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Impact of air pollution from different sources on sperm DNA methylation

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ABSTRACT

Environmental exposure is associated with increased incidence of respiratory and cardiovascular diseases and reduced fertility. Exposure to air pollution can influence gene expression through epigenetic mechanisms. In this study, we analysed gene-specific CpG methylation in spermatozoa of city policemen occupationally exposed to air pollution in two Czech cities differing by sources and composition of the air pollution. In Prague, the pollution is mainly formed by NO2 from heavy traffic. Ostrava is a hotspot of industrial air pollution with high concentrations of particular matter (PM) and benzo[a]pyrene (B[a]P). We performed genome-wide methylation sequencing using the SureSelectXT Human Methyl-Seq system (Agilent Technologies) and next-generation sequencing to reveal differentially methylated CpG sites and regions. We identified differential methylation in the region chr5:662169 – 663376 annotated to genes CEP72 and TPPP. The region was then analysed in sperm DNA from 117 policemen using targeted methylation sequencing, which proved its hypermethylation in sperm of Ostrava policemen.

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Air pollution; DNA methylation; spermatozoa

Introduction

Air pollution caused by industry, local combustion and traffic brings major health issues for human populations living in urban industrial agglomerations. Besides chronical respiratory and cardiovascular diseases and cancer, the biological impact of long-term exposure to environmental contaminants can also manifest in reduced fertility and altered offspring health. There is a growing evidence that parental effects associated with environmental factors and affecting subsequent generations can be ascribed to epigenetic inheritance (Skinner et al. 2010). DNA methylation in CpG sites of regulatory regions is an epigenetic mechanism involved in specific gene expression control and in imprinting. Being a subject of dynamic changes induced by internal and external factors, DNA methylation can serve as biomarker of environmental exposure (Martin and Fry 2018).

An increasing number of papers focusing on environmentally related DNA methylation changes in blood leukocytes has been published, including studies of effects of diesel exhaust, particulate matter (PM), polycyclic aromatic hydrocarbons (PAH) and other industrial air contaminants (Carmona et al. 2014; Jiang et al. 2014; Dai et al. 2017; Honkova et al. 2022; Holliday et al. 2022). However, only a few studies have dealt with possible epigenetic changes induced by air pollutants in

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germline, that can be detected in ejaculated spermatozoa (Consales et al. 2016; Ma et al. 2019; Yang et al. 2020; Cheng et al. 2022).

The Ostrava industrial region and the Czech capital Prague are the cities most suffering from air pollution in the Czech Republic. The main sources of air pollution in Ostrava, the town representing a hot spot of air pollution in Europe, are iron industry, coal mining and local combustion. As a result, increased concentrations of airborne dust (PM_{10}, $PM_{2.5}$), benzene and benzo[a]pyrene (B[a]P) have been detected in Ostrava for decades (Jirik et al. 2016). In contrast, mostly heavy traffic with frequent traffic jams stands behind the air pollution in Prague (Branis 2009). Increased morbidity, high prevalence of cardiovascular diseases and bronchitis in both children and adults were reported in the Ostrava region (Tomaskova et al. 2016; Jirik et al. 2016). This intensified a search for the molecular genetic basis of the impact of air pollution exposure on human health in the highly polluted areas of the Czech Republic. As a part of these efforts, global and specific gene expression studies and genome-wide methylation profiling were performed using peripheral blood samples to compare the situation in both cities (Ostrava and Prague) (Rossner et al. 2011, 2015; Honkova et al. 2022). In Ostrava, also biological effects of seasonal changes in the level of air pollution on male gametes were studied, and increased sperm nuclear and mitochondrial DNA damage was found in semen samples collected following winter season with high air pollution (Rubes et al. 2021; Vozdova et al. 2022a). However, no differentially methylated CpG loci were revealed using genome-wide methylation next generation sequencing (NGS) in semen samples collected repeatedly from the same men following winter and summer, the two seasons differing significantly by air pollution levels in Ostrava (Vozdova et al. 2022b).

In the current study, we used genome-wide methylation NGS to compare specific sperm DNA methylation in occupationally exposed men from Prague and Ostrava. Our aim was to find potential differentially methylated CpG sites and regions in the sperm DNA of policemen working outdoors in the two cities differing by major sources and composition of air pollution. Differential methylation in a CpG island located on chromosome 5, that is associated with *CEP72* and *TPPP* gene, was detected by NGS and subsequently evaluated in a larger cohort of Prague and Ostrava policemen by targeted sequencing.

Material and methods

Study participants

The study group consisted of healthy municipal policemen working in Ostrava (54 men) and Prague (63 men), Czech Republic. The mean age of the men was 40.4 ± 9.37 in Ostrava 39.5 ± 9.5 in Prague. Data on their reproductive and general health, on their exposure and lifestyle factors were collected in a questionnaire. All study participants were non-smokers without alcohol or drug abuse and without any serious health or reproductive issues, namely chronic and andrological diseases and long-term treatment, diabetes, varicocele, accessory gland infection and chlamydial infection. All the men achieved at least secondary education. An informed consent was obtained from all participants prior to the study in accord with the Helsinki II declaration. The study was approved by the Ethics Committee of the Institute of Experimental Medicine CAS in Prague (approval number: 2018/09).

Air pollution

Mean concentrations of the main air pollutants shown in Table 1 were calculated from the Czech Hydrometeorological Institute historical data for years 2016, 2017, and 2018 (available at https://www.chmi.cz/files/portal/docs/uoco/isko/tab_roc/tab_roc_EN.html) (Honkova et al. 2022). The concentrations of air pollutants were measured using stationary air pollution sensors. The concentrations of $PM_{2.5}$ and NO_2 were measured hourly by

Table 1. Long-term concentrations (mean ± SD) of main air pollutants in Prague and Ostrava in three years preceding the sample collection (2016–2018) (Honkova et al. 2022) and current limit values.

		2016		2017		2018	
Polutant	Limit value (yearly)	Prague	Ostrava	Prague	Ostrava	Prague	Ostrava
$PM_{2.5}$ (µg/m3)	20	16.5 ± 13.8	22.2 ± 18.3	16.7 ± 12.2.	21.7 ± 15.9	18.0 ± 15.2	22.9 ± 18.2
B[a]P (ng/m3)	1	0.8 ± 0.3	2.2 ± 0.9	0.9 ± 0.3	2.5 ± 0.7	0.8 ± 0.2	2.9 ± 0.9
NO_2 (µg/m3)	40	25.6 ± 24.0	16.4 ± 15.0	31.0 ± 29.1	16.2 ± 13.6	33.0 ± 31.8	17.2 ± 15.4

automatic emission monitoring, and the results of the one-hour measurements were processed to obtain daily averages. A 24-h sample was evaluated for B[a]P measurements every third day.

Semen samples and sperm isolation

The semen samples were obtained in March and October 2019. Semen samples were collected by masturbation to clean glass containers after 2–7 days of sexual abstinence. The semen was allowed to liquefy at room temperature, and standard semen parameters (sperm concentration, motility, viability and morphology) were assessed in accordance with the World Health Organization guidelines for semen analysis (World Health Organization 2010). The fresh semen samples were aliquoted, cooled, and transported for further analysis that started within 24 hours. The semen aliquots intended for the DNA methylation analysis were washed in PBS, and the sediment was processed by GuEX and Proteinase K to remove somatic cells and their DNA, and isolate sperm for DNA extraction. Briefly, the sediment was incubated in 400 µl GuEX buffer (50 mM Guanidine hydrochloride, 10.5 mM Tris pH 8.0, 10.5 mM NaCl, 10.5 mM EDTA pH 8.0, 1 mM NaOH, pH 8.0–8.5; Sigma, St. Louis, MO, USA) and 20 µl proteinase K (20 mg/ml) (Qiagen, Hilden, Germany) for 15 min at 37°C. After centrifugation at 6600 rpm for 10 min, the sediment was resuspended in 200 µl PBS. The processed sperm samples were stored frozen.

DNA isolation and Next Generation Sequencing (NGS)

Genomic DNA was isolated from spermatozoa using the QIAamp DNA Blood Mini Kit (Qiagen) with 30 µl of 1 M dithiothreitol (Sigma), and stored frozen. In this study, a total of 32 sperm DNA samples from 24 Prague policemen were analysed by methylation NGS. Briefly, 24 samples from the March collection, which followed the high air pollution winter season, were analysed. Additionally, samples from the October collection were also analysed in eight of the men. The construction of DNA libraries and sequence enrichment were performed using the SureSelectXT Human Methyl-Seq system (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instructions. EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for the bisulphite conversion. Sequencing was performed on the Illumina NextSeq 500 platform at the paired-end configuration using NextSeq 500/550 High or Mid Output v2 kits (300 cycles, 2 × 150bp) (Illumina, San Diego, CA, USA) at the CEITEC Core Facility Genomics (Brno, Czech Republic). The SureSelectXT Human Methyl-Seq platform assesses 84 Mb of the human genome, including 3.7 million CpGs (Kerachian et al., 2020). The design covers 95% of all CpG islands, 91% bases of all CpG islands, 45% bases of all CpG shores and 33% bases of all CpG shelfs. CpG shores are regions ~2 kb upstream and downstream of CpG islands, and CpG shelves are ~4 kb upstream and downstream of CpG islands.

Methylation data from sperm samples of 24 Ostrava policemen previously analysed using the same NGS protocol (Vozdova et al. 2022b), were used for comparative methylation analysis in this study.

NGS data analysis

NGS data analysis was performed as previously described (Vozdova et al. 2022b). Briefly, raw Fastq files were adaptor-trimmed and low-quality bases were filtered out using Trimmomatic 0.39 (Bolger et al. 2014) with following parameters: ILLUMINACLIP: TruSeq3-PE-2.fa: 2:30:10, LEADING: 25, TRAILING: 25, SLIDINGWINDOW: 4:20, MINLEN: 35. Percentage of cytosine methylation for individual positions in human DNA was analyzed using Bismark v.0.22.3 toolkit (Krueger and Andrews 2011). Specifically, filtered fastq files were aligned to the human reference genome (hg38) using Bowtie2 algorithm, deduplicated, and cytosine methylation was called while ignoring the first 2 bp from the 5' end of Read 2 and the last 2 bp from the 3' end of Read 2. QC reports of particular steps in the analysis were generated using fastqc tool, bismark2report tool implemented in Bismark v.0.22.3, and QaliMap v.2.2.2 (Okonechnikov et al. 2016).

Differential methylation analysis

Differential methylation of CpG dinucleotides between Ostrava and Prague was analyzed using the Bioconductor package DSS (Dispersion Shrinkage for Sequencing data) version 2.48.0 (Park and Wu 2016). Only sites covered with at least 3 reads in each sample and at least 5 reads in more than 50% of the samples were subjected to the statistical testing. A total of 2,065,266 out of 4,126,739 sites passed this filtering step. Linear model comparing the two collection seasons in Prague was fitted reflecting paired sample design. Smoothing of the data was not performed during the linear model fitting. Wald test at each CpG site was performed using the linear model fitting results, and statistical significance was evaluated based on the false discovery rate values.

Subsequently, linear model comparing the two collection localities (Prague and Ostrava) was applied using our previously published methylation NGS data available from NCBI BioProject and Sequence Read Archive under the entry PRJNA759765. Because no differences were detected between the two collection seasons, FASTQ files were merged prior data analysis in the men where multiple samplings were available. Smoothing of the data was performed during the linear model fitting. Wald test at each CpG site was performed using the linear model fitting results, and statistical significance was evaluated based on the false discovery rate values. PCA analysis was performed using R version 4.1.2 tool princomp (Venables and Ripley 2003).

Targeted DNA methylation analysis

Our analysis of NGS data revealed differential DNA methylation in the chr5:662169 – 663376 region including 79 CpG sites. A part of this region showing the highest methylation (chr5:662169–662853) was further analyzed by targeted sequencing approach in the whole Prague (63 men) and Ostrava (54 men) cohorts. Bisulfite modified DNA (EpiJET Bisulfite Conversion Kit) was amplified using two pairs of specific primers: i) 5'-ATTTCTAGGGGTTAAGATGGG-3' and 5'-AAATACTAAACACCAAAACGC-3', and ii) 5'-TGGGGATTGGGTTGTTTATG-3' and 5'-AATCACAAACGAATAACCACC-3'. All PCR reactions were performed in 25 µl reaction volume using Hot Start Combi PPP Master Mix (Top-Bio, Prague, Czech Republic) according to manufacturer's instructions. Cycling parameters were: 95°C for 4 min for initial denaturation, 40 cycles at 95°C for 60 s, 54°C for 40 s, and 72°C for 70 s, with a 5 min final extension at 72°C Amplified PCR products were analyzed by gel electrophoresis on 2% agarose gel. The PCR products were cloned into pDrive Cloning Vector (Qiagen). Screening of the recombinant clones was performed by PCR using the primer 5'-CAGCTATGACCATGATTACGCC-3' derived from pDrive Vector sequence. Methylation status of at least 8 clones per each sample was analyzed by Sanger sequencing in in each sample.

Statistical analysis

The level of sperm DNA methylation in the chr5:662169–662853 region between the Prague and Ostrava policemen was compared by non-parametric exact tests using SPSS software package, version 18 for Windows (SPSS, Inc. Chicago, IL, USA). Spearman's correlation test was used to analyze relationships between the sperm DNA methylation in the chr5:662169–662853 region, age and semen quality parameters.

Results

Air pollution

Long-term observations of the Czech Hydrometeorological Institute (2016–2018) revealed that yearly annual concentrations of the main air pollutants regularly exceed the current limits for $PM_{2.5}$ (20 μg/m^3) and B[a]P (1 mg/m^3) in Ostrava. The concentrations of NO_2 in Prague, despite not exceeding the current limits, were almost double of those in Ostrava (Table 1). In the sampling year 2019, the air pollution exceeded the current limit values for $PM_{2.5}$ in Ostrava, and for B[a]P in both Ostrava and Prague in the winter season (Figure 1). In Ostrava, the seasonal fluctuation with increased winter concentrations was detected for all monitored pollutants ($PM_{2.5}$, B[a]P, NO_2). In Prague, the winter and summer concentrations of $PM_{2.5}$ and NO_2 were comparable.

Semen analysis

The results of the conventional semen analysis are summarised in the Supplementary Table S1.

Methylation analysis

Evaluation of the NGS data quality revealed that data from six of the 32 sperm DNA samples from Prague policemen analysed by methylation NGS in this study did not meet the quality criteria. These six samples were excluded from the comparative analysis. Basic quality data for the remaining 26 samples are displayed in the Supplementary Table S2. The paired comparison of the spring and autumn samples in the eighteen Prague policemen, who had both semen samples analysed by NGS, did not reveal any differences in CpG methylation between the two collection seasons. This observation also held true for the previously analysed repeated samples from Ostrava (Vozdova et al. 2022b). Consequently, the spring and autumn data were pooled in the individual men for the bioinformatic analysis comparing Prague and Ostrava samples.

No difference in the global CpG methylation was detected between Prague and Ostrava. The average total CpG methylation detected by NGS was 41.33% in Prague samples and 41.20% in Ostrava. The frequency of CpGs with > 50% methylation was 42.64% in Prague and 42.67% in Ostrava. Results of the PCA analysis are shown in Figure 2.

The highest sperm CpG methylation difference between Prague and Ostrava was revealed in chr5:662169–663376 (AreaStat = 205.8). This differentially methylated region (DMR) comprises 79 CpGs and is annotated to the genes *CEP72* and *TPPP*. The second highest methylation difference was detected in the region chr12:78803764–788034789 (AreaStat = 111.4) but this region is not annotated to any known functional genomic element (https://genome.ucsc.edu/, Human genome GRCh38/hg38 accessed 15 September 2022). The significance of all other DMRs was lower than the study threshold (AreaStat <100, FDR < 0.05). No significant differentially methylated loci were detected.

Our NGS data revealed 13.8% difference in the mean total CpG methylation of the whole chr5:662169–663376 region between the cohorts (12.1% methylation in Prague and 25.9% in Ostrava policemen). The average difference in specific methylation in the individual CpG positions in the chr5:662169–663376 region was 13.6% (min – max 4.9–22.6% difference) (Figure 3). The

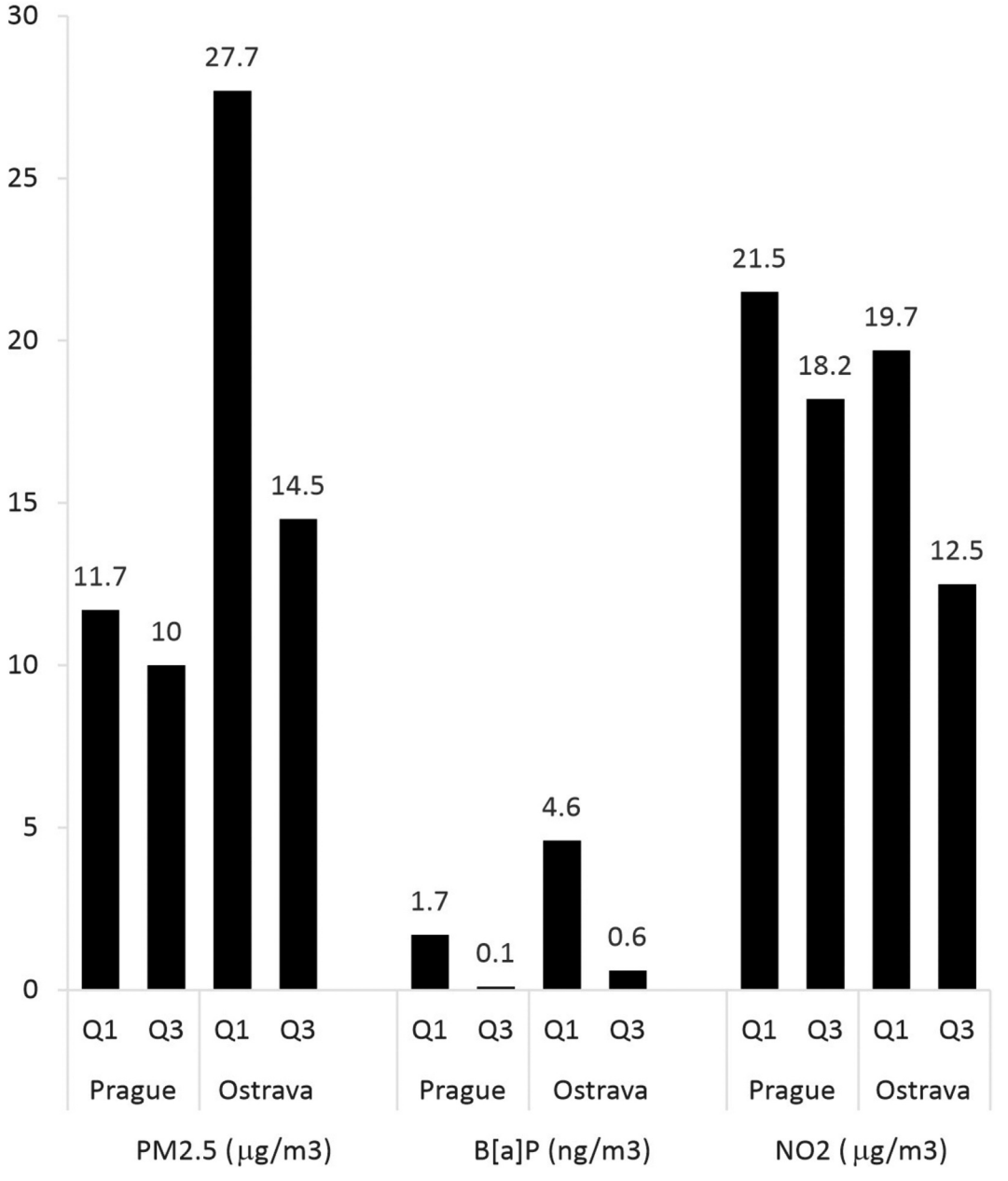

Figure 1. Mean concentrations of air pollutants related to the whole territory in three months preceding sample collections (Q1: January – March; Q3: July – September).

mean total methylation difference in the part of the region exceeding 20% methylation (chr5:-662169–662853, 28 CpGs) was 16.7% (Mann-Whitney test, $p < 0.001$) (Figure 4a). To confirm the differential methylation of the chr5:662169–662853 region in Prague and Ostrava policemen, we performed a targeted Sanger sequencing of this region in all men (63 men from Prague, 54 men from Ostrava). The mean CpG methylation difference was 11.96% (Mann-Whitney test, $p < 0.001$). The distribution of samples with low (<20%), medium (20–80%) and high (>80%) methylation is displayed in Figure 4b. The CpG methylation in this region showed a weak correlation with age

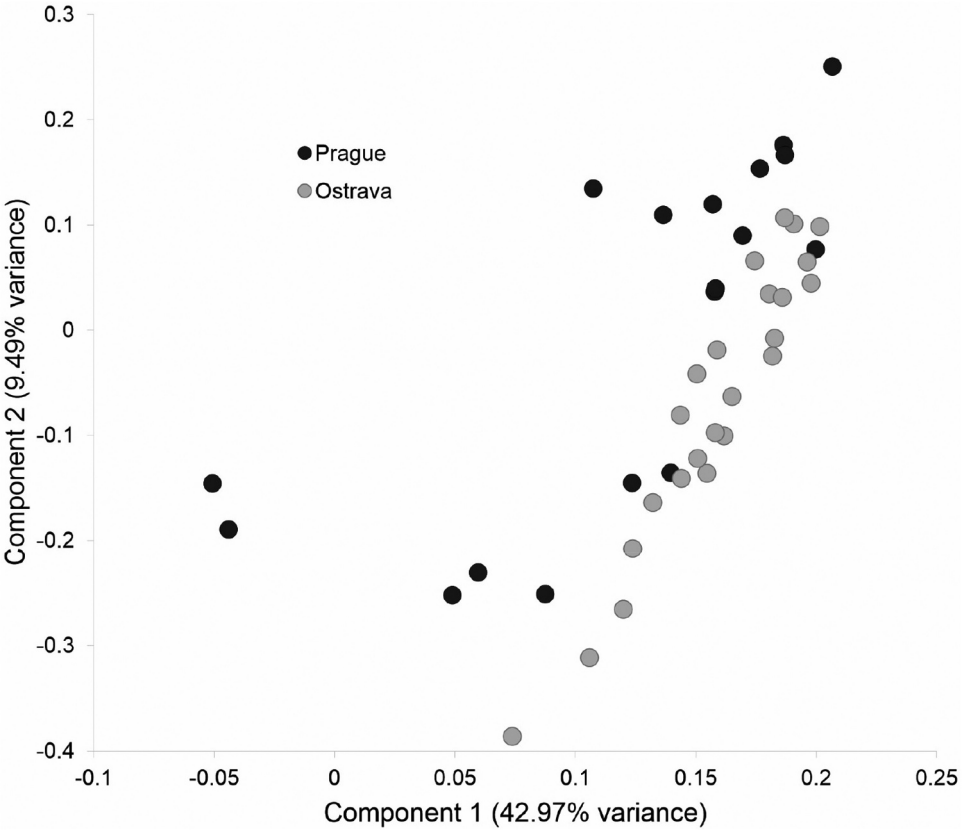

Figure 2. Principal component analysis plot of sperm CpG methylation profiles in Prague and Ostrava policemen.

(Spearman's Rho = 0.197, $p = 0.033$). No correlation between the CpG methylation level in the chr5:662169–662853 region and semen parameters was detected.

Protein-protein interaction network

Analysis of the CEP72 and TPPP protein-protein interaction networks was performed using the STRING database (https://string-db.org/; accessed 12 September 2023) (Supplementary Figure S1).

Discussion

It is known that exposure to air pollutants has a negative impact on human health. Among the obvious consequences of the exposure we can name mainly the increased incidence of respiratory and cardiovascular diseases (Tomaskova et al. 2016; Jirik et al. 2016; Manisalidis et al. 2020). Moreover, air pollution was found associated with neurodevelopmental disorders and congenital abnormalities in children, diminished ovarian reserve and a compromised semen quality (Liu et al. 2023a, 2023b; Santos et al. 2023; Feng et al. 2023). Epigenetic modifications have been recognized as an important molecular mechanism of the gene-environment interactions, and there is a growing evidence of environmentally related global and gene-specific DNA methylation changes (Martin and Fry 2018). The importance of our understanding of the effects of air pollution on the sperm DNA methylation lies primarily in the potential intergenerational transmission of the acquired changes and their impact on the offspring health. However, data on the effects of air pollution on

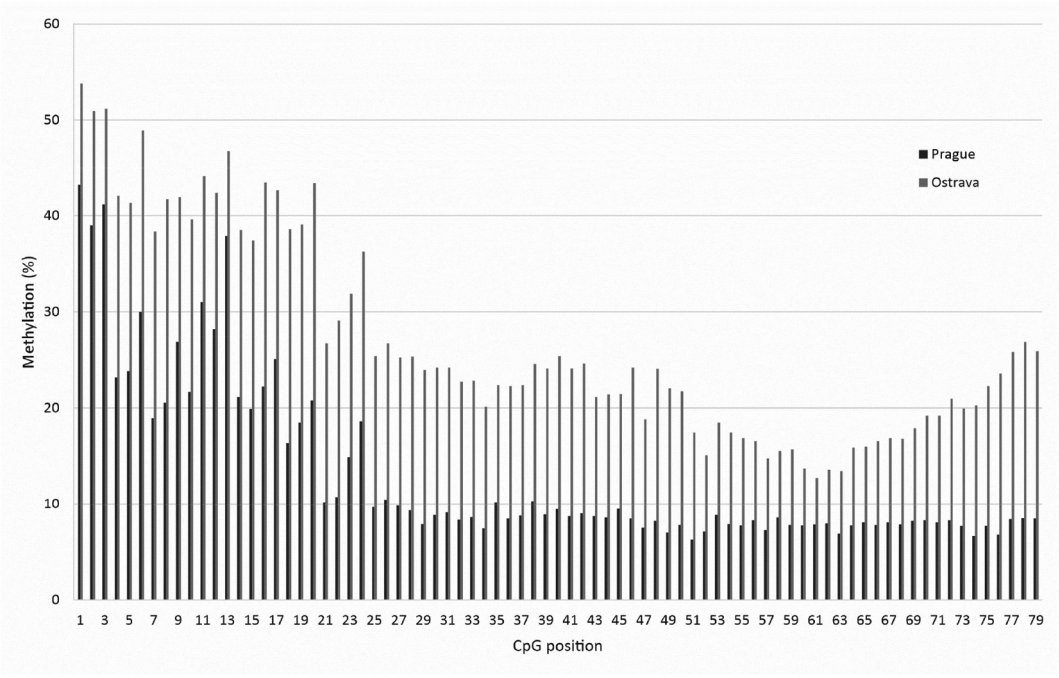

Figure 3. Methylation rates in the differentially methylated region chr5:662169 –663,376 detected by methylation NGS in sperm DNA of Prague (18 men) and Ostrava (24 men) policemen.

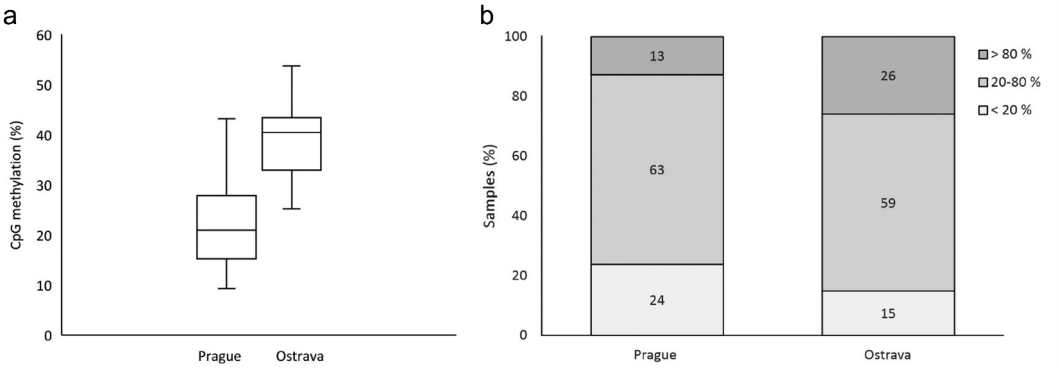

Figure 4. Methylation in the chr5:662169 –662,853 region. a. box plots comparing the CpG methylation rates in the chr5:662169 – 662,853 region detected by methylation NGS in Prague (18 men) and Ostrava (24 men) policemen. The vertical height of each box represents the 25%–75% data range, the horizontal line within each box represents the median value, and the upper and lower extensions represent the largest and smallest values that were determined to not be an outlier. b. comparison of the percentage of samples showing low (<20%), intermediate (20–80%) and high (>80%) methylation of the selected region (chr5:662169 – 662,853) in the whole Prague (63 men) and Ostrava (54 men) cohorts.

DNA methylation in the germline are still limited and contradictory (Åsenius et al. 2020). Decreased global sperm DNA methylation following a long-term exposure to air pollution was reported in fertile Chinese men (Cheng et al. 2022). In the contrast, a persistent sperm DNA hypermethylation was detected in mice exposed to traffic and industrial air pollution (Yauk et al. 2008).

In this study, we used genome-wide methylation NGS to analyse possible effects of air pollution on gene-specific sperm DNA methylation in men living and working in two Czech

cities with high air pollution (Prague and Ostrava). Both cities suffer from increased air pollution but the sources the air pollution and resulting concentrations of individual monitored pollutants are different in Prague and Ostrava. In Prague, a heavy traffic producing high levels of NO_x is the main source of air pollution throughout the year. In the contrast, $PM_{2.5}$, B[a]P and NO_2 resulting from heavy industry form the main components of the air pollution in Ostrava, and their concentrations differ between the highly polluted winter and the less polluted summer season.

The study groups of Prague and Ostrava city policemen were subjected to both occupational and lifetime exposure. We took the advantage of recruiting men working in the same profession (city policemen patrolling in the streets of the two large cities on a daily routine). This gave us a great opportunity to analyse men with comparable education, salaries, social status, and lifestyle attitudes, including diet and alcohol consumption. Both groups were homogeneous regarding the participants' occupation (active policemen), length of employment with the police (at least one year, more than 5 years in > 80% participants in both groups), diet (mixed diet including meat), attitude to smoking (only non-smokers were included) and alcohol consumption (occasional or moderate alcohol consumption). The men were healthy and without any known issues regarding their reproduction which made them optimal subjects for unbiased sperm analysis. The main limitation of the study is that the total number of city policemen serving and living in both Czech cities and eligible for the study was relatively low. Due to this limit, this study can be considered a pilot study for future research.

Our previous methylation NGS analysis revealed no significant differences in the sperm CpG methylation between the two sampling periods in Ostrava, despite seasonal air pollution variations (Vozdova et al. 2022b). In this study, no significant sperm CpG methylation differences were found between the two seasons in Prague policemen. These findings align with previously published data on blood DNA methylation in Prague and Ostrava policemen, where no seasonal changes were detected in blood CpG methylation using array CGH (Honkova et al. 2022). This supports the published hypotheses that a long-term exposure and geographical factors may have a more significant impact on sperm DNA methylation than the fluctuating seasonal exposure (Ding et al. 2016; Åsenius et al. 2020; Honkova et al. 2022).

In the long-term perspective (2016–2018), the yearly mean concentrations of the monitored air pollutants were relatively stable in the individual cities, with higher concentrations of $PM_{2.5}$ and B[a]P in Ostrava, and NO_2 in Prague (Table 1). Previous genome-wide comparisons of the blood CpG methylation in the Prague and Ostrava policemen revealed differential CpG methylation of several genes involved in Axon guidance, PIK3A-Akt and MAPK signalling pathways between the two cohorts (Honkova et al. 2022). Also a differential expression of genes participating in processes associated with transcription, translation, replication of DNA and cell division was detected in blood samples from the two cities (Rossner et al. 2015).

In our study focused on spermatozoa, we detected significant differential CpG methylation in the region chr5:662169–663376 annotated to two genes: *CEP72* and *TPPP*.

The *CEP72* gene product is centrosomal protein 72 which acts in the recruitment of key centrosomal proteins to the centrosome, and in the process of formation of a focused bipolar spindle needed for proper tension between sister chromatids. The gene is involved in cell cycle, mitotic and organelle biogenesis and maintenance pathways (https://www.genecards.org/). The *CEP 72* gene has recently been listed among candidate human imprinted genes with paternal gametic origin of methylation (Jima et al. 2022). According to the STRING database, CEP72 protein interacts with other genes involved in centrosome, centriole and spindle formation, Golgi apparatus function and vesicular trafficking (https://string-db.org/) (Supplementary Figure S1). It was reported that a loss of the *CEP72* gene affects the morphology of spermatozoa in mice (Chen et al. 2022). Our analysis did not show any correlation between the mean methylation of the analysed region of the *CEP72* gene in spermatozoa and semen parameters of the men providing the semen samples.

The *TPPP* gene encodes tubulin polymerization-promoting protein which plays a key role in the regulation of microtubule dynamics and elongation of the myelin sheath. The gene is involved in Microtubule cytoskeleton regulation and Sudden infant death syndrome (SIDS) susceptibility pathways (https://www.genecards.org/). The TPPP protein mainly interacts with proteins involved in cytoskeleton organisation, intracellular transport, exocytosis and synapsis (https://string-db.org/).

Conclusions

Our results indicate that the long-term exposure to different sources of air pollution can influence gene-specific CpG methylation in human sperm depending on the source of air pollution and the resulting composition of the emissions. Given the potential intergenerational transmission of the methylation changes, especially for imprinted genes, it is important to consider that the neurodevelopmental and other disorders frequently diagnosed in children born in the regions with elevated air pollution, and traditionally attributed to their prenatal exposure, might also result from altered parental gamete methylomes.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All NGS data obtained in semen samples of analysed Prague policemen are available from NCBI BioProject (https://www.ncbi.nlm.nih.gov/bioproject) under the entry PRJNA905922. Raw sequencing data are available from the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra). Previously published NGS data obtained in semen samples of Ostrava policemen and used for the comparative analysis in this study are available from NCBI BioProject PRJNA759765.

Geolocation information

Czech Republic.

Author contributions

Conceptualization, M.V., S.K., A.P. and J.R.; Methodology, S.K.; Investigation, S.K., M.V. and V.K.; Resources, J.R.; Writing – Original Draft Preparation, M.V.; Writing – Review & Editing, J.R., S.K., A.P.; Visualization, M.V.; Supervision, J.R.; Funding Acquisition, J.R. All authors approved the final draft.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Institute of Experimental Medicine AS CR in Prague, approval number: 2018/09.

Informed Consent Statement

Written informed consent was obtained from all subjects involved in the study.

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