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Molecular Epidemiology and Air Pollution

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1. Introduction

A new and evolving area of research termed molecular epidemiology aims to merge sophisticated and highly sensitive laboratory methods (many of them developed during the recent revolution in molecular biology) with analytical epidemiological methods. Molecular epidemiology bridges from basic research in molecular biology to studies of human cancer causation by combining laboratory measurement of internal dose, biologically effective dose, biological effects and the influence of individual susceptibility with epidemiologic methodologies [1]. The most common view is that the approach represents a natural convergence of molecular biology and epidemiology [2].

The number of biomarkers available for evaluating genetic and cancer risk in humans is quite large. Their utility for human biomonitoring is suggested by the well-known paradigm of environmentally induced cancer, which represents endpoints for assessing the entire spectrum of human-genotoxicant interactions [3]. These biomarkers begin with exposure and include absorption, metabolism, distribution, critical target interaction (i.e. DNA damage and repair), genetic changes and finally disease. Disease is the province of traditional epidemiology. The development of biomarkers has given rise to the field of molecular epidemiology, which uses these biomarkers rather than disease to assess the risk of environmental exposure [4, 5].

The paradigm of environmental cancer starts with exposure. A large number of biomarkers are available now, but to evaluate their sensitivity and to interpret the results obtained we need to know data on exposure. Previously, data on exposure were usually not published in studies using various biomarkers.

It is well established that ambient air pollution is related to human health. Increased exposure to respirable particulate matter (PM) correlates with increased mortality caused by lung cancer and cardiovascular diseases [6-8]. Pope et al. [9] suggested that a long term increase in PM_{2.5}

of $10 \mu\text{g}/\text{m}^3$ is connected with an 8% increase in lung cancer mortality in adult men. Despite the fact that other factors related to cancer incidence, such as smoking habit or inappropriate diet, are probably stronger influences, the absolute number of cancer cases related to air pollution is high due to the high prevalence of exposure [10].

Although the quantitative health risk related to air pollution is assessed by epidemiological studies [11-14], alternative types of studies are necessary for the purposes of primary prevention. On the level of human populations, such studies are first of all molecular epidemiological studies evaluating quantitative relations between external exposure and measurable biological events (biomarkers). These biomarkers form a chain from exposure to disease [15-17].

One of the most frequently used biomarkers are DNA adducts as biomarker of exposure, quantifying the biologically effective dose of genotoxic compounds that were covalently bound to DNA as a target molecule of carcinogenesis [18-22]. If DNA adducts are not effectively repaired, they might be fixed as mutations during replication. According to the well known scheme of the multi-step process of chemical carcinogenesis, an accumulation of mutations may lead to carcinogenesis. Thus, DNA adduct levels have a direct relation to mutagenesis and carcinogenesis. Data are accumulating about the relation of DNA adducts to environmental exposure to complex mixture components such as carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) [23] and to malignant tumors and other degenerative diseases [24, 25].

The Comet assay (single cell gel electrophoresis, SCGE) in its standard alkaline version allows the detection of DSB (double strand breaks), SSB (single strand breaks), alkali-labile sites and transient repair sites. DSB represent the principal lesion leading to the formation of chromosomal aberrations. The majority of chemical mutagens induce DSB indirectly via the generation of other DNA lesions such as SSB or oxidative damage that may be converted to DSB during DNA replication or repair [26]. When combined with specific bacterial repair enzymes, it identifies a broad spectrum of additional lesions including oxidized purines and pyrimidines [27]. Due to its relative simplicity, its low requirements on the number of analyzed cells as well as its capacity to detect DNA damage independently of the cell cycle, comet assay is believed to serve as an exposure biomarker assay providing information about the biologically effective dose [28].

DNA fragmentation in the mature sperm may be understood as another biomarker of exposure, measured by the sperm chromatin structure assay (SCSA) [29]. The sperm sample is stained with acridine orange, which is a metachromatic DNA dye that fluorescence green when intercalated into native DNA and shifts to a red fluorescence when associated with collapsed single-stranded DNA. These stained samples are measured by flow cytometry [30,31]. Human sperm cells can undergo DNA fragmentation or covalent modification of nucleotides. Both these types of disturbances contribute to infertility. Altered sperm chromatin structure can be significant contributing factor to subfertility that is not revealed by standard spermogram parameters, because they can range within physiological values. Consequently, fertilization failure, impaired embryo morphology, abnormal development of blastocysts, embryo implantation failure or repeated spontaneous abortions can occur.

Chromosomal aberrations in human peripheral lymphocytes are recognized as a valuable biomarker of effect, probably the only one that has been internationally standardized and validated [32]. While classic cytogenetic analysis (conventional method, CCA) is the method of choice for determining unstable types of aberrations, the fluorescent in situ hybridization technique (FISH) seems to be a rapid, sensitive, and reliable method for the detection of stable structural rearrangements that remain undiminished over time, such as translocations [33-37]. The FISH painting technique appears to be more sensitive than the conventional technique for detecting the genomic frequency of translocations induced by various chemical agents or irradiation [38].

The measurement of micronuclei (MN) in human peripheral blood lymphocytes is one of the methods frequently used in molecular epidemiology. MN represent a measure of both chromosome breakage and chromosome loss. Therefore, an increased frequency of micronucleated cells, used as a biomarker of genotoxic effects, can reflect exposure to agents with clastogenic or aneugenic modes of action [39]. Currently, the MN assay is one of the preferred methods for assessing chromosomal damage as a result of environmental mutagen exposure as well as a tool for genotoxicity testing.

Another important aspect demonstrating the advantages of molecular epidemiology studies over classical epidemiology is the possibility of identifying the genetic susceptibility of individuals to the action of various compounds [17]. Biomarkers of susceptibility mostly take into account the role of genetic makeup of the organism in the response to the exposure to xenobiotics. The role of genetic polymorphisms on the metabolic activation of xenobiotics (oxygenases of cytochromes P450 such as *CYP 1A1*) and also their detoxification (glutathione-S-transferases) is well known. Further biomarkers of individual susceptibility are polymorphisms in genes encoding DNA repair enzymes (*XRCC1*, *XPD*, *hOGG1*) [40-42]. One more factor affecting susceptibility to the genotoxic and carcinogenic effects of xenobiotics is the saturation of the organism by vitamins A, C, E, folic acid etc., which are known to play a significant role as free radical scavengers and antioxidant agents and which also affect the synthesis of DNA repair enzymes [43-46].

Inhalation of PM, particularly PM of aerodynamic diameter < 2.5 μm (PM_{2.5}) and smaller, leads to inflammation and subsequent production of reactive oxygen species (ROS) [47]. The production of ROS, that include e.g. the hydroxyl radical, superoxide anion, or hydrogen peroxide, is caused by both the physical effects of PM (PM is phagocytosed by macrophages that consequently produce ROS), and the presence of various chemicals on the surface of PM (e.g. metals, PAHs) with pro-oxidant properties. ROS may arise from exogenous or endogenous sources. The latter are mostly physiologic and include various metabolic processes and inflammation, whereas exogenous sources are environmental factors such as smoking, diet [46, 49], ultraviolet radiation, ionizing radiation or exposure to environmental pollution [50].

ROS can attack lipids, proteins and nucleic acids simultaneously [51]. The highly reactive hydroxyl radical reacts with DNA by addition to the double bonds of DNA bases, and by abstraction of a hydrogen atom from the methyl group of thymine and C-H bonds of 2'-deoxyribose [52]. This reaction yields several modified bases but, as a biomarker of oxidative DNA damage, 8-oxodeoxyguanosine (8-oxodG) is most often studied. The oxidized base is

highly mutagenic and, if unrepaired, its presence in DNA causes GC>TA transversions. If repaired, 8-oxodG is excreted in urine where it can be assessed by various techniques as a general biomarker of oxidative stress. Urinary levels of 8-oxodG are also believed to reflect the total DNA excision repair capacity of an organism [53]. Other sources of 8-oxodG, including oxidation of the nucleotide pool, may affect urinary levels of 8-oxodG. Numerous reports have indicated that urinary 8-oxodG is not only a biomarker of generalized, cellular oxidative stress, but may also imply the risk of cancer, atherosclerosis and diabetes [51, 54].

Several metabolic pathways of PAH activation have been described [55] and one of them, activation through PAH-*o*-quinones, leads to ROS generation and oxidative stress [56]. The modification of DNA molecules represents the most serious form of impact of ROS on the organism because it may lead to base changes, mutations, and/or DNA breaks. If ROS attack both DNA strands, double-strand DNA breaks may appear. These breaks may lead either to unstable chromosomal aberrations, or, if homologous or non-homologous end-joining repair seals the breaks, to stable chromosomal translocations. Translocations are more serious because they are usually fixed in the genome and may lead to rearrangements of regulatory elements and genes, including oncogenes thus increasing cancer risk [57]. Another, indirect mechanism of DNA double strand breaks induction is associated with DNA adducts formation. Adducts may cause persistent blockage of one DNA strand during its synthesis and uncoupling of the other strand which may result in the formation of double strand breaks [58].

The attack of ROS on lipids leads to lipid peroxidation. This reaction may have potentially serious consequences, as it may damage cellular membrane and inactivate membrane-bound receptors or enzymes. In addition, secondary products of lipid peroxidation, such as aldehydes, are highly reactive and may propagate oxidative stress by reacting with other cellular molecules including proteins [59]. Currently, isoprostanes are considered the most reliable markers of lipid peroxidation. These prostaglandin-like compounds, first described in the 1990s, are formed by free-radical induced peroxidation of arachidonic acid, independent of cyclooxygenase enzymes. Lipid peroxidation products, including isoprostanes, play a role in the pathogenesis of many diseases [60].

New omics biomarkers: mRNA expression. Although the effect of air pollutants on humans may be monitored by the analysis of mRNA expression of individual selected genes [61], the current trend is to use transcriptomics as a tool for studying genome-wide responses of the organism to environmental exposures [62]. It has been concluded that transcriptome is a dynamic entity that is highly responsive to environmental exposures [62]. But studies of the effects of environmental pollutants on gene expression profiles are scarce [63, 64, 65].

2. Czech studies – Fig. 1

The Northern Bohemia was in late eighties one of the most polluted regions in Europe. It was therefore believed that such specific situation is just the location to study the sensitivity of biomarkers to detect genetic damage [15]. As the exposed region was selected the Teplice district, as the control region the Prachatice district.

Later EXPAH project (Effects of polycyclic aromatic hydrocarbons in environmental pollution on exogenous and oxidative DNA damage) tried to evaluate the hypothesis that PAHs are the major source of genotoxic activities of organic mixtures associated with air pollution, one of the studied cities was Prague [66-72].

Ostrava Region is area highly polluted by benzo[a]pyrene, nowadays one of the highest exposure in EU [73].

Molecular epidemiology studies in those regions are examples of the use of biomarkers to identify genetic damage induced by air pollution as well as which biomarkers may be used to evaluate the health risk for exposed populations.

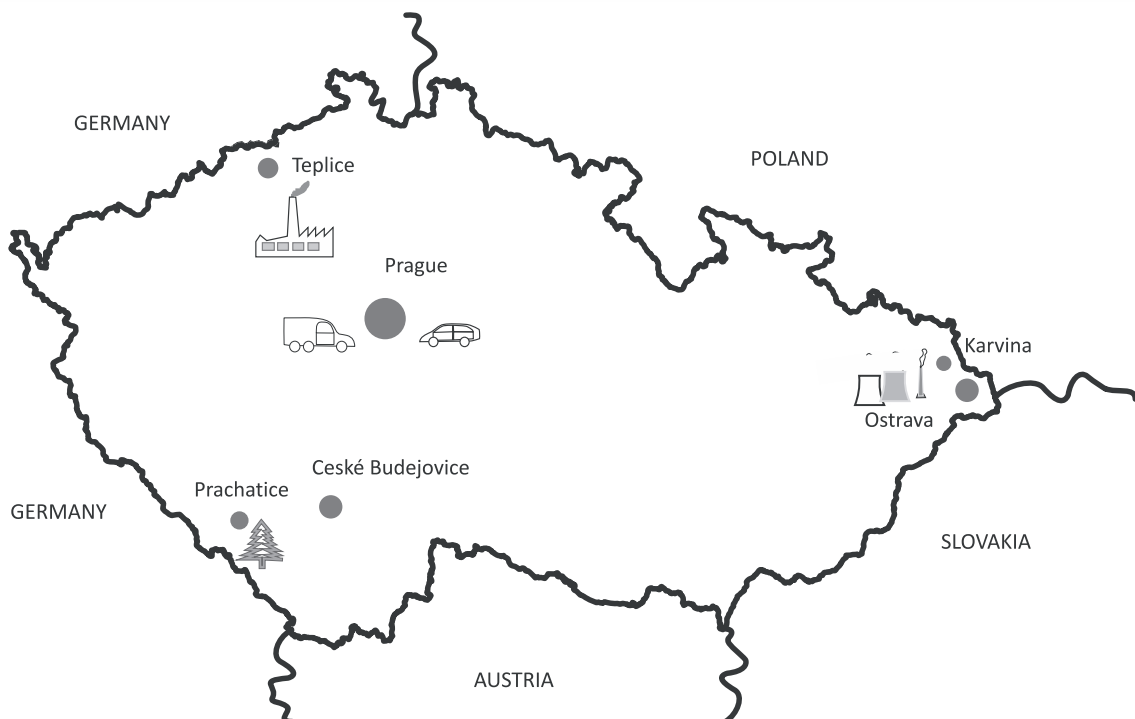


Figure 1. Map of the Czech Republic with the locations of molecular epidemiology studies.

3. Teplice program

The first studies in which biomarkers detected seasonal differences in exposure and genetic damage from environmental pollution were conducted in a population from a highly polluted area in Silesia (Poland) [73-76]. Perera et al. [74] found that the exposure to environmental pollution was associated with a significant increase of DNA adducts, SCE (sister chromatid exchanges) and chromosomal aberrations, as well as with frequency of *ras* oncogene overexpression. In these studies, the investigators were not able to determine the relationship between individual exposure to air pollution and biomarkers of exposure and genetic damage because personal exposure data were not collected.

The methods of molecular epidemiology were widely used in studies on the impact of air pollution to genetic damage in Czechoslovakia. Mining districts in the northwestern region of the Czech Republic were polluted by combustion from power plants and heavy industry, which resulted in one of the worst environmental pollution in Europe [77]. The Teplice Program was initiated by the Czech Ministry of the Environment in 1990 to provide scientifically valid information needed to assess environmental health problems in the Northern Bohemian mining districts. The program was successful thanks to collaboration with U.S. Environmental Protection Agency; it included the air pollution monitoring, human exposure, biomarker, and health effects studies [15]. An air quality monitoring and receptor modeling study conducted in the Teplice district during 1992 to 1994 identified emissions from residential heating and vehicles as the major sources of organic carbon, including c-PAHs. c-PAHs and their nitroderivatives associated with the respirable particle fraction PM₁₀ have been identified as a major source of carcinogenic risk in urban areas [78, 79]. Therefore this situation was understood as a convenient model to check the relationship between c-PAHs in the polluted air and biomarkers.

Polycyclic aromatic hydrocarbons (PAHs) adsorbed onto respirable air particles (<2.5 μm) are mainly derived from incomplete combustion, including mobile sources such as motor vehicles and stationary sources such as power plants, residential heating etc. Some of these compounds exhibit carcinogenic and/or mutagenic properties [80]. Molecular epidemiology studies using biomarkers of exposure and early biological effects could provide invaluable information about the genotoxic effects of environmental exposure to such PAHs mixtures. The measurement of DNA adducts was selected as a promising biomarker of exposure, since such measurements take into account individual differences in exposure, absorption, distribution, metabolic activation and detoxification of PAHs in the body as well as cell turnover and the repair of DNA damage [22].

The first study compared a group of women working as postal workers or gardeners from a highly polluted Teplice district with the group of women working as postal workers and nursery school teachers in the town of Prachatice, a control district in southern Bohemia in winter 1993/1994. At that time personal exposure to B[a]P in Teplice reached up to 7.5±3.6 ng/m³. DNA adducts by ³²P-postlabeling in WBC (white blood cells) were significantly affected by personal exposure to c-PAHs ($r=0.710$; $p<0.001$). Percentage of DNA in tail measured by Comet assay correlated significantly ($r=0.304$; $p<0.05$) with personal exposures to respirable particles (PM_{2.5}). No effect was observed on the frequency of chromosomal aberrations or SCE [18]. A group of 10 women non-smokers also participated in a follow up study during 5 samplings in November 1992, October 1993-February 1994. Personal exposure to B[a]P during 24 h sampling was during this period between 2.0±1.1 ng/m³ and 7.5±3.6 ng/m³. Analyzing data from the follow up study, a significant effect of personal exposure on DNA adduct levels and their relationship with short-term exposure to c-PAHs was found ($r=0.621$, $p<0.001$). No other variables as age, passive smoking and consumption of fried and smoked food during the 24 h of personal exposure monitoring had significant effect on DNA adduct levels [19].

Svecova et al. [81] analyzed the effect of air pollution, particularly PM₁₀, PM_{2.5}, c-PAHs and B[a]P, on urinary levels of 8-oxodG in children from the districts of Teplice and Prachatice.

The urine was collected in the year 2004 from 894 children born in the period 1994-1998 [82]. 8-oxodG was determined by ELISA [83]. Stationary monitoring of PM_{2.5}, PM₁₀ and c-PAHs was done continually during the entire sampling period as well as before this period. Collected data on air pollution allowed to correlate individual urinary 8-oxodG levels with levels of pollutants measured at different times and for different periods before collection of urine samples. In multivariate models, they found that exposure to PM₁₀ and PM_{2.5} measured in a 3-day interval 4–6 days before sampling, PM₁₀, PM_{2.5}, and B[a]P in a 7-day period before sampling, c-PAHs and B[a]P in a 3-day interval 1–3 and 7–9 days before urine collection were significant factors positively affecting 8-oxodG levels in urine. It may be generalized that PM₁₀, PM_{2.5}, and c-PAHs increase oxidative damage within one week of exposure. Increased level of 8-oxodG was observed also in children exposed to environmental tobacco smoke (ETS). It may be hypothesized that ETS exposure and short-term exposure to fine particles and c-PAHs induce oxidative stress, and therefore may be starting point for respiratory and allergic morbidity in children.

Rubes et al. [84] studied the impact of air pollution in Teplice in the period 1995-1997 to sperm DNA damage repeatedly in the same donors, measuring the sperm chromatin structure assay (SCSA), when the percentage of mature sperm with abnormal chromatin/fragmented DNA was determined and expressed as % DNA fragmentation index (DFI). In the study 36 semen donors participated, 21 men gave seven samples, 10 gave six samples. Air pollution levels over the last 90 days before sampling ranged from 28.7- 67.8 µg/m³ (for PM₁₀), and from 0.3-7.9 ng/m³ (for B[a]P). None of other semen outcomes (sperm concentration, semen volume, sperm morphology and sperm motility) showed significant associations with air pollution. Only mean % DFI was significantly associated with exposure (p<0.05). It was the first study reporting association between exposure to ambient air pollution and DNA fragmentation in human sperm. Rubes et al. [85] further studied the hypothesis, if men homozygous null for *GSTM1* (*GSTM1*-) are less able to detoxify reactive metabolites of c-PAHs found in air pollution. Using a longitudinal study design, this study revealed a significant association between *GSTM1* null genotype and increased DNA damage in sperm, defined as % DFI. This study shows for the first time that endogenous DNA fragmentation in human sperm can be modulated by polymorphism in *GSTM1*, a gene involved in c-PAH metabolism. Men who are homozygous null for *GSTM1* exhibit increased susceptibility to sperm DNA damage associated with exposure.

4. Prague

The capital city of Prague has become one of the most polluted localities in the Czech Republic, especially due to traffic. Therefore, the effect of exposure to carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) adsorbed onto respirable air particles (PM_{2.5}, <2.5 µm) on DNA adducts and chromosomal aberrations was repeatedly studied in groups of city policemen working in the downtown area as well as in bus drivers [86].

Studied groups of a total of 950 subjects from three studies were used for analyses of associations between c-PAHs exposure, DNA adducts and chromosomal aberrations in 1) city

policemen in 2001 (exposed N = 53, controls N = 52) [36], 2) city policemen in 2004 (exposed N = 480, 120 x 4) [87], 3) bus drivers in 2005-2006 (N = 120 x 3, exposed I N = 50, exposed II N = 20, controls N = 50) [83]. All volunteers ad 1 and 2) were male city policemen working in the Prague downtown and spending >8 h outdoors daily. Controls from study 1) were age- and sex-matched healthy males spending >90% of their daily time outdoors and working in a suburban area. Study 3) involved 50 bus drivers working in the center of Prague (I), 20 garagemen (II) and, as controls, 50 healthy administrative workers spending >90% of their daily time indoors.

Ambient air particles (PM₁₀, PM_{2.5}) and c-PAHs (benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, benzo[a]pyrene (B[a]P), chrysene, dibenz[ah]-anthracene and indeno[cd]pyrene) were monitored using VAPS samplers, while personal exposure to c-PAHs was evaluated using personal samplers during working shifts. Quantitative chemical analysis of c-PAHs was performed by HPLC with fluorimetric detection according to the EPA method [19].

DNA adducts were analyzed in lymphocytes by the ³²P-postlabeling assay, which was performed according to a standardized procedure – Fig. 2 [22].

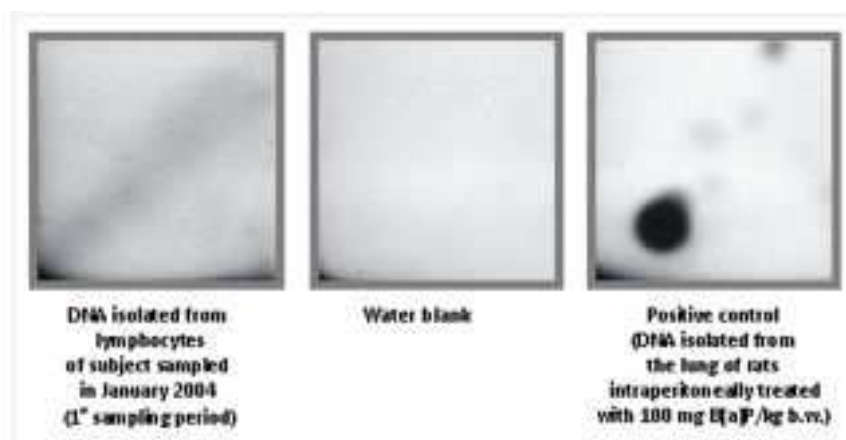


Figure 2. DNA adducts by ³²P-postlabeling.

Based on the personal monitoring data, during their working shifts the city policemen were exposed to significantly higher concentrations of c-PAHs as well as B[a]P than the controls (median): 9.7 vs. 5.8 ng/m³ (p<0.01) and 1.6 vs 0.8 ng/m³ (p<0.01), respectively. The level of B[a]P-like DNA adducts was higher in the exposed group (0.122±0.036 vs. 0.099±0.035 adducts/10⁸ nucleotides, p=0.003). The results of multivariate regression analysis showed smoking, vitamin C levels and polymorphisms of the *XPD* repair gene in exon 23 and the *GSTM1* gene to be significant predictors for total DNA adduct levels. Exposure to ambient air pollution, smoking, and polymorphisms of the *XPD* repair gene in exon 6 were significant predictors for B[a]P-like DNA adducts [22].

Using the FISH technique and probes for chromosomes 1 and 4 (Fig. 3), the genomic frequency of translocations calculated as $F_C/100$ was 1.72 and 1.24 for exposed and controls (p<0.05),

respectively. *CYP1A1*2C (Ile/Ile)*, *XPD 23 (Lys/Lys)*, and *XPD 6 (CC)* genotypes were associated with an increase in the number of aberrant cells as determined by the conventional method. Factors associated with an increased level of translocations determined by FISH included age, smoking, B[a]P-like DNA adducts, folate, and polymorphisms of *CYP1A1*2C*, *GSTP1*, *EPHX1*, *p53* and *MTHFR* genes. Ambient air exposure to c-PAHs significantly increased FISH cytogenetic parameters in nonsmoking city policemen [36].

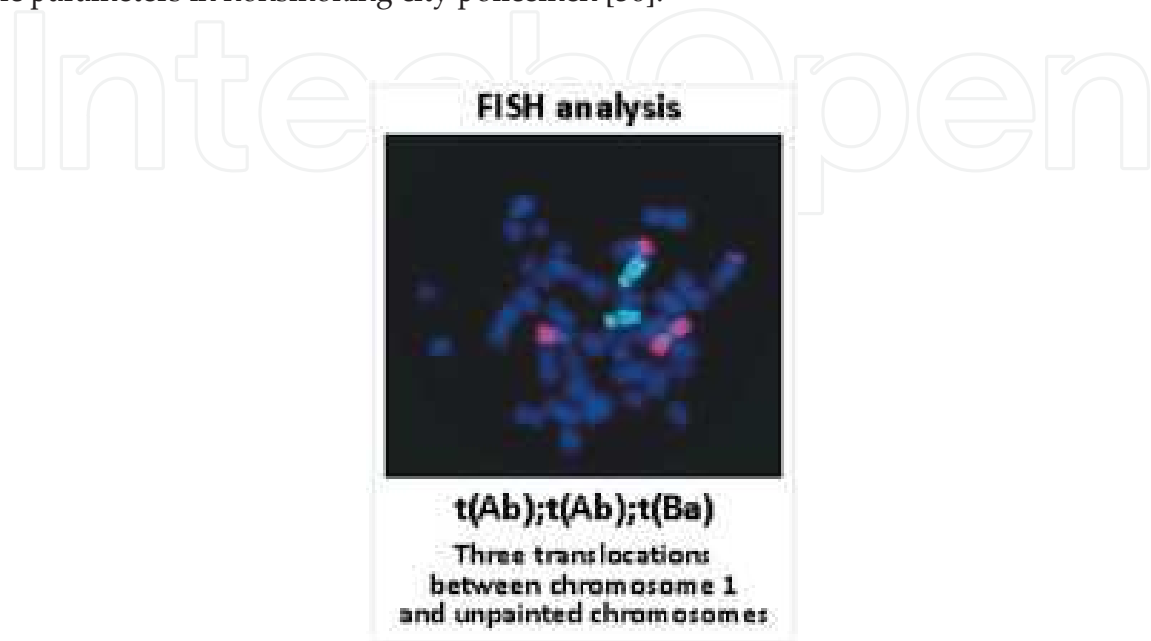


Figure 3. Chromosomal aberrations by fluorescence in situ hybridization (FISH) – whole chromosome painting #1 and #4. (t(Ab);t(Ab);t(Ba); three translocations between chromosome 1 and unpainted chromosomes.

Total DNA adducts, B[a]P-like DNA adducts and the genomic frequency of translocations in the study conducted in the year 2001 were significantly affected by smoking – an effect of air pollution was observed only in nonsmokers [22, 36]. Therefore, later studies used only nonsmokers as volunteers.

The obtained results were confirmed in a subsequent study in which city policemen were sampled in January, March, June, and September 2004. Using personal monitoring, the concentrations of c-PAHs were 1.58 ng/m³ for B[a]P and 9.07 ng/m³ for c-PAHs during January, 0.39 ng/m³ for B[a]P and 3.46 ng/m³ for c-PAHs during March, 0.18 ng/m³ for B[a]P and 1.92 ng/m³ for c-PAHs during June, and 0.45 ng/m³ for B[a]P and 3.08 ng/m³ for c-PAHs during September. Total DNA adducts were only slightly elevated in January (2.08 ± 1.60) compared to March (1.66 ± 0.65), June (1.96 ± 1.73) and September (1.77 ± 1.77). B[a]P-like DNA adducts, however, were significantly higher in January than in the March and June sampling periods (0.26 ± 0.14 vs. 0.19 ± 0.12 and 0.22 ± 0.13, respectively; p<0.0001 and p=0.017), indicating that c-PAH exposure probably plays a crucial role in DNA adduct formation in lymphocytes [80]. In those periods, the mean frequency of translocations measured by FISH (F_C/100) was 1.32±1.07, 0.85±0.95, 0.87±0.81, and 1.08±0.94, respectively. The frequency of chromosomal aberrations determined by CCA was 2.07±1.48, 1.84±1.28, 1.84±1.42, and 1.64±1.46 % AB.C., respectively [86].

In another study bus drivers were sampled in winter 2005, summer 2006 and winter 2006. Using personal monitoring, the concentrations of B[a]P for the exposed group were 1.25 ng/m³ during winter 2005, 0.20 ng/m³ during summer 2006 and 1.04 ng/m³ during winter 2006; for controls the concentrations were 1.75 ng/m³ during winter 2005, 0.24 ng/m³ during summer 2006, and 0.75 ng/m³ during winter 2006. The total DNA adducts in the exposed group were 1.72 ± 0.56 during winter 2005, 1.22 ± 0.45 during summer 2006, and 1.62 ± 0.59 adducts/10⁸ nucleotides during winter 2006; in garagemen the totals were 1.24 ± 0.41 during winter 2005, 1.27 ± 0.48 during summer 2006, and 1.70 ± 0.08 adducts/10⁸ nucleotides during winter 2006; in controls the totals were 2.15 ± 0.61 during winter 2005, 1.18 ± 0.36 during summer 2006, and 1.90 ± 0.79 adducts/10⁸ nucleotides during winter 2006. In those periods the mean frequencies of translocations measured by FISH ($F_C/100$) were 1.62 ± 1.17 , 2.18 ± 1.75 , and 1.77 ± 1.31 in the group of bus drivers, 1.20 ± 1.24 , 0.88 ± 1.11 , and 1.01 ± 0.78 in garagemen, and 1.65 ± 1.49 , 1.34 ± 1.01 , and 1.87 ± 1.29 in controls, respectively. The frequencies of chromosomal aberrations determined by CCA were 1.30 ± 1.15 , 1.43 ± 1.01 , and 1.30 ± 1.04 % AB.C. in the group of bus drivers, 0.95 ± 0.76 , 1.15 ± 1.09 , and 1.55 ± 0.97 % AB.C. in garagemen, and 1.17 ± 0.93 , 1.50 ± 0.99 , and 1.52 ± 1.12 % AB.C. in controls, respectively [86].

The levels of DNA adducts were significantly affected by stationary exposure to B[a]P within the last 30 days before samples collection. Data obtained for biomarkers of exposure and effect were used for pooled analysis. Using multivariate logistic regression, the relationship between personal exposure to B[a]P and DNA adducts measured by ³²P-postlabeling was calculated (DNA adducts = $1.042 + B[a]P \times 0.077$, $p < 0.001$, Fig. 4).

These results indicate that c-PAH exposure plays a crucial role in DNA adduct formation in lymphocytes. A similar relationship was observed between personal exposure to B[a]P and the genomic frequency of translocations measured by FISH ($F_C/100 = 1.255 + B[a]P \times 0.082$, $p < 0.05$, Fig. 5) [86].

In the subset of 54 city policemen (exposed) and 11 controls (working indoors) genetic damage was analyzed by Comet assay, the sampling was performed during January and September 2004. The exposed group displayed significantly higher levels of unspecified DNA damage than controls during both seasons, oxidative DNA damage was significantly higher in the exposed group only in January. Correlation analysis revealed a strong association in the exposed group between the level of oxidative DNA damage and personal exposure to c-PAHs during January. Regression analysis of the influence of genetic polymorphism on the level of DNA damage suggested higher oxidative DNA damage with variant alleles of genes *CYP1A1*2C (Ile/Val)*, *MTHFR*, *MS*; variant allele of *p53MspI* polymorphism increased both unspecific and oxidative DNA damage [88].

In the same groups of bus drivers Rossner et al. studied the effect of air pollution to oxidative damage to DNA [83] and oxidative damage to lipids [89]. 8-oxodG in urine was used as the biomarker of DNA oxidative damage. Increased level of urinary 8-oxodG in bus drivers was observed in all three sampling periods, as well as a protective effect of vitamin C on oxidative DNA damage. Multivariate logistic regression analysis identified PM_{2.5} and PM₁₀ levels, measured by stationary monitoring during a 3-day period before urine collection, as the only

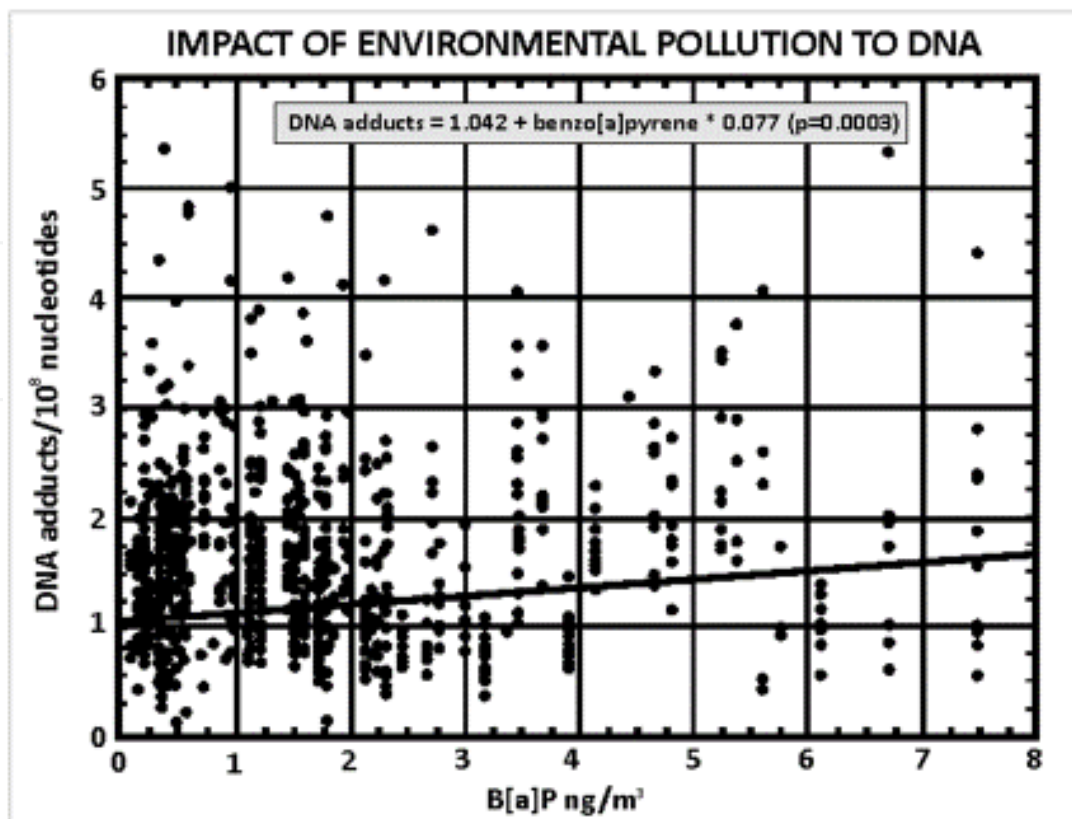


Figure 4. Impact of B[a]P exposure to DNA adducts.

factors significantly affecting 8-oxodG levels, while the levels of c-PAHs had no significant influence [83].

Lipid peroxidation was determined by 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) in urine. It was significantly higher in bus drivers than in controls in both winter seasons. Personal c-PAHs and B[a]P exposure 48 h before sample collection significantly increased 15-F_{2t}-IsoP levels in urine. When data from stationary monitors were used, c-PAHs and B[a]P had a significant effect on 15-F_{2t}-IsoP levels for the 3-day period immediately preceding sample collection, but exposure to PM_{2.5} and PM₁₀ affected lipid peroxidation at least 25-27 days before sampling [89].

Another study on city policemen in February and May 2007 analyzed the impact of air pollution on the level of micronuclei measured by automated image analysis (MetaSystem Metafer 4) [90] (Fig. 6).

Using multivariate logistic regression, the relationship between personal exposure to B[a]P and micronuclei expressed as MN/1000 cells was calculated ($MN = 5.18 + B[a]P \times 1.11$, $p=0.002$, Fig. 7).

These results indicate that MN frequencies, when measured by the automated scoring system, are significantly affected by higher levels of air pollutants. Others have published similar observations in general populations living in polluted regions [91] as well as in heavily exposed workers [92, 93]. A recent meta-analysis of data from a 25-year period also indicates increased MN frequencies in environmentally exposed subjects, specifically children. Results of Ross-

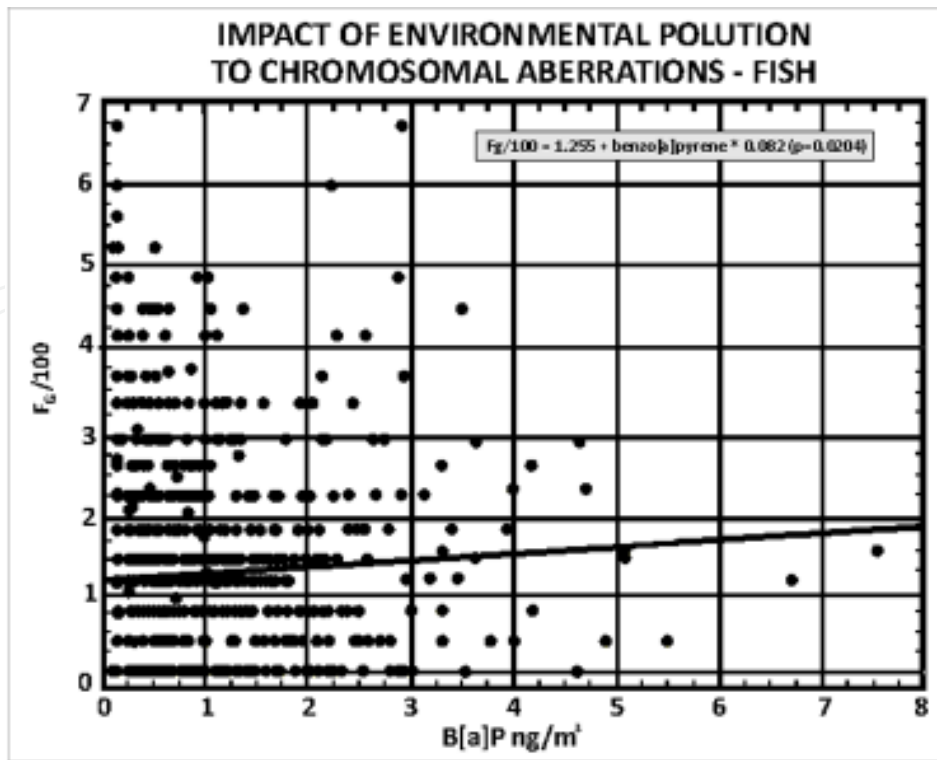


Figure 5. Impact of B[a]P exposure to genomic frequency of translocations (Fg/100).

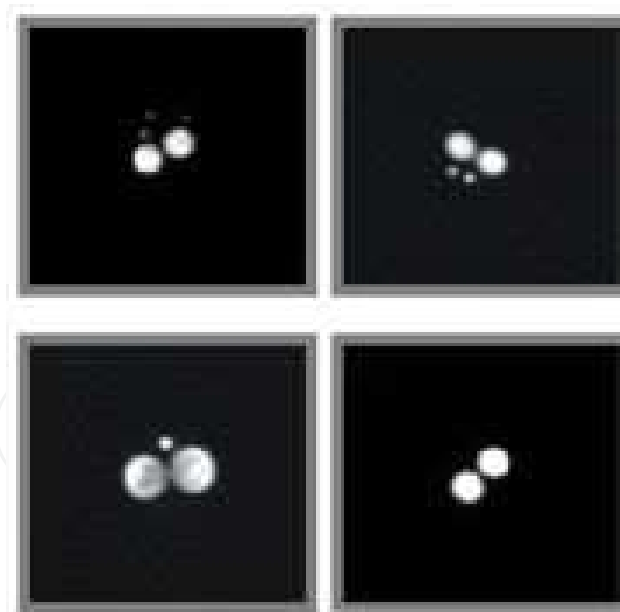


Figure 6. Micronuclei by automated image analysis in binucleated cells (BNC): a) BNC with three MN; b) BNC with two MN; c) BNC with one MN; d) BNC without MN.

nerova et al. [95] also showed that MN frequency was affected by exposure to c-PAHs up to 60 days before sample collection. Concentrations of c-PAHs measured more than 60 days before the collection of samples had no effect on MN formation. Similar results were obtained

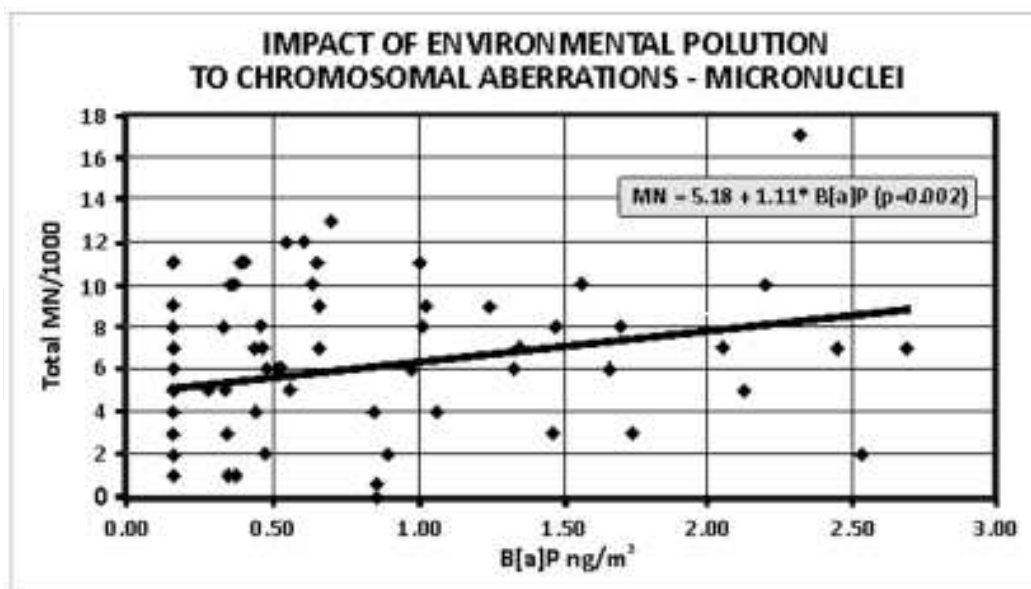


Figure 7. Impact of B[a]P exposure to micronuclei.

using conventional cytogenetic analysis, where the frequency of aberrant cells corresponded to the exposure to chemical carcinogens during the periods of 3 months [95].

The work of Rossnerova et al. [90] was the first human biomonitoring study focused on the measurement of MN by automated image analysis for assessing chromosomal damage as a result of environmental mutagen exposure. The results demonstrate the ability of c-PAHs to increase MN frequency, even if the environmental exposure to c-PAHs occurred up to 60 days before collection of biological material. Further, those findings indicate the ability of the automated image analysis system to analyze easily slides with a low density of cells that would be very laborious to score using visual analysis. Considering the possibility to analyze more BNC more quickly, it may be suggested to analyze 2000 to 3000 binucleated cells in future studies to obtain more statistically powerful data.

Comparing stationary exposure in the center of Prague as well as the personal exposure of city policemen to c-PAHs in February 2007 [90] vs. February 2001 [22] or January 2004 [96], exposure to c-PAHs in February 2007 was lower due to meteorological conditions, e.g. personal exposure to B[a]P was only 1.04 ± 0.76 ng/m³. It is important to note that even this B[a]P concentration increased the frequency of MN.

In the same study population of 47 city policemen DNA fragmentation in mature sperm by SCSA, relationship between air pollution and genetic polymorphism in metabolic genes (*CYP1A1*, *EPHX1*, *GSTM1*, *GSTP1*, *GSTT1*), folic acid metabolism genes (*MTR*, *MTHFR*) and DNA repair genes (*XRCC1*, *XPB6*, *XPB23*, *hOGG1*) was analyzed. DNA fragmentation index (DFI) was significantly higher in February vs. May 2007. Rubes et al. [97] observed that concentration of 1 ng B[a]P/ m³ induces DNA fragmentation in mature spermatozoa, which may be modulated by a polymorphism in metabolic (*CYP1A1MspI*, *GSTM1*) and DNA repair genes (*XRCC1*, *XPB6*, *XPB23*). Sperm DNA fragmentation seems to be a sensitive biomarker of air pollution.

The effect of exposure to air pollution to biomarkers in newborns was analyzed in two locations with different level of pollution: Prague vs. Ceske Budejovice in winter season 2008/2009. The levels of B[a]P, benzene and PM_{2.5} for both locations for the years 2008 and 2009 were obtained from the Czech Hydrometeorological Institute. The mean concentration of these pollutants 3 months before birth were calculated to estimate the individual exposure of each mother-newborn pair: B[a]P concentration in Prague was 1.9 ± 0.5 ng/m³ vs. 3.2 ± 0.2 ng/m³ in Ceske Budejovice ($p < 0.001$), PM_{2.5} 27.0 ± 2.5 µg/m³ vs. 24.5 ± 0.7 µg /m³ ($p < 0.001$), benzene 2.5 ± 0.5 µg / m³ vs. 2.1 ± 0.1 µg /m³, respectively [98].

As biomarkers, umbilical cord blood (UCB) was used to determine DNA adducts by ³²P-postlabeling [22] and micronuclei using automated image analysis [90]. DNA adducts were analyzed as B[a]P-like adducts and total adducts, both categories were significantly higher in Ceske Budejovice vs. Prague ($p < 0.001$). Higher frequencies of micronuclei in newborns in Ceske Budejovice vs. Prague ($p < 0.001$) were observed. Multivariate logistic regression showed a significant impact of 3 months mean B[a]P exposure before birth.

The changes in the transcriptome of newborns from UCB were studied [92]. Non-smoking mothers were selected: 52 from Ceske Budejovice and 35 from Prague. Total RNA was isolated from leukocytes, gene expression profiles were determined by HumanRef-8 Expression BeadChips (Illumina, San Diego, CA, USA) containing 24 526 transcript probes. Genes with $|\log_2FC| > 0.58$ (binary logarithm of fold change) and $P < 0.01$ were considered as differentially expressed between Ceske Budejovice and Prague. Leukocytes from newborns showed different expression of 104 genes (37 up-regulated and 67 down-regulated genes). Down-regulated biological processes were immune and defense response (*KIR2DL3*, *KIR3DL3*, *KIR3DL4*, *KIR2DS5*, *KLRC3*, *CLTA4*), negative regulation of proliferation (*CNDKN1A*, *CTLA4*, *TGFBR3*), apoptosis (*PRF1*, *NR4A2*, *GZMB*, *TNFAIP3*, *PP2R2B*, *DDIT4*), response to oxygen levels, cell migration, organ regeneration, signal transduction (*RGS1*, *SOCS1*, *THBS*) and cell differentiation (*FLT3*, *ZBTB16*), up-regulated gene encoding *SERPINA1* (which is considered as biomarker of exposure to genotoxic agents). Down regulated signaling pathways were natural killer cell mediated cytotoxicity, antigen processing and presentation, autoimmune thyroid disease, graft vs. host disease, up-regulated MAPK signaling pathway [98].

The results were surprising because air pollution in Prague was understood to be higher than in Ceske Budejovice. Results of analysis of DNA adducts, micronuclei and transcriptome indicate in the same direction the significance of exposure to B[a]P in Ceske Budejovice to induce genetic damage in newborns, when PM_{2.5} and benzene was higher in Prague. It seems to be a coincidence that changes observed in Ceske Budejovice were observed when exposure to B[a]P was 3.2 ng/m³, corresponding to the effect of exposure inducing IUGR 2.8 ng/m³ [100].

5. Ostrava program

The Ostrava Region (OSTR) is an industrial and heavily populated area situated in the easternmost part of the Czech Republic. Concentrations of PM₁₀ (particulate matter < 10 µm) were continuously above 40 µg/m³ daily average in the years 2002-2011 and considerably

higher than urban background in the largest city of CR - Prague. Similarly, population in this region is exposed to high concentrations of PM_{2.5} (particulate matter < 2.5 μm). Concentrations of B[a]P in the OSTR are the highest in the Czech Republic as well as in the European Union. Standard of 1 ng/m³/year B[a]P has been exceeded on all OSTR monitoring stations in all years 2004-2011 [72].

Sram et al. [72] investigated the impact of high level of environmental air pollution on selected biomarkers. Exposure was measured as follows: PM_{2.5} by stationary monitoring, c-PAHs (B[a]P) and VOC (benzene) by personal and stationary monitoring. Personal exposure to c-PAHs was defined using outdoor concentration, ETS exposure (environmental tobacco smoke), indicator of home heating by coal, wood or gas, frequency of exhaust fan use, cooking habits, and commuting by a car [101]. Cotinine in urine, triglycerids, total, HDL and LDL cholesterols, and vitamins A, C, E in plasma were used as life-style indicators.

The following parameters were analyzed: DNA adducts by ³²P-postlabeling as biomarkers of effect, chromosomal aberrations by FISH (fluorescent in situ hybridization) and MN as biomarkers of effect, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) as a marker of oxidative DNA damage, 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) as a marker of lipid peroxidation, protein carbonyls as a marker of protein oxidation, and genetic polymorphisms as biomarkers of susceptibility. Sampling was done in winter 2009, summer 2009 and winter 2010. Volunteers were recruited from office workers in Ostrava city, city policemen from Havirov and Karvina (N=98), and in 2010 also from general population of Ostrava-Radvanice (N=28). City policemen from Prague (N=65) served as a control group.

During all sampling periods, the study subjects from OSTR were exposed to significantly higher concentrations of B[a]P and benzene than subjects in Prague as measured by personal monitoring. Taken separately, B[a]P levels were lowest in Prague in 2009, Prague winter 2010 concentrations were about equal to the lower Ostrava 2009 levels, and levels in Ostrava in winter 2010 were 5-fold higher. Despite higher B[a]P air pollution in OSTR during all sampling periods, the levels of B[a]P-like DNA adducts per 10⁸ nucleotides were significantly higher in the Ostrava subjects only in winter 2009 (mean ± SD: 0.21 ± 0.06 versus 0.28 ± 0.08 adducts/10⁸ nucleotides, *p* < 0.001 for Prague and Ostrava subjects, respectively) (Table 1; controls - unexposed subjects from [22]). During the other two sampling periods, the levels of B[a]P-like DNA adducts were significantly higher in the Prague subjects (*p* < 0.001). Multivariate analyses done separately for subjects from Ostrava and from Prague, combining all sampling periods in each location, revealed that exposure to B[a]P and PM_{2.5} significantly increased levels of B[a]P-like DNA adducts only in the Ostrava subjects [102].

Despite several-fold higher concentrations of air pollutants in the Ostrava Region, the levels of stable aberrations (genomic frequency of translocations per 100 cells (F_G/100), percentage of aberrant cells (% AB.C.) were comparable (Table 2; controls - unexposed subjects from [36]).

The frequency of unstable aberrations measured as number of micronuclei was unexpectedly significantly lower in the Ostrava Region subjects in both seasons of 2009. Urinary excretion of 8-oxodG did not differ between locations in either season.

	Exposure				DNA adducts (adducts/10 ⁸ nucleotides)		
	N	B[a]P (ng/m ³)		B[a]P - "like"		Total	
		2009	2010	2009	2010	2009	2010
Prague	60	0.80 ± 0.55	2.86 ± 1.87	0.21 ± 0.06	0.25 ± 0.12	1.30 ± 0.41	1.37 ± 0.41
Ostrava	98	2.73 ± 2.60	14.8 ± 13.3*	0.28 ± 0.08	0.16 ± 0.06	0.37 ± 0.37	1.03 ± 0.33*
Controls	42	0.80 ± 0.62		0.10 ± 0.03		0.76 ± 0.20	

* P < 0.05

	Exposure				FISH		
	N	B[a]P (ng/m ³)		% AB.C.		F _G /100	
		2009	2010	2009	2010	2009	2010
Prague	60	0.80 ± 0.55	2.86 ± 1.87	0.27 ± 0.18	0.25 ± 0.15	1.43 ± 1.15	1.39 ± 1.03
Ostrava	98	2.73 ± 2.60	14.8 ± 13.3*	0.26 ± 0.19	0.22 ± 0.18	1.44 ± 1.23	1.25 ± 1.18
Controls	42	0.80 ± 0.62		0.21 ± 0.16		1.13 ± 1.01	

* P < 0.05

Lipid peroxidation measured as levels of 15-F2t-IsoP in blood plasma was elevated in the Ostrava subjects sampled in 2009, similarly increased in Prague samples in 2010 (Table 3). Multivariate analyses conducted separately for subjects from Prague and Ostrava showed a negative association between the frequency of micronuclei and concentrations of B[a]P and PM_{2.5} in both regions. A positive relationship was observed between lipid peroxidation and air pollution [103]. Plasma levels of 15-F2t-IsoP increased linearly with increasing concentration of B[a]P in the ambient air up to concentration of 9 ng B[a]P/m³ – higher doses of B[a]P did not increase plasma 15-F2t-IsoP levels any further [103].

	Exposure				Oxidative stress 15-F2t-isoprostane (pg/mL)	
	N	B[a]P (ng/m ³)		15-2Ft-IsoP		
		2009	2010	2009	2010	
Prague	60	0.80 ± 0.55	2.86 ± 1.87	165.9 ± 41.7	265.5 ± 104.7*	
Ostrava	98	2.73 ± 2.60	14.8 ± 13.3*	279.3 ± 303.6*	279.5 ± 124.5	

* P < 0.05

In contrast to the above results, changes were observed in a group of 4 subjects from Prague who spent 3 weeks in Ostrava just in the period of inversion in winter 2010, when the average daily concentration of B[a]P reported by stationary monitoring was 14.7 ± 13.3 ng/m³. The frequency of micronuclei in peripheral lymphocytes in those individuals increased approx. 50% (Table 4) [104], and similar increase was observed for genomic frequency of translocations.

Groups	Sampling	N (6000c./person)	Frequency of micronuclei	
			% AB.C. with MN	MN/1000
Controls	(i)	4	0.81 ± 0.15	8.32 ± 1.63
	(ii)	4	0.80 ± 0.14	8.47 ± 1.55
Exposed	(i)	4	0.74 ± 0.43	7.96 ± 4.92
	(ii)	4	1.14 ± 0.55	12.91 ± 6.49*

* P < 0.05

The relationship between exposure to B[a]P and the level of DNA adducts and chromosomal aberrations in winter 2010 in Ostrava inhabitants was surprising, as the results did not correspond with the expected dose effect relationship. Therefore Rossner et al. [61] put forward a hypothesis about a possible adaptive response, indicating that this outcome may be affected by DNA repair. In 64 subjects from Prague and 75 subjects from Ostrava they investigated the levels of oxidative stress markers (8-oxodG, 15-F2t-IsoP, protein carbonyls) and cytogenetic parameters [$F_C/100$, % AB.C. and acentric fragments (ace)], and their relationship with the expression of genes participating in base excision repair (BER) and non-homologous end-joining (NHEJ) by quantitative PCR. Multivariate analyses revealed that subjects living in Ostrava had increased odds of having above-median levels of *XRCC5* expression (OR; 95% CI: 3.33; 1.03–10.8; $q = 0.046$). Above-median levels of 8-oxodG were associated with decreased levels of vitamins C (OR; 95% CI: 0.37; 0.16–0.83; $p = 0.016$) and E (OR; 95% CI: 0.25; 0.08–0.75; $p = 0.013$), which were elevated in subjects from Ostrava. They suggest that air pollution by c-PAHs affects *XRCC5* expression, which probably protect subjects from Ostrava against the induction of a higher frequency of translocations; elevated vitamin C and E levels in the Ostrava subjects decrease the levels of 8-oxodG. Such changes in gene expression were not observed in the 4 subjects from Prague after 3 weeks stay in Ostrava, their reaction differed from subjects with long residence time in OSTR.

Global gene expression analysis in a group of total 312 exposed subjects from OSTR and 154 controls from Prague [98] was conducted with the aim to characterize molecular response of the organism exposed to heavy air pollution [105]. A combination of geographical and meteorological conditions (a valley affected by frequent atmospheric inversions), heavy industry and the fact that industrial production exists in the OSTR region continually for almost three centuries creates a specific situation suitable for research on environmental air pollution and human health. Given these characteristics a higher number of differentially expressed genes was expected to be found in subjects living in the polluted region. The rationale behind this hypothesis was that the protection of the organism against deleterious effects of air pollution would require greater changes in the transcriptome than in the control subjects. Unexpectedly, despite lower concentrations of air pollutants a higher number of deregulated genes and affected KEGG pathways was found in subjects from Prague. In both locations differences between seasons were observed. The quantitative real-time PCR (qRT-

PCR) analysis showed a significant decrease in expression of *APEX*, *ATM*, *FAS*, *GSTM1*, *IL1B* and *RAD21* in subjects from Ostrava, in a comparison of winter and summer seasons. In the control subjects, an increase in gene expression was observed for *GADD45A* and *PTGS2*. The Rossner et al. [105] concluded that high concentrations of pollutants in Ostrava do not increase the number of deregulated genes. This may be explained by adaptation of humans to chronic exposure to air pollution. To further explain this phenomenon analyses focused on regulation of mRNA expression are necessary.

For the first time, this study measures the levels of biomarkers in subjects exposed to air pollutants in this region. Simultaneous assessment of oxidative stress markers, DNA adducts, chromosomal aberrations and transcriptomics is a new approach that can bring more clarity to the mechanisms of pollution effects.

6. In vitro studies

A wide variety of in vitro systems was developed in order to study the genotoxicity of chemicals and their mixtures, including complex mixtures of environmental pollutants adsorbed onto respirable air particles (PM_{2.5}). Complex mixtures of organic compounds to which humans are exposed through air pollution are only partially characterized with respect to their chemical composition due to difficulties with chemical analysis of the individual components. Therefore, assays based on biological effects of complex mixture components may be a suitable alternative to a circumstantial chemical analysis. Using rat liver microsomal fraction (S9), it has been repeatedly shown that PAHs formed DNA adducts after metabolic activation by P450 enzymes to diol epoxides. This activation system may be used in acellular assay coupled with ³²P-postlabeling to assess genotoxic potential of complex environmental mixtures via the analysis of DNA forming activity of the mixtures in native DNA [106-109].

The first study comparing the biological activities of complex mixtures from urban air particles PM₁₀ was published by Binkova et al. [110]. HiVol samples were collected during the winter (October-March) and summer (April-September) seasons in the years 1993-1994, extracts (EOM-extractable organic matter) were analyzed in several fractions using in vitro acellular assay (calf thymus DNA with/without rat liver microsomal S9 fraction) with DNA adduct analysis by ³²P-postlabeling (Teplice district: winter: PM₁₀ 69.3 µg/m³, B[a]P 7.4 ng/m³, summer: PM₁₀ 36.5 µg/m³, B[a]P 0.8 ng/m³; Prachatice district: winter: PM₁₀ 29.6 µg/m³, B[a]P 5.4 ng/m³; summer: PM₁₀ 23.6 µg/m³, B[a]P 0.7 ng/m³). The highest total DNA adduct levels were observed in the neutral fraction, especially in the aromatic subfraction with metabolic activation, which contained mainly PAHs and their methyl derivatives. The major PAH-DNA adducts contributed about 50% of the total DNA adducts resulting from all of the crude extracts using S9-metabolic activation. These results indicate that PAHs are a major source of genotoxic activities of organic mixtures associated with urban air particles.

This approach was later repeatedly used in different localities with different level of PM₁₀ and PM_{2.5} pollution. When EOM extracted from these particles was analyzed, total PAH-DNA adducts highly correlated with concentrations of B[a]P and c-PAHs [111-113]. All studies

showed that a cell-free system in conjunction with the sensitive ^{32}P -postlabeling is a suitable model to detect genotoxic potential of EOMs, particularly those containing c-PAHs, as well as to distinguish between direct and indirect genotoxicants in the complex environmental pollutants. Those results indicate that c-PAHs contribute predominantly to the total genotoxicity of various EOMs. The strong correlation between B[a]P and other c-PAH content in all EOMs tested in these studies ($r = 0.98$; $p < 0.001$) suggests that B[a]P may be used as an indicator of other c-PAHs in mixtures.

Topinka et al. [114] used acellular assay for the DNA adduct analysis of EOM according to the size fraction of particulate matter: 1-10 μm , 0.5-1 μm , 0.17-0.5 μm and $<0.17 \mu\text{m}$ and the concentration of c-PAHs. The fraction of 0.5-1 μm , that formed 37-46% of total PM mass, was the major carrier of c-PAHs, and induce highest genotoxicity detected as DNA adducts by ^{32}P -postlabeling.

Numerous studies analyzing the effect of c-PAHs, particularly B[a]P, on oxidative damage in cell lines *in vitro* or in animals *in vivo* have been published [115-121], but the results are conflicting, probably because of differences in the experimental protocols. Gabelova et al. [115] did not find any significant increase of oxidative DNA damage measured by a single cell gel electrophoresis assay (the Comet assay) in HepG2 cells treated with 7.5 μM B[a]P for 2, 24 or 48 h. In the study by Park et al. [116], B[a]P at concentrations up to 10 μM induced nonspecific DNA damage measured by the Comet assay after 24 h treatment of HepG2 cells. Lipid peroxidation, analyzed as malondialdehyde (MDA) levels, was increased but showed no dose-response. The authors concluded that oxidative DNA damage is probably related to B[a]P toxicity. In the A549 cell line, Garcon et al. [117] tested the effect of B[a]P treatment on lipid peroxidation measured as MDA levels. They treated the cells for 72 h with 0.05 μM B[a]P but found no increase. They hypothesized that the antioxidant defenses of the cells prevented the induction of lipid peroxidation by B[a]P [118, 119].

Rossner et al. [122] investigated the ability of organic extracts of size segregated aerosol particles (EOM; three fractions of PM, aerodynamic diameter 1-10 μm , 0.5-1 μm and 0.17-0.5 μm) to induce oxidative damage to DNA in an *in vitro* acellular system of calf thymus (CT) DNA with and without S9 metabolic activation. PM was collected in the Czech Republic at four places with different levels of air pollution. Levels of 8-oxodG tended to increase with decreasing sizes of PM. S9 metabolic activation increased the oxidative capacity of PM.

These results indicate that smaller size fractions are more potent inducers of oxidative damage to DNA. This observation is in agreement with other studies [123-125]. In these studies, however, water-soluble PM extracts were used. Moreover, end-point parameters for measuring the potency of PM to induce oxidative damage differed from Rossner's approach. There are only two other reports that used organic PM extracts in the acellular CTDNA system [124, 126] and only one that tested S9 metabolic activation of extracts [126], but none of these analyzed the oxidative capacity of individual size fractions. Thus, Rossner's results [122]) are probably the first showing that the ability of organic PM extracts to induce oxidative damage to DNA also increases with decreasing sizes of particles. This trend seems to be less pronounced after S9 metabolic activation of EOM. Thus, the presence of PAHs in EOM is probably not the only factor responsible for oxidative damage induction by PM organic extracts.

On the other hand, results of Rossner et al. [122] showed that metabolic activation of PAHs plays at least a partial role in the induction of oxidative damage to DNA because 8-oxodG levels in CT-DNA incubated with S9 fraction were significantly higher than in samples without S9 metabolic activation. Also, they observed a positive correlation between c-PAHs concentrations and 8-oxodG levels induced by PM. This correlation was stronger and statistically significant when PM extracts were incubated with S9 metabolic fraction.

The oxidative capacity of PM extracts increases with increasing levels of air pollution. Smaller size fractions of PM induce higher oxidative damage, which is caused partly by higher content of c-PAHs and partly by other unidentified factors.

7. Discussion

The average personal environmental exposure to approx. 10 ng/m³ of c-PAHs (or 1.6 ng/m³ of B[a]P) [86], or approx. twice as high c-PAHs concentrations by stationary monitoring – according to outdoor/indoor ratio) during winter increases DNA adduct formation and decreases repair efficiency, which may be further affected by genetic polymorphisms. This concentration of c-PAHs and its impact on increased DNA adduct formation seems to be important for re-evaluating the risk assessment of c-PAHs. PAH-DNA adduct formation represent one of the key first steps in carcinogenesis [127]. According to Phillips [128] the use of DNA adducts as a measure of exposure, several years prior to the onset (or clinical manifestation) of disease, can identify individuals at higher probability of subsequently developing cancer. Another study demonstrated significant correlations between DNA adduct levels and atherosclerosis [25]. Both examples demonstrate the potential of DNA adducts as biomarkers of risk.

DNA adducts determined by the ³²P-postlabeling method are sensitive biomarkers of environmental exposure to c-PAHs, if the study simultaneously includes personal and stationary monitoring, information on life style, determination of cotinine, vitamin and lipid levels, as well as genetic polymorphisms of metabolic and DNA repair genes.

Nowadays it is generally accepted that a high frequency of chromosomal aberrations in peripheral lymphocytes is predictive of an increased risk of cancer [32]. Therefore, it may be hypothesized that environmental exposure to c-PAHs that increases the genomic frequency of translocations represents a significant health risk. Surprising results were observed in the group of city policemen who were examined in January and March: the genomic frequency of translocations decreased similarly as did their exposure to c-PAHs. This reveals that these chromosomal aberrations are not so stable as originally expected [79]. When Binkova et al. [22] studied the relationship between chromosomal aberrations and DNA adducts in the same subjects using FISH, multiple regression analysis indicated that B[a]P-like DNA adducts are a significant predictor of the genomic frequency of translocations. Whole chromosome painting using the FISH technique is more sensitive than the originally used conventional cytogenetic method, which was not affected by the studied concentrations of c-PAHs.

Studies in the Czech Republic [22, 36, 90, 97] suggest that environmental exposure to concentrations higher than 1 ng B[a]P/m³ represent a risk of DNA damage as indicated by an increase in DNA adducts and an increase in translocations detected by FISH, increase of micronuclei as well as the increase of DNA fragmentation in the mature sperm.

A relationship between DNA adducts (biomarker of exposure) and chromosomal aberrations as determined by FISH (biomarker of effect) was observed for the first time [22].

Studies by Binkova et al. [18, 19, 22] and Topinka et al. [87] suggest that DNA adducts in the lymphocytes of subjects exposed to increased c-PAH levels are an appropriate biomarker of a biologically effective dose, directly indicating whether or not the extent of exposure to these compounds is related to an increased mutagenic and carcinogenic risk. All results indicate that molecular epidemiology studies should be done in a very complex manner, simultaneously using biomarkers of exposure, effect and susceptibility. Air pollution, and specifically c-PAHs, induce genetic damage and may significantly affect human health.

Although PAHs may induce oxidative stress during their metabolism by the formation of reactive quinones, the results of epidemiological studies on 8-oxodG levels after occupational, as well as environmental, PAH exposure are contradictory. Several authors reported a positive correlation between PAH exposure, measured by urinary levels of 1-hydroxypyrene, and 8-oxodG levels, either in lymphocytes or in urine [129-132]. Others did not see any correlation between 8-oxodG levels and urinary 1-hydroxypyrene and/or PAH levels in the air [133-135]. Similarly, Rossner et al. [83] did not find any effect of either personal or stationary c-PAH exposure on 8-oxodG levels in urine. These observations suggest that while c-PAHs may have the potential to induce oxidative DNA damage, other factors probably play a role in the response of the organism to environmental pollution.

Unlike c-PAHs and B[a]P, Rossner et al. [89] did not observe any effect of stationary PM_{2.5} and PM₁₀ on levels of 15-F_{2t}-IsoP when PM concentrations over a 3-day period immediately preceding urine collection were used in the statistical analysis. Only when the multivariate model was applied did the effect of PM_{2.5} exposure reach borderline significance. Interestingly, when concentrations of PM_{2.5} and PM₁₀ over various 3-day periods before sampling were included in the statistical analysis, they found significant effects of PM exposure on 15-F_{2t}-IsoP levels. This observation suggests that 15-F_{2t}-IsoP levels, unlike 8-oxodG, are not markers of immediate effect; rather, they reflect the exposure to pollutants that occurred at least 3-4 weeks before sampling. The possible explanation may be the existence of DNA repair mechanisms that remove damage to DNA shortly after it occurs. Damage to lipids is not repaired; rather the lesions accumulate and thus allow the detection of exposure to pollutants longer period before sampling.

Among other factors that seem to affect the levels of oxidative stress markers, a positive association between cotinine (considered as a marker of passive smoking) and 15-F_{2t}-IsoP levels was observed [136-138], although negative reports have also been published [139].

In vitro studies reveal that the biological activity of PM_{2.5} may differ according to the sources and the content of c-PAHs. The genotoxic risk may be substantially different when comparing industrial emissions vs. traffic related emissions or emissions from the local heating using coal.

Review of *in vitro* studies allows to propose the cell free system with CT-DNA to be used as a system to evaluate the biologic activity of organic compounds extracted from particulate matter, detecting genetic damage as DNA adducts or oxidative damage as 8-oxodG, as a first information about a possible genetic risk of air pollution, and the different quality of respirable particles according to the content of c-PAHs adsorbed on their surface as well as oxidative damage induced according to the size of PM.

8. Conclusion

All discussed studies indicate, that DNA adducts, Comet assay and DNA fragmentation in sperm are sensitive biomarkers of exposure to c-PAHs in polluted air, chromosomal aberrations by FISH and micronuclei as biomarkers of effect, and 8-oxodG and 15-F2t-IsoP as biomarkers of oxidative damage.

It seems that when using these biomarkers the dose-effect is seen only in a certain range, probably up to 10 ng B[a]P/m³.

It is important to identify simultaneously the gene susceptibility, especially the genetic polymorphisms of metabolic genes and genes encoding DNA repair enzymes. DNA damage may be further affected by life style as smoking, ETS, diet – intake of vitamins A, C, E, folic acid, oxidative metabolism by lipid metabolism (triglycerides, cholesterol, HDL, LDL) – it is therefore pertinent to analyze all these endpoints in the biological material in the course of molecular epidemiology studies.

New perspectives may be seen in using the microarray methods, e.g. studying the gene expression of genes coding DNA repair enzymes.

Summing up, molecular epidemiology studies on the environmental exposure to c-PAHs in ambient air should be very complex: determining personal exposure, DNA and oxidative damage, gene susceptibility and life style factors. It will bring new results, which may specify new information important to evaluate properly c-PAHs human health risk.

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