Chapter 7
Molecular Epidemiology Focused on Airborne Carcinogens

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7.1 Introduction

7.1.1 Air Pollutants and Their Impact on Human Health

Humans are constantly exposed to thousands of xenobiotics, that are present in the ambient air, soil, water, as well as in food and various products of human activity. Routes of exposure include inhalation, ingestion and/or dermal contact. Ambient air pollution is considered the most serious in terms of its effect on human health, because it is ubiquitous in both industrialized and developing countries and thus a vast majority of human population suffers from its negative impact. Combustion of fossil fuels due to traffic, local heating and/or industrial production represent a predominant source of air pollution. It has been shown that air pollution has both acute and chronic effects on human health affecting different organs and systems, particularly the respiratory, cardiovascular and nervous systems (Kampa and Castanas 2008). Even though air pollutants are a diverse group of xenobiotics, they can be classified into four categories: gaseous pollutants [SO_2, NOx, CO, ozone and volatile organic compounds (VOCs)], persistent organic pollutants (POPs; e.g. dioxins), metals, and particulate matter (Kampa and Castanas 2008). Biological effects of these compounds may be exerted either through the interaction of the chemicals with biomolecules (nucleic acids, lipids and proteins) thus hampering their function or, in case of nucleic acids, inducing mutations, or by generation of reactive oxygen species (ROS) that cause oxidative damage. In the following text, we will discuss health effects of some of the most important air pollutants.

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Volatile organic compounds (VOCs) include a class of organic compounds generated mostly as by-products of fuel combustion in road transportation. Most studies concerning VOCs focused on benzene which is a known human carcinogen responsible for hematological malignancies. The harmful effects of benzene on human health are linked to the formation of ROS that induce oxidative stress, and thus damage DNA and other macromolecules (Barreto et al. 2009). Readers are referred to Chap. 6 for more detailed understanding on the mechanisms involved in benzene-induced carcinogenesis.

Dioxins, polychlorinated biphenyls (PCBs) and pesticides are among the class of persistent organic compounds. These chemicals are characterized by their stability in the environment and by the ability to increase their effect as they move through food chain. Dioxins are produced while burning chlorine-containing material; PCBs were widely used in many industrial products including e.g. coolants, plasticizers, flame retardants or lubricating oils. Many of the compounds are carcinogenic/suspected carcinogens to humans. Dioxins and some of the PCBs interact with aryl hydrocarbon receptor and may thus affect expression of various genes.

Many metals are natural components of the Earth's crust but may be released into the air during combustion or industrial processes. Some of the metals (Ca, Zn, Mg, Fe) at low doses are important cellular components forming specific protein domains (e.g. Zn fingers, hemoglobin molecules). However, at higher concentrations metals may induce ROS formation or interfere with enzyme functions by replacing naturally-occurring ions (e.g. Zn) in protein domains. In this way, carcinogenic properties of some heavy metals may be manifested.

Particulate matter (PM) is a broad class of air pollutants that encompasses particles of various sizes (coarse particles of aerodynamic diameter $\leq$10 μm, fine particles of aerodynamic diameter $\leq$2.5 μm, ultrafine particles of aerodynamic diameter $\leq$100 nm) and chemical composition. PM is mostly emitted during activities associated with burning organic material (local heating, industrial production, power plants, road traffic), but may also arise from natural sources including windblown dust. After inhalation, PM is deposited in upper airways, but smaller particles penetrate to the lungs, some of them reaching alveoli. Ultrafine particles may even enter the bloodstream and may thus be carried to distant parts of the body. Depending on the source of PM, particles may contain metals, reactive gases, material of biological origin or various organic compounds including polycyclic aromatic hydrocarbons (PAHs). PAHs have been identified to be responsible for most of the genotoxic activity of PM (Binkova et al. 1999) causing damage to DNA and proteins by inducing DNA and protein adducts. Inhalation of fine and ultrafine PM also leads to inflammation and subsequent production of reactive oxygen species (Mazzoli-Rocha et al. 2010). 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 phagocyted by macrophages that consequently produce ROS), and the presence of various chemicals on the surface of PM (e.g. metals, PAHs) with prooxidant properties. It has been repeatedly shown that exposure to PM correlates with increased mortality caused by lung cancer and cardiovascular diseases (Dockery et al. 1993; Pope et al. 1995; Sarnat et al. 2001). Pope et al. suggested that a long term increase in PM2.5 of 10 μg/m^3 is associated with an 8 % increase in lung

cancer mortality in adult men (Pope et al. 2002). Despite the fact that other factors related to cancer incidence, such as smoking habits 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 (Beaglehole et al. 1993).

After entering the organism, some xenobiotics are metabolized and form active compounds that may interact with cellular macromolecules. Other chemicals do not require metabolic activation and act as direct mutagens/carcinogens.

Many polycyclic aromatic hydrocarbons, products of incomplete combustion of organic material, are typical examples of compounds requiring metabolic activation. Three principal pathways of PAHs metabolism have been proposed (Xue and Warshawsky 2005). **The Bay region dihydrodiol epoxides pathway** involves three enzymatic reactions: oxidation of a double bond catalyzed by cytochrome P450 enzymes to unstable arene oxides, their hydrolysis by microsomal epoxide hydrolases to trans-dihydrodiols and cytochrome P450-catalyzed oxidation to diol-epoxides that can bind to DNA. **The radical cation pathway** includes one electron oxidation catalyzed by P450 peroxidase. In this pathway PAHs are oxidized independently of molecular oxygen; organic or lipid hydroperoxides are used as the oxidant source instead. Radical cations are electrophilic and capable of interacting with nucleophilic centers in cellular macromolecules including DNA. Both pathways lead to formation of reactive intermediates that bind to macromolecules and form adducts. Adducts negatively impact the function of macromolecules and in case of DNA may result in induction of mutations and thus increase the risk of cancer. **Activation through PAH-*o*-quinones** is the third major pathway of PAH metabolism. In this pathway dihydrodiol dehydrogenases catalyze the oxidation of trans-dihydrodiols to PAH *o*-quinones. PAH *o*-quinones are electrophilic metabolites that enter redox cycles and generate ROS thus leading to oxidative damage of DNA and other macromolecules.

Apart from this reaction ROS may be generated by other metabolic processes or by inflammation. These processes are among the endogenous sources of ROS. Exogenous sources include environmental factors such as smoking, diet (Loft et al. 1992; Klaunig and Kamendulis 2004), ultraviolet radiation, ionizing radiation or exposure to environmental pollution (Wu et al. 2004). ROS can attack lipids, proteins and nucleic acids (Cooke et al. 2003). 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 recombination or non-homologous end-joining repair seal the breaks, to stable chromosomal translocations. The attack of ROS on lipids that leads to lipid peroxidation may have also potentially serious consequences, as it may damage cellular membranes 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 (Slade et al. 2010). Oxidation of proteins generates carbonyl groups mostly on side chains of protein molecules. These modifications affect the function of proteins and interfere with enzymatic activity and structural properties of proteins (Dalle-Donne et al. 2006).

7.2 Molecular Epidemiology and Biomarkers

From the health of human population point of view, it is very important to estimate damage to the organism caused by xenobiotics in early stages before exposure-related diseases are manifested. This requirement can be fulfilled by the implementation of human biomonitoring into healthcare practice. In general, human biomonitoring may either concentrate on measurement of xenobiotic levels in body fluids or on analyses of changes of biomolecules. While the former approach is analytically less demanding, it does not address the question of the biological effect of xenobiotics on human organism. This is why the latter approach is preferable. Its application in the recent decades has developed into the field of molecular epidemiology.

Molecular epidemiology aims to merge sophisticated and highly sensitive laboratory methods with analytical epidemiological methods. It 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 (Perera and Whyatt 1994). The most common view is that this approach represents a natural convergence of molecular biology and epidemiology (Perera et al. 1998).

Molecular epidemiology focuses on analyses of biomarkers as parameters that allow for quantitative differentiation of subjects exposed to harmful compounds/factors from a normal population. Thus, the biomarkers rather than the disease are used to assess the risk of environmental exposure (Albertini et al. 1996; Albertini 1998). The number of biomarkers available for evaluating genetic and cancer risk in humans is quite large and they may be broadly classified into three groups: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility (Fig. 7.1). Their utility for human biomonitoring is based on the paradigm of environmentally induced cancer (Committee on Biological Markers of the National Research Council 1987). The biomarkers encompass processes of interaction of xenobiotics with the organism starting with exposure and absorption, followed by their metabolism, distribution, critical target interaction (i.e. damage to macromolecules and repair), and finally resulting in genetic changes and disease. In relation to the recent technical development and emergence of omics technologies new biomarkers, also called omics biomarkers (Bonassi et al. 2013), have appeared (Fig. 7.1). These biomarkers, that can be classified as intermediate omics biomarkers of effect (Vineis et al. 2013), include e.g. analyses of mRNA expression (transcriptomics), DNA methylation (epigenomics) and microRNA (miRNA) expression (micromics). Analyses of expression of selected individual genes have been expanded to gene expression profiling of the whole genome. The biomarkers that strive to address mechanisms of regulation of gene expression include methylation profiles of the genome and miRNA analyses. Although the studies that apply the new biomarkers in the biomonitoring are still relatively scarce, the results are promising and indicate that new avenues have opened in biomarkers research. However, an ultimate answer to the

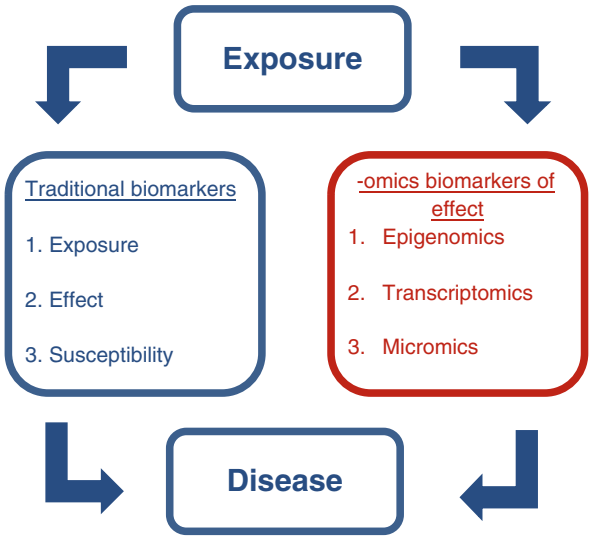

Fig. 7.1 Overview of traditional and omics biomarkers applied in molecular epidemiology and their incorporation into the exposure-disease link

question how xenobiotics impact human health should be provided on the protein level. This answer may be solved in the future when the emerging field of proteomics becomes more advanced and available for researches worldwide.

An ideal biomarker should meet certain criteria. It should be: (1) sensitive enough to be detectable even at low levels of exposure; (2) specific so that it reflects exposure to compounds of interest; (3) standardized and validated so that its analysis is reproducible in both intra- and interlaboratory settings; (4) its analysis should be inexpensive and technically relatively easy to perform; (5) collection of samples for the biomarker analysis should be non-invasive; and (6) its detection method should be high-throughput so that analyses of larger sample sets can be easily performed.

In the following text, we will discuss individual groups of biomarkers, give examples of some of the most commonly used biomarkers and report the results of studies in which the biomarkers have been analyzed.

7.2.1 Biomarkers of Exposure

The concentration of xenobiotics, their metabolites, or levels of modified macromolecules formed as a result of interactions between xenobiotics and target tissue/cell/molecule are included among the biomarkers of exposure. The concentrations of xenobiotics and their metabolites may be measured in body fluids (urine, blood). These biomarkers typically include detection of metals in urine or blood plasma, analyses of metabolites of PAHs, PCBs, pesticides and other xenobiotics in urine and/or blood plasma. However, as mentioned above, these parameters do not reflect the actual effect of the compounds on human organism; they simply serve as

information on the amount of xenobiotics that entered/left the organism. For this reason, biomarkers of biologically effective dose that includes levels of modified cellular macromolecules (proteins, lipids and DNA) are a parameter of choice for molecular-epidemiological studies. DNA or protein adducts have been of particular interest in many studies.

DNA adducts quantify the biologically effective dose of genotoxic compounds that were bound to DNA as a target molecule of carcinogenesis (Binkova et al. 1995, 1996, 1998, 2007; Phillips and Castegnaro 1999). Typical examples of genotoxic compounds include carcinogenic PAHs (c-PAHs) that form bulky DNA adducts or reactive oxygen species (ROS) that induce formation of e.g. 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) as a result of interaction with DNA. If DNA adducts are not effectively repaired, they might be fixed as mutations during replication. Thus, DNA adduct levels have a direct relation to mutagenesis and carcinogenesis. Data are accumulating about the relation of DNA adducts induced by environmental exposure to complex mixture components such as carcinogenic polycyclic aromatic hydrocarbons (Georgiadis et al. 2001; Lewtas 2007) and incidence of malignant tumors and other degenerative diseases (Migliore and Coppede 2002; Binkova et al. 2002).

7.2.1.1 Bulky DNA Adducts

Bulky DNA adducts are markers of exposure to genotoxic aromatic compounds and the ability of an individual to metabolically activate carcinogens and repair DNA damage (Phillips 2005). The use of DNA adducts as a measure of exposure can identify individuals at higher probability of subsequently developing cancer several years prior to the onset (or clinical manifestation) of the disease (Phillips 2005). Bulky DNA adducts determined by the standardized ^{32}P-postlabeling method (Fig. 7.2) are also sensitive biomarkers of environmental exposure to c-PAHs, if the study simultaneously includes personal and stationary monitoring, information on the life style, determination of cotinine, vitamin and lipid levels, as well as genetic

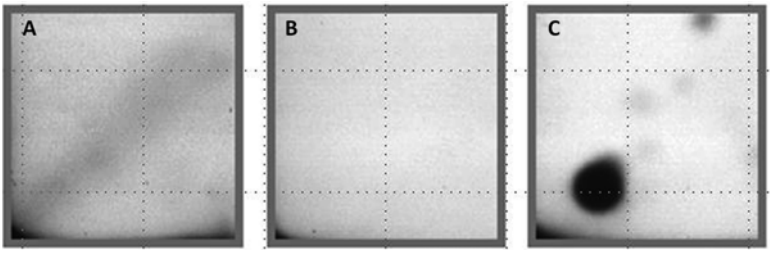

Fig. 7.2 A typical result of bulky DNA adducts analysis by ^{32}P-postlabeling in human peripheral blood lymphocytes. (**a**) DNA isolated from peripheral blood lymphocytes of a human subjects exposed to air pollution; (**b**) A negative control (water blank); (**c**) A positive control (DNA isolated from the lungs of a rat treated with B[a]P)

polymorphisms of metabolic and DNA repair genes (Phillips and Castegnaro 1999; Palli et al. 2001, 2003; Godschalk et al. 2001; Binkova et al. 2007; Georgiadis et al. 2001; Kyrtopoulos et al. 2001; Peluso et al. 1998; Autrup et al. 1999).

^{32}P-postlabeling was widely used in the Czech Republic simultaneously with the personal monitoring of exposure to c-PAHs. The studies included subjects exposed to high levels of air pollutants in Northern Bohemia (B[a]P concentrations up to 7.5 ± 3.6 ng/m^3) (Binkova et al. 1995, 1996), capital city of Prague (groups of city policemen and bus drivers of a total of 950 subjects) (Sram et al. 2011) and in highly polluted Ostrava region (Rossner et al. 2013b). The data obtained for biomarkers of exposure and effect from these studies were used for the pooled analysis. Using multivariate logistic regression, the relationship between personal exposure to B[a]P and DNA adducts measured by ^{32}P-postlabeling was calculated (DNA adducts $= 1.042 + \text{B[a]P} \times 0.077$, $p < 0.001$) (Sram et al. 2011). These results indicate that c-PAH exposure plays a crucial role in DNA adduct formation in lymphocytes.

A pooled analysis of bulky DNA adducts in white blood cells of 3,600 subjects from several European countries was published in 2010 (Ricceri et al. 2010). Lowest DNA adduct levels were observed in spring, followed by summer, autumn and winter. Bulky DNA adduct levels were significantly lower in Northern Europe than in Southern Europe. Authors observed weak associations between bulky DNA adducts and exposure variables. The effect was more pronounced, if DNA adducts were determined in peripheral lymphocytes. In a review of 18 studies that analyzed traffic-associated bulky DNA adducts, including exposure assessment, differences between exposed and control subjects were observed; in nine studies an association between DNA adducts and exposure was detected (DeMarini 2013).

However, the relationship between the exposure to PAHs (B[a]P) and bulky DNA adduct levels is not linear. As Lewtas et al. pointed out, in case of higher occupational exposure to PAHs, as in coke oven workers, the exposure-DNA adduct relationship does not follow dose-response curve. This superlinear response is consistent with saturation of enzyme activity, as would be expected at high doses for carcinogens that require metabolic activation (Lewtas et al. 1997).

7.2.1.2 Oxidative Damage Markers

As mentioned above, all cellular macromolecules may be a target for ROS attack. Although oxidative DNA damage is the most serious because it may lead to mutations (e.g. the presence of 8-oxodG in DNA may result in GC to TA transversions), lipid peroxidation yields highly reactive intermediates that cause secondary damage to cellular structures. Unlike DNA, no repair systems exist for oxidized proteins; they can be either recognized by the proteolytic system and degraded by proteasomes or accumulate in the organism as dysfunctional molecules (Dunlop et al. 2009).

8-oxodG is the most often studied product of DNA oxidation and its presence in urine or lymphocyte DNA was used as a marker of disease or environmental exposure in numerous studies (reviewed e.g. in Loft et al. 2008; Rossner and Sram

2012). Urine is particularly suitable matrix for the analysis of 8-oxodG levels: the samples can be obtained non-invasively, in sufficient quantity, 8-oxodG in urine is very stable and there is no risk of artifactual DNA oxidation during the sample handling. Many studies have shown the effect of traffic-related exposures on 8-oxodG excretion in urine. Traffic exhaust contains a complex mixture of chemicals with the ability to induce ROS production and subsequently oxidative DNA damage. In agreement with this fact, urinary 8-oxodG levels were significantly elevated in taxi drivers (Chuang et al. 2003), highway toll station workers (Lai et al. 2005), city and long-distance bus drivers (Rossner et al. 2007, 2008a; Han et al. 2010) and diesel exhaust emission inspectors (Lee et al. 2010). Interestingly, in another study that followed city policemen in the winter and spring season which differed in levels of air pollutants no effect of seasonal variability was observed (Rossner et al. 2011a).

Air pollution not directly related to traffic resulted in elevated urinary 8-oxodG levels in firefighters (Hong et al. 2000), coke-oven workers (Wu et al. 2003) and boilermakers (Kim et al. 2004). Svecova et al. analyzed the effect of PM10, PM2.5, c-PAHs and B[a]P, on urinary levels of 8-oxodG in 894 children aged 6–10 years living in the Czech Republic. All analyzed pollutants increased oxidative damage within one week of exposure (Svecova et al. 2009). On the other hand, no effect of environmental air pollution on 8-oxodG excretion was observed in a group of office workers and city policemen living in heavily polluted region of the Czech Republic (Rossner et al. 2013a).

Products of lipid peroxidation may be formed by three different mechanisms: free-radical mediated, nonradical-nonenzymatic and enzymatic (Niki 2009). These reactions give rise to a number of products that in low concentrations are important redox signaling mediators. However, at higher concentrations they cause damage to the organism and have been implicated in pathogenesis of various diseases. From the molecular epidemiology point of view, several lipid peroxidation products (LPO) are commonly analyzed: conjugated diens, lipid hydroperoxides, malondialdehyde (MDA)/thiobarbituric acid-reactive substances (TBARS) and F_2-isoprostanes (Moller and Loft 2010). The levels of 15-F_{2t}-isoprostane (15-F2t-IsoP), a commonly used biomarker of lipid peroxidation that is formed from arachidonic acid by a free radical-mediated peroxidation of arachidonic acid independent of cyclooxygenase (Morrow et al. 1990), have been consistently shown to be elevated after exposure to air pollutants including cigarette smoke (Kato et al. 2006), ozone (Chen et al. 2007), c-PAHs and PM (Rossner et al. 2007, 2008b, 2011a, 2013a; Barregard et al. 2006; Nuernberg et al. 2008). On the other hand, levels of lipid hydroperoxides, that are formed as a product of reaction between oxygen and carbon radical in lipids, did not differ between traffic officers and controls sampled in Catania, Italy (Bonina et al. 2008). TBARS levels, that are usually considered a non-specific marker of lipid peroxidation, were positively associated with exposure to $PM_{2.5}$ in a group of senior subjects (Liu et al. 2009). This marker was also affected in subjects who moved to a highly polluted location (Mexico City). Interestingly, the levels of TBARS dropped to normal levels after a 16 weeks stay in the city (Medina-Navarro et al. 1997).

Protein carbonyl groups, used as a marker of protein oxidation, are relatively difficult to induce and thus they probably reflect more severe cases of oxidative stress associated with protein dysfunction (Dalle-Donne et al. 2003). The use of this

marker in molecular-epidemiological studies is not very common and the results are conflicting (Bagryantseva et al. 2010; Ceylan et al. 2006; Rossner et al. 2007, 2008b, 2011a, 2013a). The usefulness of this marker in biomonitoring of the effect of air pollutants on human organism remains to be clarified.

7.2.1.3 Comet Assay

The comet assay (single cell gel electrophoresis, SCGE) is widely used in human biomonitoring to measure DNA damage as a marker of exposure to genotoxic agents or to investigate genoprotective effects (Collins et al. 2014). The comet assay allows the detection of both single and double strand breaks (DSB) depending in assay conditions; 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 single strand DNA breaks or oxidative damage that may be converted to DSB during DNA replication or repair (Obe et al. 2002). When combined with specific bacterial repair enzymes, it identifies a broad spectrum of additional lesions including oxidized purines and pyrimidines (Collins 2004). The comet assay is characterized by relative simplicity, low requirements on the number of analyzed cells as well as ability to detect DNA damage independently of the cell cycle.

DeMarini reviewed the use of the method to detect DNA damage induced by traffic in seven exposure groups. In all groups, the higher level of DNA damage was observed in the exposed versus the control populations; the association between exposure levels and DNA damage was observed in all, but one study (DeMarini 2013). Collins et al. further reviewed studies focusing on the effect of air pollution, especially PAHs, on DNA damage. In all ten studies, comet assay detected higher DNA damage in exposed groups (Collins 2004).

Novotna et al. used the comet assay to analyze genetic damage in 54 city policemen (exposed) and 11 controls (working indoors); the sampling was performed in two seasons (January and September). 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 in January only. The correlation analysis revealed a strong association in the exposed group between the level of oxidative DNA damage and personal exposure to c-PAHs in January (Novotna et al. 2007).

All these studies strongly suggest that the data obtained from the comet assay may serve as an important biomarker of exposure to air pollution.

7.2.2 Biomarkers of Effect

These biomarkers are characterized as measurable biochemical, or physiological alterations within the organism that are known to negatively affect health or are associated with progression of a disease. They include parameters that characterize chromosomal changes or DNA breaks.

7.2.2.1 Chromosomal Aberrations

Chromosomal aberrations in human peripheral blood lymphocytes are recognized as a valuable biomarker of effect in molecular epidemiology. Three basic cytogenetic techniques have been used over time for evaluation of genetic damage – conventional cytogenetic analysis (CCA), analysis of micronuclei (MN) and fluorescent in situ hybridization (FISH).

CCA, as a method focused mainly on unstable aberrations such as chromosomal and chromatid breaks (Fig. 7.3a), has been accepted as a technique suitable for the biological monitoring of genetic damage in somatic cells since the early 1970s. This method was frequently used in various studies to investigate the levels of damage in people exposed to clastogenic agents in the workplace (Natarajan and Obe 1980; Sram et al. 2004). Pooled European data (22,358 subjects) proved that chromosomal aberrations are a valuable standardized and validated biomarker of effect

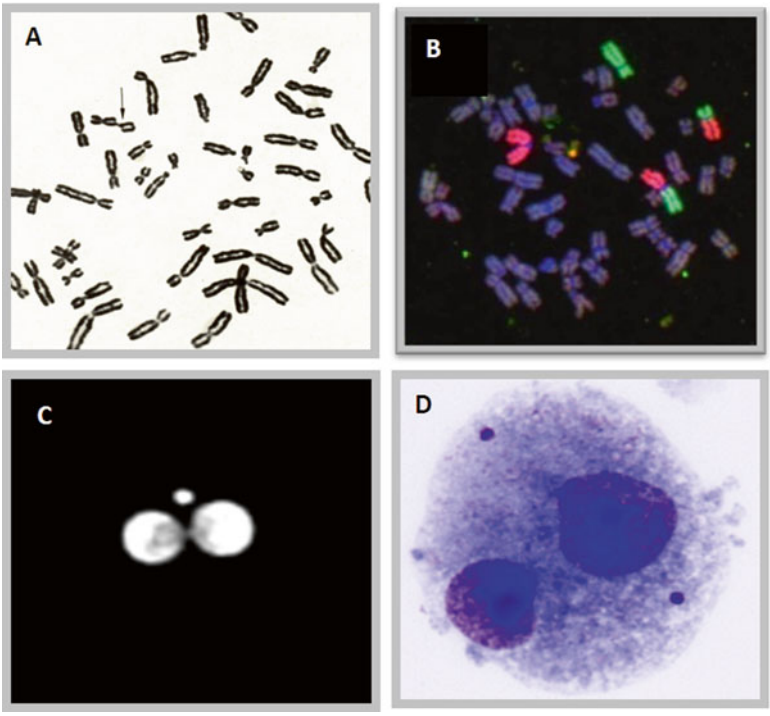

Fig. 7.3 Examples of cytogenetic findings in human peripheral blood lymphocytes, detected by various methodological approaches used in genetic toxicology: (**a**) Chromatid break identified by conventional cytogenetic analysis in metaphases chromosomes; (**b**) Reciprocal translocation between chromosomes #1 (painted *red*) and #4 (painted *green*) identified by fluorescent *in situ* hybridization in metaphases chromosomes; (**c**) Cytochalasin-B-blocked binucleated cell with one micronuclei stained by DAPI, identified by automated image analysis; (**d**) Cytochalasin-B-blocked binucleated cell with two micronuclei stained by Giemsa, identified by visual technique

(Hagmar et al. 2004; Rossner et al. 2005; Bonassi et al. 2008). Low cost of the analysis of Giemsa stained slides is an important advantage of the method; the disadvantage is its laboriousness. This method usually involves evaluation of 100 or 200 well-spread metaphases per subject depending on the size of exposed and/or control groups. There were some efforts for automation of this method, but current state of the art allows scanning metaphases and evaluation of dicentric chromosomes only; both chromosomal and chromatid breaks are not recognized by any automation system yet.

The analysis of **MN** in human peripheral blood lymphocytes that has been used since 1976 (Countryman and Heddle 1976) is the most frequently applied cytogenetic method in molecular epidemiology (Fig. 7.3c, d). Current assay procedure focused on evaluation of MN in binucleated cells (BNC) dates its origin to 1985, when cytochalasin-B was first used to inhibit cytokinesis (Fenech and Morley 1985). 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 (Fenech and Morley 1985). 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 (Kirsch-Volders et al. 2014).

The HUman MicroNucleus international collaborative project (HUMN), established in 1997, pooled data from more than 6,700 subjects and confirmed that an elevated MN frequency is predictive of an increased cancer risk (Bonassi et al. 2007). Another analysis in this project confirmed that MN frequency is not elevated in moderate smokers and only heavy smokers showed a significant increase in genotoxic damage as measured by the micronucleus assay (Bonassi et al. 2003). Though the visual scoring of MN is relatively easy for a trained person, the scoring of thousands of cells is very time-consuming and tiring work, moreover affected by interpersonal and interlaboratory variability (Fenech et al. 2003). Unlike CCA, there are some validated options for automation and image analysis of MN based on scanning and scoring of MN on Giemsa or DAPI stained slides (Fenech et al. 2013). One of the current biomonitoring studies, the first one that used automated image analysis, showed the impact of season variability of air pollution (concentration of B[a]P) on the frequency of MN in BNC in moderately polluted area (Rossnerova et al. 2009). Using multivariate logistic regression, the relationship between personal exposure to B[a]P and micronuclei expressed as MN/1,000 cells was calculated ($MN = 5.18 + B[a]P \times 1.11$, $p = 0.002$) for this location. Another study performed in highly polluted Ostrava region in the Czech Republic generally failed to show biomarker changes. These results were explained by differences in gene expression between locations and a possibility of adaptive response for population living in highly polluted area was suggested (Rossner et al. 2013a, b, 2014b). This results opened new course for future research.

FISH technique is another method having been used in genetic toxicology since the late 1980s. It is focused mainly on identification of stable chromosomal aberrations like non-reciprocal translocations, reciprocal translocations or insertions,

which are not easily recognized by conventional method (Fig. 7.3b). This method also allows identification of unstable chromosomal aberrations represented e.g. by acentric fragments, but the results are generally limited to the painted chromosomes.

Due to the fact that different laboratories paint different chromosomes by the whole chromosome painting, two important tools were suggested for comparison of results between studies: (1) aberrant cells are classified according to the Protocol for Aberration Identification and Nomenclature (PAINT) (Tucker et al. 1995); (2) the genomic frequencies of translocations (F_G) are calculated by formula suggested by Lucas et al. (1992) where exchange frequencies obtained from each chromosome are calculated for the whole genome by dividing the observed frequencies by the factor of 2.05 f_p (1 − f_p), where f_p is the fraction of painted DNA converted by individual chromosomes.

The background translocation frequency by age, gender, race and smoking status were assessed in pooled data from healthy humans (Sigurdson et al. 2008). FISH technique was successfully used in various human studies for evaluation of the effect of exposure e.g. carcinogenic polycyclic aromatic hydrocarbons (c-PAHs), metals or radiation (Beskid et al. 2007; Palus et al. 2003; Edwards et al. 2004).

Surprising results were observed in the group of city policemen who were examined in two seasons with different concentrations of air pollutants (January and March 2004): the genomic frequency of translocations decreased similarly as did the subjects' exposure to c-PAHs. This suggests that chromosomal aberrations are not so stable in time as originally expected (Sram et al. 2007b). Using multivariate logistic regression the relationship between personal exposure to B[a]P and the genomic frequency of translocations measured by FISH was calculated ($F_G/100 = 1.255 + B[a]P \times 0.082$, $p < 0.05$). When Binkova et al. studied the relationship between chromosomal aberrations and bulky DNA adduct levels in the same subjects, multiple regression analysis indicated that B[a]P-like DNA adducts are a significant predictor of the genomic frequency of translocations (Binkova et al. 2007).

Whole chromosome painting using the FISH technique is more sensitive than the conventional cytogenetic method, which was not affected by the studied concentrations of c-PAHs. Nevertheless this cytogenetic method is generally based on the fact, that the sensitivity of each chromosome to DNA damage is the same. However, some studies discussed different sensitivity of individual chromosomes (Orjuela et al. 2010; Rossner et al. 2014a).

7.2.2.2 Sperm DNA Fragmentation

Sperm DNA fragmentation can be attributed to various pathological conditions including cancer, fever, age, or infection. Many environmental conditions, such as chemotherapy, radiation, air pollution, smoking, as well as ROS can also affect DNA fragmentation in sperm. It is now recognized that elevated sperm DNA fragmentation has a significant effect on reproductive outcome (Evenson et al. 2002;

Larson-Cook et al. 2003). As illustrated below, sperm DNA fragmentation was identified as a sensitive biomarker of air pollution.

Sperm DNA fragmentation is determined by the sperm chromatin structure assay (SCSA). The sperm sample is stained with acridine orange, which is a metachromatic DNA dye that fluoresces 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 (Evenson et al. 2007; Evenson 2013).

Using the SCSA, Rubes et al. studied the impact of air pollution to sperm DNA damage repeatedly in the same donors living in the polluted Northern Bohemian region (Rubes et al. 2005). DNA fragmentation index (DFI), defined as a percentage of mature sperm with abnormal chromatin/fragmented DNA, was significantly affected by the exposure. Other parameters (sperm concentration, semen volume, sperm morphology and sperm motility) were not associated with air pollution. It was the first study reporting association between exposure to ambient air pollution and DNA fragmentation in human sperm. These results were further confirmed by another study (Rubes et al. 2010), in which DNA fragmentation was observed in mature spermatozoa in subjects exposed to concentration of 1 ng B[a]P/m^3.

7.2.3 Biomarkers of Susceptibility

Biomarkers of susceptibility mostly take into account the role of genetic makeup of the organism in the response to the exposure to xenobiotics. These biomarkers are represented by single nucleotide polymorphisms (SNPs), studied particularly in genes proven to be critical e.g. for metabolic activation of xenobiotics (oxygenases of cytochromes P450), their detoxification (glutathione-S-transferases), or repair pathways (e.g. *XRCC1*, *XPD*, *hOGG1*) (Tuimala et al. 2002; Thacker and Zdzienicka 2003; Kelada et al. 2003). The saturation of the organism by vitamins (e.g. A, C, E, folic acid) is also regarded as a factor affecting susceptibility to the genotoxic and carcinogenic effects of xenobiotics. Vitamins are known to play a significant role as free radical scavengers and antioxidant agents; they also affect the synthesis of DNA repair enzymes (Zijno et al. 2003; Ames 2001; Fenech 2001; Fenech and Ferguson 2001).

It has been shown that levels of biomarkers of exposure and effect are modulated by genetic polymorphisms in relevant genes. Palli et al. demonstrated the effect of polymorphisms in *XPD*, a DNA repair gene, on bulky DNA adduct levels of traffic workers and general population exposed to high levels of genotoxic agents related to vehicle emissions (Palli et al. 2001). The study of Godschalk et al. provided the evidence for combined effects of genetic polymorphisms in *GSTM1*, *GSTT1*, *NAT1* and *NAT2*, genes encoding proteins responsible for detoxification of xenobiotics, on bulky DNA adduct formation in smoking individuals and indicated that simultaneous assessment of multiple genotypes may identify individuals at higher cancer risk (Godschalk et al. 2001). Another study of Palli et al. confirmed that biomarkers of

dietary intake of antioxidants as well as genetic susceptibility markers (*GSTM1*) modulate bulky DNA adduct levels in healthy adults (Palli et al. 2003). Binkova et al. demonstrated that smoking, vitamin C and polymorphisms in *XPD*, *XRCC1* and *GSTM1* are significant predictors for total bulky DNA adduct levels (Binkova et al. 2007). Rubes et al. showed for the first time that men who are homozygous null for *GSTM1* exhibit increased susceptibility to sperm DNA damage associated with exposure to air pollutants (Rubes et al. 2007). In another study, DNA fragmentation index in mature spermatozoa increased after B[a]P exposure and was modulated by a polymorphism in metabolic (*CYP1A1MspI*, *GSTM1*) and DNA repair genes (*XRCC1*, *XPD6*, *XPD23*) (Rubes et al. 2010). In a study by Novotna et al. that used the comet assay to analyze genetic damage in city policemen and controls, regression analysis revealed the influence of genetic polymorphism in *CYP1A1*, *MTHFR*, *MS* and *p53* genes on the level of oxidative and unspecified DNA damage (Novotna et al. 2007). The frequency of stable chromosome aberrations analyzed by the FISH technique was modified by genetic polymorphisms in *CYP1A1*2C*, *GSTP1*, *EPHX1*, *p53* and *MTHFR* genes (Sram et al. 2007a).

In the last couple of years genome-wide association studies (GWAS) showed that many common genetic variants of small, additive effect (McHale et al. 2010) located both in genes and regulatory elements probably play a decisive role in the overall susceptibility of the organism to negative effects of xenobiotics. Thus, nowadays, studies focusing on a small number of SNPs in pre-selected genes are not regarded as sufficient to address the role of genetic susceptibility to e.g. exposure to xenobiotics or to a certain disease; the research in this field has shifted towards large studies that analyze SNPs in thousands of samples using genome-wide approaches (Evangelou and Ioannidis 2013).

7.2.4 Omics Biomarkers

The central dogma of molecular biology that states: “DNA makes RNA makes protein” describes the principle of gene expression. It was formulated by Nobel Prize winner Francis Crick in 1970. The central dogma says “how?” the genetic information flows, but does not answer the question “how many?”, i.e. how many RNAs and proteins are produced during gene expression. For this reason it is important to study the regulatory elements that control intensity of transcription by DNA methylation and intensity of translation by microRNAs binding to the target messenger RNA.

Technical progress and new technologies that became available in the last couple of years made sophisticated genomic methods accessible for a large number of laboratories. As a result, many analyses that would not been possible in the past became a regular part of laboratory routine. Therefore, in the following paragraphs we will focus on new, omics biomarkers: mRNA expression, DNA methylation and miRNA expression. It should be noted that other omics biomarkers exist (e.g. metabolomics and proteomics markers) but they will not be discussed in this text.

7.2.4.1 mRNA Expression

Although the effect of air pollutants on humans may be monitored by the analysis of mRNA expression of individual selected genes (Rossner et al. 2011b), the current trend is to use transcriptomics as a tool for studying genome-wide responses of the organism to environmental exposures (Wild et al. 2013). It has been concluded that transcriptome is a dynamic entity that is highly responsive to environmental exposures (Wild et al. 2013).

Most of the studies analyzing transcriptome changes in exposed subjects use peripheral blood cells. During the last ten years a number of authors reported the effect of air pollutants on global mRNA expression, but a vast majority of them focused on occupational exposures or tobacco smoking (reviewed in Wild et al. 2013). Exposure to benzene (Forrest et al. 2005; McHale et al. 2009), metal fumes (Wang et al. 2005) and diesel exhaust (Peretz et al. 2007) resulted in differential expression of a large number of genes. Studies on the impact of tobacco smoking showed that it is possible to distinguish between subjects exposed and unexposed to tobacco smoke on the basis of transcriptome (Lampe et al. 2004; van Leeuwen et al. 2008a; Wright et al. 2012).

However, studies of the effects of environmental pollutants on gene expression profiles are scarce (van Leeuwen et al. 2006, 2008b; De Coster et al. 2013). In two such studies, higher exposure to air pollutants, which included c-PAHs, was associated with an increased number of deregulated genes (van Leeuwen et al. 2006, 2008b). In the study by De Coster et al. a significant correlation between gene expression modulation and excretion of 1-hydroxypyrene, a marker of PAH exposure, was found (De Coster et al. 2013). In none of these studies detail information from personal monitoring on exposure to environmental pollutants was provided. In addition, these studies were small and included a maximum of 71 subjects from both genders (van Leeuwen et al. 2008a).

Recently, global gene expression analysis in a group of total 312 exposed subjects and 154 controls was conducted with the aim to characterize molecular response of the organism exposed to heavy air pollution (Rossner et al. 2014b). To control for the seasonal variability the samples were collected repeatedly in three different seasons. The exposed group originated from the Ostrava region, a location in the Northeastern part of the Czech Republic that is affected by very high concentrations of air pollutants, particularly c-PAHs. The Ostrava region is one of the most polluted parts of the European Union. 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 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 dysregulated genes and

affected KEGG pathways was found in subjects from the control region. 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 authors conclude that high concentrations of pollutants in Ostrava do not increase the number of deregulated genes. This may be explained by adaption of humans to chronic exposure to air pollution. To further explain this phenomenon analyses focused on regulation of mRNA expression are necessary.

7.2.4.2 DNA Methylation

Methylation of cytosine ring at position 5 in CpG sites of DNA leading to formation of 5-methyl-cytosine (5-mC) is an important event in epigenetic changes of cells linked to the control of gene function (Hayatsu 2008). Studies on nuclear DNA methylation changes in white blood cells are rapidly emerging, and thus methylation profiles can serve as a useful biomarker. Molecular epidemiological studies have reported associations between global methylation and several different cancers as well as selected factors including age, gender, race, various environmental exposures or life style factors (Terry et al. 2011).

The level of DNA methylation and changes of methylation profiles can be identified by various methods (Fraga and Esteller 2002; Laird 2010), some of which provide quantitative information about global DNA methylation, while others render qualitative data about gene-specific DNA methylation. Global DNA methylation is most commonly quantified by analyses of highly repetitive sequences like long interspersed nucleotide elements (LINE, e.g. LINE-1), short interspersed nucleotide elements (SINE, e.g. Alu), and pericentromeric satellites (Sat2). For gene-specific methylation, e.g., array methodologies, including the Illumina Infinium Human Methylation 450 K BeadChips interrogating <485,000 CpG sites at single-nucleotide resolution, may be used (Sandoval et al. 2011). The most advanced technology, Whole-Genome Bisulfite Sequencing by Next Generation Sequencing that uses bisulfite treatment combined with high-throughput sequencing is today a top of methodology approaches which allow obtaining both global and gene specific picture of DNA methylome, but due to the price, this method is not used routinely yet. Generally, obtained data can vary by assay types according to their focus on various CpG sites in the genome (Wu et al. 2012; Flom et al. 2011). Moreover, the type of tissue, even different blood cell types can affect global methylation profile, which underlines the functional significance of methylation (Wu et al. 2011; De Bustos et al. 2009).

Currently, there is evidence that DNA methylation in both adults and children is influenced by exposure to environmental pollutants (Terry et al. 2011; Baccarelli and Bollati 2009; Bollati and Baccarelli 2010; De Prins et al. 2013). Several studies suggested that exposure to metals can affect the epigenome (Cheng et al. 2012).

Other study found an inverse correlation between global DNA methylation of Alu, but not of LINE-1 repeated elements, and plasma levels of persistent organic pollutants (POPs) (Rusiecki et al. 2008). Furthermore, a study focused on the changes in DNA methylation patterns in subjects exposed to low doses of benzene showed an association with decreased methylation of LINE-1 and Alu sequences (Bollati et al. 2007). Long-term exposure to PM10 was inversely correlated with methylation in above mentioned repeated elements and demethylation within the promoter of inducible nitric oxide synthase gene (*iNOS*) (Tarantini et al. 2009). *iNOS* methylation was also decreased after acute exposure to PM2.5 (Madrigano et al. 2012). PM10 and PM2.5 exposure have recently been associated with hypomethylation of selected tandem repeats in Beijing, China study groups (Guo et al. 2014). Differences in methylation pattern in children from two regions with various levels of air pollution have recently been analyzed by using the Human Methylation 27 K BeadChips (precursor of 450 K BeadChips) (Rossnerova et al. 2013).

Furthermore, there is a evidence that altered DNA methylation is an important epigenetic mechanism in prenatal programming and that developmental periods are sensitive to environmental stressors. A recent study showed a lower degree of placental global DNA methylation in association with exposure to particulate air pollution in early pregnancy (Janssen et al. 2013). Results of another study that followed non-smoking women during pregnancy suggested that prenatal air polycyclic aromatic hydrocarbons (PAH) exposure was associated with lower global methylation in umbilical cord blood cells and confirmed that global methylation levels were positively associated with the presence of detectable DNA adducts in cord blood (Herbstman et al. 2012). Moreover, a set of genes, *AHRR* (aryl hydrocarbon receptor repressor), *CYP1A1* (cytochrome P450 1A1), and *GFI1* (growth factor independent 1 transcription repressor), with methylation differences present at birth in children whose mothers smoked during pregnancy were each identified by Infinium Illumina Methylation 450 K arrays (Joubert et al. 2012).

7.2.4.3 microRNA Expression

microRNAs are RNA molecules that have been intensively studied in the last few years. The first miRNA, named lin-4, was discovered by Victor Ambros in *Caenorhabditis elegans* in 1993 (Lee et al. 1993). The latest miRBase database [http://www.mirbase.org/, release (v20, June 2013)] contains 24,521 miRNAs identified in 206 various species processed to produce 30,424 mature miRNA products. miRNAs are a class of small (19–25 nucleotides) non-coding RNAs with important role in regulation of gene expression by binding to a target mRNA (Ambros 2004). Various analytical methods like qRT-PCR, Northern blot, microarray or sequencing are used for validation of miRNAs and identification of most altered of them. Mice, rats, and human tissues as well as a various human cell lines are prevalently used in research. Commonly used sources of human samples for this type of analysis are mainly cancer tissues, bronchial tissue, placental cells or peripheral blood lymphocytes. Alternatively, urine or plasma are used for miRNAs profiling, due to the fact, that

cells-derived microvesicules containing miRNAs are released into the plasma and transfer miRNAs between tissues (Bollati et al. 2015). Since significantly different miRNA profiles can be assigned to various types of tumors, miRNAs became important diagnostic, prognostics and therapeutics markers of various types of cancer (Berger and Reiser 2013). Moreover, specific miRNAs are associated with various diseases including pulmonary diseases, such as asthma (Sessa and Hata 2013). The changes of miRNA expression became an established mechanism by which chemical carcinogens induce alterations in target cells (Izzotti and Pulliero 2014).

The important evidence that miRNAs expression is altered by exposure to carcinogens in healthy organisms was obtained in rodents exposed to cigarette smoke (Izzotti et al. 2009a). In another study, a 1 month exposure of mice to cigarette smoke was followed by physiological miRNA expression after 1 week of smoking cessation in comparison with mice that were exposed for 4 months, where alteration of miRNA persisted and resulted in the irreversible loss-of-function of miRNA-base suppression of the expression of oncogenes (Izzotti et al. 2009b; Izzotti et al. 2011). An in vitro study indicated that exposure to maternal cigarette smoke during pregnancy is associated with downregulation of miR-16, –21 and –146a (Maccani et al. 2010). Interestingly, miRNAs were 5.67-fold more sensitive than DNA to the formation of adducts induced by exposure to cigarette smoke (Izzotti and Pulliero 2014). Another study shows association between specific miRNAs (miR-1, –9, –21, –126, –135a, –146a, –155, and –222) and exposure to ambient particulate matter (PM) in a group of elderly males (Fossati et al. 2014). The analysis of the miRNA expression profiles in benzo[a]pyrene (B[a]P)-treated mice revealed the downregulation of miR-122, –142-5p and –150 and the upregulation of miR-29b, –34b-5p and 34c expression (Halappanavar et al. 2011). Other researchers reported associations between overexpression of miR-638 in connection with B[a]P-induced DNA damage (Li et al. 2012). Also association between other airborne carcinogens like diesel exhaust particles, volatile organic compounds, black carbon dust, dimethylbenz[a]anthracene, asbestos or radon and miRNAs were published (Izzotti and Pulliero 2014).

7.3 Conclusions

All discussed studies indicate that bulky DNA adduct levels, the comet assay and analyses of DNA fragmentation in the sperm may be considered sensitive biomarkers of exposure to c-PAHs in polluted air. Stable chromosomal aberrations and unstable chromosomal aberrations measured as frequencies of micronuclei, as well as markers of oxidative damage to DNA and lipid peroxidation can be recommended as reliable biomarkers of effect. However, under specific circumstances, the exposure to environmental pollution may not be reflected on the level of biomarkers. These circumstances are not fully understood yet, but it seems that chronic exposure to intermediate levels of air pollutants and subsequent adaptation of the organism to environmental pollution may play a role. Moreover, to fully understand the

environment-organism relationship it is important to simultaneously identify the gene susceptibility, especially the genetic polymorphisms of metabolic genes and genes encoding DNA repair enzymes. It should be also taken into account that DNA damage may be further affected by life style factors as smoking, environmental tobacco smoke exposure, dietary intake of vitamins (e.g. A, C, E, folic acid), or oxidative damage associated with 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.

Studies in the Czech Republic suggest that exposure to air pollution exceeding B[a]P concentrations of 1 ng/m^3 represent a risk for DNA damage as indicated by the increase in levels of bulky DNA adducts, the increase of the frequency of stable translocations and micronuclei as well as the increase of DNA fragmentation in the mature sperm. It should be noted, though, that when using biomarkers of exposure and effect, the dose-response effect is detectable only in a certain range of concentrations of xenobiotics; for B[a]P the limit is probably around 10 ng B[a]P/m^3.

New perspectives may be seen in using the omics techniques, e.g. studying mRNA expression as well as regulatory processes, including DNA methylation and miRNA profiles. The ultimate direction in biomarker research should be the application of proteomics.

Summing up, molecular epidemiology studies on environmental exposures to c-PAHs and other airborne carcinogens should be planned as very complex exercises: they should include determination of personal exposure, analyses of damage to DNA and other macromolecules, assessment of gene susceptibility and life style factors. If planned in this way they have a potential to bring new results, which may specify new information important for proper evaluation of human health risk associated with c-PAHs and other airborne carcinogens exposure.

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