeding were performed using the IBM SPSS Software (Chicago, IL, USA), version 20.0. Depending on the distribution of the data, *t*-test or the nonparametric Mann–Whitney Sum *U*-test were used for comparison of individual groups. Advanced statistical analyses related to methylation were carried out by Genedata AG (Switzerland) using Genedata Expressionist® version 7.5.

Beta values for the determination of the level of methylation as the ratio of the fluorescent signals from the methylated vs. unmethylated sites were calculated by the Illumina software and were not further normalized. Most of the beta values were either <0.2 or >0.7 but the results show a high variance among the individuals. Striking differences in the distribution of beta values per region or disease could not be observed. As example, the experiment 5366044015.A from a child from the Ostrava region with asthma shows 62% of beta values <0.2 and 21% with beta values >0.7. The experiment 5366044015.H from a child from the control region without asthma shows 62% with beta values <0.2 and 20% with beta values >0.7. Analyses were performed to study the distribution of beta values and the variation of methylation across all samples, in particular to detect differences in methylation patterns between children from the different regions and children with or without asthma.

Principal component analysis (PCA) was performed to identify directions of maximum variance in the data using the covariance matrix. PCA with the beta values was applied to detect potential regional effects (polluted Ostrava region vs. Prachatic control region); for the evaluation of the association between bronchial asthma and methylation pattern and effect of gender on methylation. Partial least squares (PLS) analysis was performed for searching for variables that explain covariance, in particular to evaluate the impact of cotinine level, length of gestation, birth weight and length of full breastfeeding on methylation. PLS was performed using 3 latent factors and cotinine (ng/mg), length of gestation (weeks), birth weight (grams), and length of fully breastfeeding (months) as response variables. For PCA and PLS imputation was performed using the row means.

Beta values are not normal distributed across an experiment but normal distribution was found for each site across all samples, therefore, ANOVA and *t*-tests including the estimation of false discovery rates using the Storey–Tibshirani (ST *q*-value) and the Benjamini–Hochberg methods (BH *q*-value) were used to study the region and/or disease groups. Because the ST *q*-value is only applicable if the fraction of truly null features can be estimated, this value could not be determined in all analyses. ANOVA and *t*-tests were also performed with *M*-values which were transformed from the beta values using the formula $M = \log_2(\text{beta}/(1 - \text{beta}))$. However, the results did not differ significantly from the results obtained with the beta values.

In addition, ranking tests were also applied (Wilcoxon and Kruskal–Wallis, respectively) (data not shown). In order to exclude potential confounding effects, linear models were applied to detect region and/or disease effects/associations. The age of the children, the birth weights, the gestation and the breastfed length as covariates, and the gender as factor were included in the models. In one type of the models smoking in the family was also included as factor and in another the cotinine level as covariate. All these tests produced very similar or even identical results compared with the results obtained by ANOVA and *t*-test.

Most analyses were performed after exclusion of samples like smokers or not unambiguously matching gender samples. The replicated experiment (5366044015.L) used for QA/QC was excluded from all analyses, therefore averaging of beta values across replicates was not necessary.

All CpG sites which are presented in the Results were verified by comparison with the list of non-specific probes downloaded from <http://www.sickkids.ca/Research/Weksberg-Lab/Publications/index.html> [36].

3. Results

This study compares the DNA methylation patterns of the children from the two locations with significantly different types of air pollution. The main characteristics of the selected pollutants (B[a]P, benzene, NO₂, PM_{2.5}, PM₁₀ and metals: arsenic, cadmium, nickel a lead) were measured in both locations by the nearest stationary monitoring station (Ostrava Bartovice for Ostrava and Ceske Budejovice for Prachatic) in March 2010 and are summarized in Table 1. This overview shows that the frequency of measurements ranges from 5 to 31 times per month for all monitored pollutants, excluding benzene in Ostrava with a total of 94 measurements pooled from four Ostrava monitoring stations. The concentration of B[a]P and metals were measured less frequently in Ostrava Bartovice, the most polluted place in the Czech Republic, than in the control region. The mean and median values for all pollutants were higher in Ostrava, which differed significantly from the control region. The most marked differences in the mean concentration of the pollutants were observed for B[a]P (5.6-fold higher concentration in Ostrava) and metals in PM₁₀: As, Cd, Ni, Pb (4.9-fold, 6.3-fold, 7.3-fold and 7.14-fold higher concentration in Ostrava, respectively).

Moreover, we compared changes in concentrations of PM₁₀ during the entire life, including the prenatal period of the study children. Concentration data are available from the Czech Hydrometeorological Institute website from 1997 to present for both locations (details not shown). Data were divided into two periods: I. 1997–2003 (during this period all prenatal exposures of the children involved in this study occurred) and II. 2004–2010. We observed a significantly higher exposure to PM₁₀ in the second period in Ostrava. The increase in mean concentrations was almost 30% compared to the first period ($p < 0.05$). However, there was no significant difference in levels between periods in the control region ($p = 0.76$).

Table 2 summarizes the main characteristics of the study group (200 participants) and individual subgroups analyzed in this study (mean ± standard deviation (SD); [median: (minimum–maximum)]) for the urinary cotinine level, length of gestation, birth weight and length of full breastfeeding. Urinary cotinine levels were in a wide range (1.8–3345.1 ng cotinine/mg creatinine), especially because of five children with cotinine levels comparable with active smokers. This resulted in increased mean cotinine concentrations in comparison with median values. Moreover, the characteristics related to smoking obtained from

Table 1

Mean month concentrations of the selected air pollutants obtained by stationary monitoring in the studied locations in March 2010.

Exposure variable	Location (nearest monitoring station)						<i>p</i>
	Ostrava (Ostrava Bartovice)			Prachatice (Ceske Budejovice)			
	<i>N</i>	Mean ± SD	Median (range)	<i>N</i>	Mean ± SD	Median (range)	
<i>A. Selected pollutants</i>							
B[a]P (ng/m ³)	5	10.1 ± 2.4	9.2 (8.2–13)	10	1.8 ± 0.7	1.7 (0.8–2.9)	=0.001
Benzene (μg/m ³)	94	4.8 ± 3.5 ^a	3.7 (1.2–20)	31	1.0 ± 0.4	1.0 (0.4–1.8)	<0.001
NO ₂ (μg/m ³)	31	27.4 ± 8.9	25 (10.9–45.2)	31	18.6 ± 6.9	16.6 (6.6–32.6)	<0.001
PM2.5 (μg/m ³)	31	40.2 ± 17.2	39.2 (11.4–75.5)	20	16.4 ± 8.5	14.0 (4.9–33.9)	<0.001
PM10 (μg/m ³)	31	57.6 ± 21.0	55.6 (24.5–99.1)	29	21.9 ± 10.5	21.0 (2–43)	<0.001
<i>B. Metals in PM10</i>							
As (ng/m ³)	5	4.9 ± 5.7	3.4 (0.6–14.8)	20	1.0 ± 0.7	0.8 (0.1–2.5)	=0.019
Cd (ng/m ³)	5	1.9 ± 0.0	1.9 (1.9–1.9)	20	0.3 ± 0.2	0.3 (0.1–0.7)	<0.001
Ni (ng/m ³)	5	4.4 ± 2.7	2.5 (2.5–7.6)	20	0.6 ± 0.3	0.6 (0.2–1.3)	=0.001
Pb (ng/m ³)	5	49.3 ± 46.3	25.0 (22.9–131)	20	6.9 ± 5.7	4.2 (0.8–16.7)	=0.001

N, number of individual measurements in March 2010; SD, standard deviation.

^a Data from four various locations in Ostrava.

questionnaires (data not shown) indicate that up to 8 smokers shared the same household with children in this study and at least 1 smoker was presented in 46% of all families (41 from Ostrava and 51 from Prachatice). Early life parameters, which are factors potentially affecting the DNA methylation pattern, show that the groups were relatively equal in means and medians of these parameters. Length of gestation was normal (≥ 37 weeks) in 94% of children. Low birth weight (LBW) (less than 2500 g) was found in 11 children and very low birth weight (VLBW) (less than 1500 g) was reported in 2 cases. Data on full breastfeeding showed that 35 children were not breastfed, 76 were breastfed for up to 3 months and 89 for more than 3 months.

The principal findings showing the impact of the studied parameters on the DNA methylation pattern are summarized in Figs. 1, 1S (in the Supplementary material) and 2. Fig. 1 includes graphs a–d (a – comparison between regions Ostrava vs. Prachatice in pooled data, b – comparison between asthmatics and no asthmatics in pooled data, c – comparison between boys and girls for CpG sites located on both autosomes and gonosomes, d – comparison between boys and girls for CpG sites located on autosomes only), which were generated by PCA. Data showed in Fig. 1(a) shows that there is a tendency in DNA methylation pattern toward a separation by region. In contrast, in Fig. 1(b) no clear association between disease and methylation pattern can be found. However, if we take into consideration the effect of region, we can observe differences in methylation between asthmatics from Ostrava and from the control region Prachatice. Similarly, comparison between no asthmatics from both regions (Ostrava vs. Prachatice) also shows a tendency in DNA methylation pattern toward a separation by region. In opposite, comparison between asthmatics and no asthmatics from the Ostrava region, as well as from the Prachatice region shows no clear disease association with methylation pattern (Figure S1 – in the Supplementary material). Fig. 1(c) and (d) shows the effect of gender, which is strongly associated with differences in DNA methylation in gonosomes. Boys and girls are mostly separated into two clusters with the exception of five samples that were excluded from further analyses [Fig. 1(c)]. Fig. 1(d) demonstrates no trend for clustering by gender, when we analyzed the samples without gonosomes. Some specific CpG sites differed between genders in the level of methylation in the autosomes (data not shown). Eight specific sites were selected with differences in DNA methylation between gender >0.10 . Six of them (CpG loci in *TLE1*, *GLUD1*, *C6orf68*, *TDGF1*, *LRRC2* and *RAB9P1* genes) with highest difference were identified previously as technical artifacts (sites whose probes cross-hybridize either to X chromosome or both gonosomes); however, the previously described CpG site in the *DDX43* gene is not considered the technical artifact [36]. Moreover, in our study we

identify another CpG locus in the *SLC9A2* gene, which also does not seem to be the technical artifact (based on the BLAST search and the list of non-specific probes).

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mrfmmm.2013.02.003>.

Fig. 2 includes graphs a–d (a – association between cotinine level and DNA methylation pattern, b – association between length of gestation and DNA methylation pattern, c – association between birth weight and DNA methylation pattern, d – association between length of full breastfeeding and DNA methylation pattern), which were generated by PLS. The impact of cotinine levels [Fig. 2(a)] shows outstanding DNA methylation pattern in five children (circled) characterized by very high cotinine levels (626–3345 ng cotinine/mg creatinine). These children were excluded from most of the analyses in this study. The gradient in DNA methylation pattern was still visible for children with cotinine levels 1.8–309 ng/mg creatinine (data not shown). The length of gestation, birth weight and time of full breastfeeding are probably other factors that can affect the DNA methylation pattern for future life [Fig. 2(b–d)].

Table 3 complements the results shown in Fig. 1(a–b) and Figure 1S (a–d) in the Supplementary material with the results of ANOVA and *t*-test for individual groups. Using these tests, we obtained a number of CpG sites with significantly different methylation levels (beta values) between tested groups. All obtained CpG sites were checked and corrected to non-specific probes and polymorphic CpG sites. The overlap between results of ANOVA and *t*-test in comparison of regions with significantly different methylation (beta values) was 9916 CpG sites, and with significantly different methylation (beta values) and difference $>10\%$ was 58 CpG sites. These results supported the PCA data (strong association between region and DNA methylation pattern). Data in Table 3 are graphically supported by Venn diagram in Figure S2 (in the Supplementary material). The list of 58 gonosomal CpG loci analyzed as overlap between ANOVA and *t*-test results is presented in Table S1 (in Supplementary material), where the sites are divided to 21 CpG loci differing between regions observed simultaneously in asthmatics and controls and 37 CpG loci differing between regions observed in asthmatics only (this separation is also visible from Venn diagram in Figure S2 showed in Supplementary material). The methylation of all these 58 CpG sites was lower in children from the polluted Ostrava region.

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mrfmmm.2013.02.003>.

Table 4, part A, shows selected data from Table S2 (individual CpG sites in Supplementary material) with genes relevant to

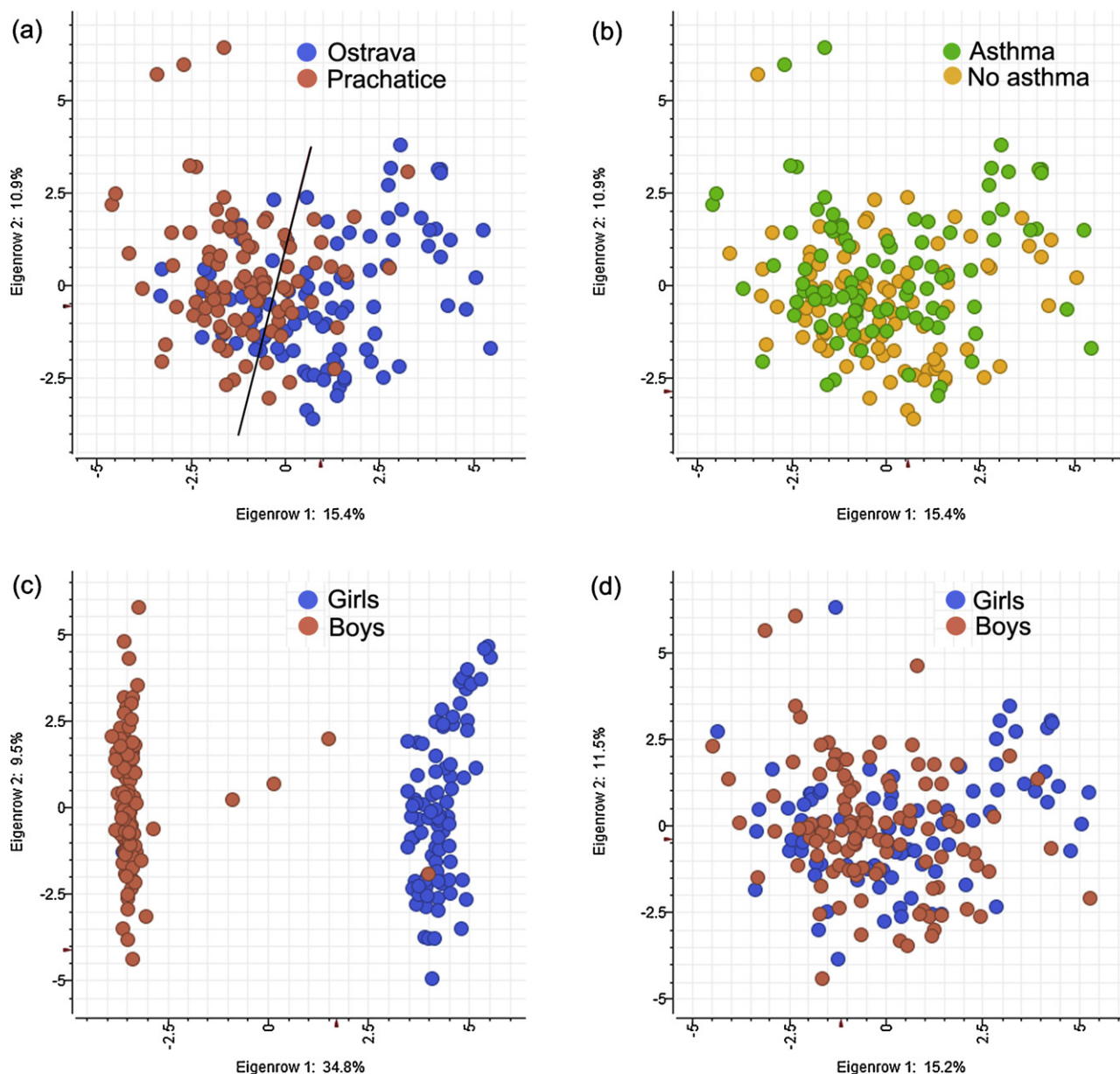


Fig. 1. Impact of the studied parameters on the DNA methylation pattern analyzed by PCA. (a) Comparison between regions (Ostrava vs. Prachatice) in pooled data shows a tendency in DNA methylation pattern toward a separation by region; (b) comparison between asthmatics and no asthmatics in pooled data from both regions shows no clear association between disease and methylation pattern; (c) comparison between boys and girls for CpG sites located on both autosomes and gonosomes shows clear separation by gender; (d) comparison between boys and girls for CpG sites located on autosomes only shows no effect of autosomal CpG sites on clustering by gender.

immune system, DNA-protein binding, signaling pathways and metabolism of xenobiotics. Part B shows results of differences in methylation of CpG sites which are presented in *ACSL3* and *ADRB2* genes previously published as candidate biomarkers of exposure and asthma [23,24].

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mrfmmm.2013.02.003>.

4. Discussion

Human studies focusing on the impact of air pollution on the DNA methylation pattern are still relatively scarce. To the best of our knowledge, no study analyzing at the same time both the effect of environments differing in ambient levels of carcinogenic B[a]P (IARC Group 1) [37] and benzene (IARC Group 1) [38] on

methylation, as well as the associations between concentration of PM or metals with epigenetic markers has been published. Even though it seems to be evident that environmental exposure plays an important role in position of epigenetic marks, none of the studies published so far that excluded the effect of tobacco-smoking used Illumina Infinium Human Methylation 27K or 450K BeadChips to investigate DNA methylation patterns. Thus, it is difficult to find relevant reports for comparison with our results. Significant differences in air pollution in both study locations were the main reason for the variance in DNA methylation patterns of children from those two regions.

A recent study examined the effect of prenatal ambient air pollutants (eight carcinogenic PAHs) on DNA methylation patterns in genes potentially associated with the asthma phenotype of the offspring, and suggested that the *ACSL3* gene could serve as a biomarker of PAH exposure and putative predictor of

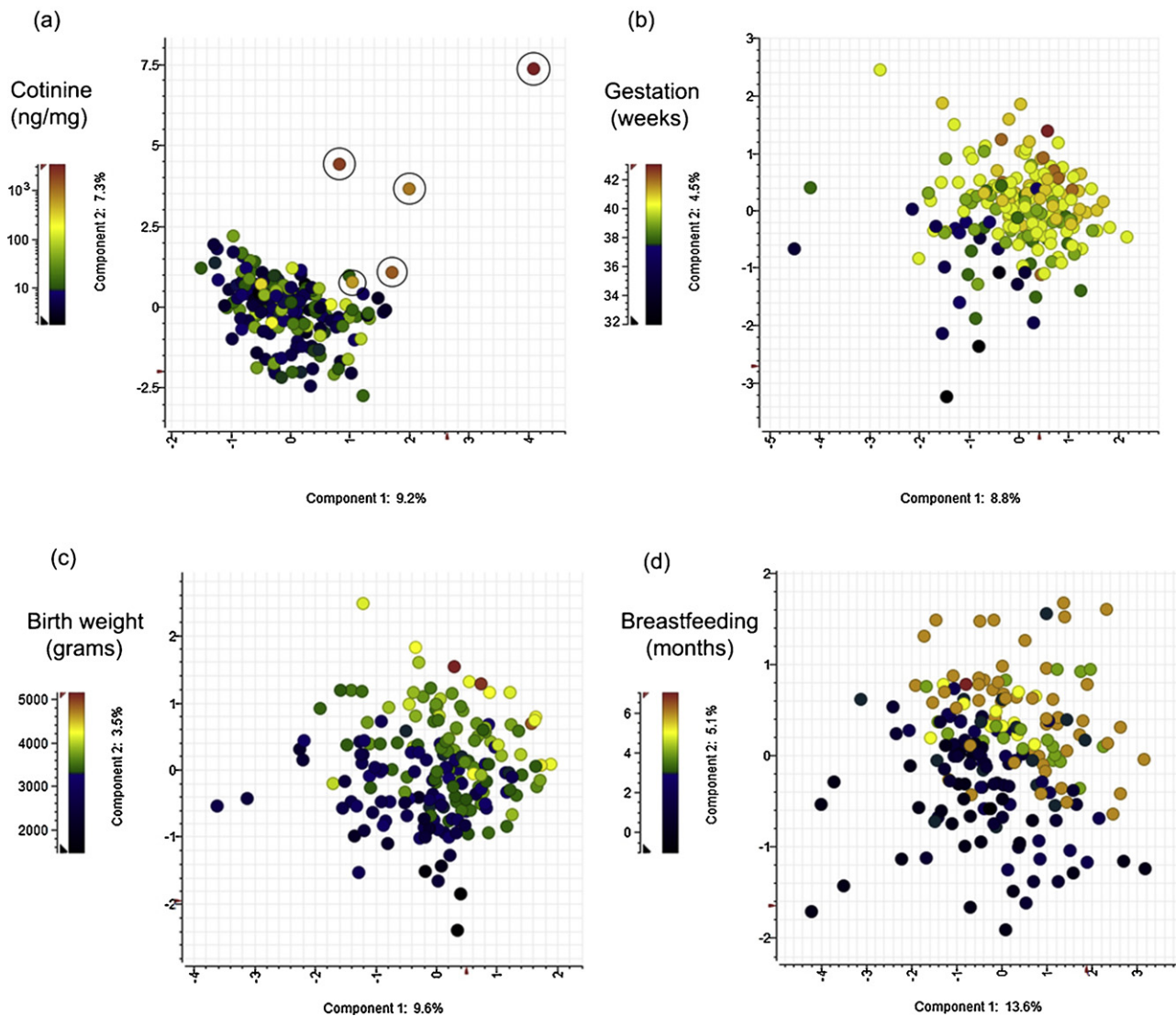


Fig. 2. Impact of the studied parameters on the DNA methylation pattern analyzed by PLS. (a) Various cotinine levels show outstanding DNA methylation pattern in five children (circled) characterized by high cotinine levels (625–3345 ng cotinine/mg creatinine); (b–d) various length of gestation (in weeks), birth weight (grams) and length of full breastfeeding (in months) shows gradient in DNA methylation pattern.

PAH-associated childhood asthma [23]. The authors made this observation in study groups with median exposure to carcinogenic PAHs of 2.3 ng/m³, which is almost 24 times lower than for the group of adults from Ostrava (median exposure 54.7 ng/m³) studied using personal monitoring [29]. In our study, we obtained information on methylation of only two specific CpG sites located in the *ACSL3* gene and two CpG sites located in the *ADRB2* gene, recently suggested as a candidate biomarker of asthma severity [24]. Despite this limitation, we observed a borderline significance for methylation of the cg22111003 CpG locus in the *ACSL3* gene for comparison of asthmatics with healthy controls from Ostrava. This result was not shown for comparison between asthmatics and healthy control from Prachatice. This observation, based only on one specific CpG site, supported previously suggested suitability of this gene as a biomarker of PAH exposure and PAH-associated childhood asthma [23]. Moreover, high concentrations of carcinogenic PAHs, including B[a]P, underline the uniqueness and suitability of the Ostrava region for human biomonitoring exploration. Concentrations of B[a]P, PM_{2.5} and PM₁₀ in our study were higher than standards for the exposure in the Czech Republic. However, we should also mention that

concentrations of metals (As, Cd, Ni, Pb) were lower than these standards.

In our study, the impact of bronchial asthma on DNA methylation pattern was not observed in a group of asthmatics pooled from both regions vs. controls. However when we divided this group by region, we concluded that asthmatic children differed in methylation pattern between regions. These results suggest a strong impact of region on the methylation pattern. This observation is in agreement with our previous study focused on gene expression profiles in the same groups that were followed in November 2008 when a distinct phenotype of asthma in children living in Ostrava compared to children living in Prachatice was observed [28,30]. Totally, we found 9916 CpG sites with significantly different methylation (beta value) between children from Ostrava vs. Prachatice from which 58 CpG sites had differences >10%. These 58 CpG sites are e.g. located in genes related to immune system, DNA-protein binding, metabolism of xenobiotics or signaling pathways. Moreover, we observed lower methylation in all of these 58 CpG sites in polluted Ostrava, which indicates a higher gene expression in comparison with the control Prachatice region. Even though, we obtained highly significant results for these unique CpG sites, we cannot

Table 2
Characteristics of the study subjects by urinary cotinine levels and by the early life parameters.

Group	N	Gender (boys/girls)	Cotinine/creatinine (ng/mg)	Length of gestation (weeks)	Birth weight (grams)	Full breastfeeding (months)
Mean $\pm$ standard deviation; [median: (minimum – maximum)]						
<i>Ostrava + Prachatice</i>						
All	200	111/89	63.0 $\pm$ 303.9; [9.3: (1.8 – 3345.1)]	39 $\pm$ 1.7; [40: (32 – 43)]	3311 $\pm$ 575; [3350: (1470 – 3350)]	3 $\pm$ 2.3; [3: (0 – 7)]
Asthmatics	100	55/45	59.7 $\pm$ 256.6; [8.9: (2.3 – 2115.2)]	39 $\pm$ 1.5; [40: (35 – 42)]	3236 $\pm$ 477; [3300: (1920 – 4350)]	3 $\pm$ 2.4; [2: (0 – 7)]
Controls	100	56/44	66.3 $\pm$ 346.0; [9.4: (1.8 – 3345.1)]	40 $\pm$ 1.8; [40: (32 – 43)]	3387 $\pm$ 652.5; [3425: (1470 – 5150)]	3 $\pm$ 2.2; [3: (0 – 6)]
<i>Ostrava</i>						
All	100	50/50	83.6 $\pm$ 369.9; [9.6: (2.9 – 3345.1)]	40 $\pm$ 1.4; [40: (35 – 43)]	3326 $\pm$ 521; [3350: (2150 – 4900)]	3 $\pm$ 2.3; [4: (0 – 6)]
Asthmatics	50	25/25	47.1 $\pm$ 194.7; [9.2: (2.9 – 1383)]	39 $\pm$ 1.5; [40: (35 – 42)]	3214 $\pm$ 439.8; [3325: (2150 – 4250)]	3 $\pm$ 2.3; [3: (0 – 6)]
Controls	50	25/25	120.1 $\pm$ 485.6; [11.2: (2.9 – 3345.1)]	39 $\pm$ 1.3; [40: (36 – 43)]	3438 $\pm$ 574; [3500: (2300 – 4900)]	4 $\pm$ 2.1; [4: (0 – 6)]
<i>Prachatice</i>						
All	100	61/39	42.4 $\pm$ 218.8; [8.9: (1.8 – 2115.2)]	39 $\pm$ 1.8; [40: (32 – 42)]	3297 $\pm$ 627; [3365: (1470 – 5150)]	3 $\pm$ 2.3; [2: (0 – 7)]
Asthmatics	50	30/20	72.3 $\pm$ 307.7; [8.0: (2.3 – 2115.2)]	39 $\pm$ 1.5; [40: (35 – 40)]	3258 $\pm$ 515.6; [3300: (1920 – 4350)]	3 $\pm$ 2.3; [2: (0 – 6)]
Controls	50	31/19	12.5 $\pm$ 12.6; [9.2: (1.8 – 76.9)]	39 $\pm$ 2.1; [40: (32 – 40)]	3336 $\pm$ 724.8; [3400: (1470 – 5150)]	3 $\pm$ 2.3; [2: (0 – 7)]

N, number of subjects.

explicitly suggest entire genes as candidate biomarkers of PAH exposure, because this observation was limited only to one CpG/gene.

Furthermore, based on data archives from stationary monitoring for PM10 we estimated that children from Ostrava are exposed to higher pollutant exposure conditions compared to lower prenatal air pollution exposure. It is likely that these differences in concentrations of PM10 also corresponded to significantly different exposures to PM2.5 and PAHs. This is in contrast to conditions in Prachatice where air pollution seems to be more stable over the years, and probably represents an important difference in comparison with children from Ostrava. Prenatal exposure plays a crucial role in development of the immune system and in early childhood respiratory illnesses [39]. Setting of DNA methylation pattern during the prenatal development is an important factor for future life [10]. Recently, Hochstenbach et al. [40] suggested that maternal exposure to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) and polychlorinated biphenyls (PCBs) lead to gender-specific epigenetic changes in the fetus, which subsequently influences immune functionality. Moreover, this exposure may represent a persistent effect on the genome. These facts support the idea that substantial changes in air pollution observed in our study could underline the negative effect of air pollutants and in the case of population from Ostrava Radvanice/Bartovice they could be considered as a possible factor responsible for the increase in the prevalence of asthma, shown in a previous study [41].

We observed differences in DNA methylation between boys and girls which are in agreement with repeatedly published data for global methylation analyzed by pyrosequencing in LINE-1 [42]. Due to the different method used in this study, our data demonstrate that differences between genders can be analyzed by comparison of gene specific DNA methylation pattern in all chromosomes including gonosomes, which are responsible for this variation. In our study we did not find the differences in methylation of autosomes between genders by PCA.

The effect of smoking in family on the DNA methylation pattern was another point of interest analyzed in this study. The role of tobacco smoking in changes of methylation in CpG sites was previously studied using 27 K [12,43] and 450K BeadChips [13]. Despite the assay similarity, there are important differences between the studies in the use of biological material (peripheral blood, placenta and cord blood), which leads to identification of different CpG loci or genes. Our study was focused mainly on a comparison of DNA methylation patterns in general, not on the association with some specific sites/genes, due to the fact that the study was not originally designed for studying of the effect of smoking and other factors.

The length of gestation and birth weight are two other studied factors which are usually correlated. Due to the progressive methylation during the prenatal development we can anticipate the differences in DNA methylation pattern in newborns in relation to these factors. In our study we analyzed differences in methylation associated with these factors in a group of children aged 11.6 years. The fact that obtained DNA methylation pattern reflects the length of gestation and birth weight also provides important evidence supporting the importance of progressive methylation during the prenatal period for future life. Last but not least, a factor which impacted DNA methylation pattern in children involved in this study was the length of full breastfeeding that, to the best of our knowledge, has not been studied yet, despite the fact that the effect of diet on DNA is central to many studies.

The application of Infinium Illumina Methylation 27K arrays was first reported in 2009 [44]. Several studies were published up to now, but some technical issues were reported recently (nonspecific probes detect up to 6–10% CpG sites, the above mentioned cross-hybridization of the autosomal sex-specific probes to the X chromosome and overlap of a small number of microarray probes

Table 3Differences in methylation between the studied groups; comparison by ANOVA and *t*-test.

Groups	Studied variable	N	Test	p-value	ST q-value	Difference in methylation ^a	Difference in methylation ^b
Ostrava, Prachatice Asthmatics, Controls	Region and disease	190	ANOVA	<0.080	<0.05	10,738	114
Ostrava vs. Prachatice Asthmatics vs. Controls	Region Disease	190	<i>t</i> -test	<0.052	<0.05	9916	58
		190	<i>t</i> -test	<0.001	<0.05	22	0
Asthmatics vs. Controls Prachatice Asthmatics vs. Controls Ostrava	Disease Disease	95	<i>t</i> -test		<0.05	0	0
		95	<i>t</i> -test		<0.05	0	0
Ostrava vs. Prachatice No asthma Ostrava vs. Prachatice Asthma	Region Region	94	<i>t</i> -test	<0.025	<0.05	5169	21
		96	<i>t</i> -test	<0.020	<0.05	5890	113

N, number of subjects.

^a Number of CpG sites with significantly different methylation (beta value).^b Number of CpG sites with significantly different methylation (beta value) and differences >10%.**Table 4**

Overview of the selected results.

Groups	Studied variable	CpG locus	Gene	Relevance	p-value	BH q-value	ST q-value
A. CpG sites with significantly different methylation and differences of beta values >10%							
Ostrava vs. Prachatice	Region	cg08460026	CTLA4	Immune response	2.25E-09	9.32E-08	3.79E-08
		cg02901679	IL10	Immune response	1.57E-08	4.64E-07	1.89E-07
		cg12809314	ZNF445	DNA-protein binding	8.77E-11	7.94E-09	3.23E-09
Ostrava vs. Prachatice Asthma	Region and Disease	cg17142183	IL1R2	Immune response	1.41E-10	1.15E-08	4.67E-09
		cg24833176	PHF20L1	DNA-protein binding	2.54E-10	1.71E-08	6.98E-09
		cg21312090	TRPS1	DNA-protein binding	6.42E-09	2.20E-07	8.94E-08
		cg10731149	CYBRD1	Metabolism of xenobiotics	9.88E-10	4.95E-08	2.02E-08
		cg26771217	CLK2	Signaling pathways	1.66E-09	7.49E-08	3.05E-08
		cg05191071	MAP3K7	Signaling pathways	1.45E-11	2.16E-09	8.78E-10
		cg08779777	PIK3CG	Signaling pathways	2.45E-07	4.66E-06	1.90E-06
B. Methylation of CpG sites/genes selected based on the published data							
Ostrava Asthma vs. Ostrava No asthma	Disease	cg08147886	ACSL3 ^a	Biosynthesis of lipids	0.0969	0.1327	N/A
		cg22111003			0.0167	0.0670	N/A
		cg15415509	ADRB2 ^b	Signaling pathways	0.0996	0.1327	N/A
		cg19110523			0.4842	0.4842	N/A
Prachatice Asthma vs. Prachatice No asthma	Disease	cg08147886	ACSL3 ^a	Biosynthesis of lipids	0.7129	0.9622	N/A
		cg22111003			0.9438	0.9622	N/A
		cg15415509	ADRB2 ^b	Signaling pathways	0.9622	0.9622	N/A
		cg19110523			0.7541	0.9622	N/A

N/A, not applicable.

^a Candidate biomarker of PAH exposure and putative predictor of PAH-associated childhood asthma [23].^b Candidate biomarker of asthma severity [24].

with polymorphic CpG sites) [36,45]. Due to these difficulties, the strict control of all significant results obtained from Infinium Illumina Methylation 27K arrays as well as from advanced 450K BeadChips is needed.

In conclusion, our observation, that differences in DNA methylation pattern between locations differing significantly in air pollution levels, validates the assumption and confirms our previous data obtained by studying gene expression profiles [28,30]. Moreover, we demonstrated the possibility of analyzing the effect of gender and cotinine levels on the DNA methylation pattern by 27K BeadChips. Other variables like length of gestation, birth weight and length of full breastfeeding were suggested as possible factors which can impact the DNA methylation pattern in future life. These results should be confirmed in future studies employing the 450K BeadChips which cover nearly 18 times more CpG sites than 27K BeadChips [5,46] and can thus offer a better association with studies focused on whole genome expression profiles.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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