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Mechanisms of oxidative stress induction by environmental pollutants in exposed human populations and in *in vitro* cell models

Komise pro obhajoby doktorských disertací v oboru „Biomedicína“

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Contents

Abbreviations

8-oxodG	8-Oxo-7,8-dihydro-2'-deoxyguanosine
8-oxoGua	8-Oxo-7,8-dihydroguanine
A549	Adenocarcinoma of alveolar basal epithelial cells
AA	Arachidonic acid
AhR	Aryl hydrocarbon receptor
BaP	Benzo[a]pyrene
BEAS-2B	Immortalized lung epithelial cells
BER	Base excision repair
CT-DNA	Calf thymus DNA
CYP	Cytochrome P450
ELISA	Enzyme-linked immunosorbent assay
EOM	Extractable organic matter
ETS	Environmental tobacco smoke
GC/MS	Gas chromatography/mass spectrometry
HEL	Human embryonic lung fibroblasts
HPLC-MS/MS	High-performance liquid chromatography–tandem mass spectrometry
IARC	International Agency for Research on Cancer
IsoP	15-F_{2t}-isoprostane
IUGR	Intrauterine growth restriction
LBW	Low birth weight (< 2500 g)
LC/GC-MS	Liquid/gas chromatography coupled with mass spectrometry
LPO	Lipid peroxidation
NER	Nucleotide excision repair
NHEJ	Nonhomologous end-joining repair
OGG1	8-Oxoguanine DNA glycosylase
PAHs	Polycyclic aromatic hydrocarbons
PBL	Peripheral blood lymphocytes
PGE_2	Prostaglandin E2
PM	Particulate matter
PTGS	Prostaglandin-endoperoxide synthase
ROS	Reactive oxygen species
S9 fraction	Microsomal fraction enzymes
SNPs	Single nucleotide polymorphisms
UGT	UDP-glucuronosyltransferases
XRCC5	X-Ray Repair Cross Complementing 5

Summary

Environmental air pollution is an inevitable consequence of human activity. As a result, populations in both developed and developing countries are constantly exposed to airborne contaminants originating from industrial production, traffic or heating. Over the years, evidence has accumulated that ambient air pollutants negatively affect human health, contributing to increased incidence of numerous diseases and decreased life expectancy. Particulate matter (PM) of various size and polycyclic aromatic hydrocarbons (PAHs) bound to it belong among the pollutants widespread in the environment, exposure to which may result in serious health issues, including cancer.

Ideally, potentially negative health impacts of ambient air pollution are detected before the onset of a disease. To achieve this goal, the levels of biomarkers in samples collected from subjects at risk are assessed. Biomarkers are defined as parameters that (i) reflect biochemical changes in the organism following the exposure to toxic compounds, (ii) are easily accessible and (iii) their analysis is fast and cost-effective. Upon entering the organism, PM and PAHs induce various biochemical changes, including oxidative damage to macromolecules (DNA, lipids, proteins). This modification affects the function of these cellular structures and in case of DNA it may potentially cause mutations. 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), 15-F_{2t}-isoprostane (IsoP) and protein carbonyl groups are the well-established biomarkers of oxidative damage of DNA, lipids, and proteins, respectively. They can be easily assessed in urine/blood plasma and their elevated levels may indicate possible health risks associated with toxic compounds exposure. In-depth molecular investigation of mechanisms of air pollutants impacts may be further performed using genomic methods, including whole genome gene expression analysis and/or single nucleotide polymorphisms (SNPs) detection.

This thesis summarizes results of research spanning almost 15 years. In the studies reported here, the biomarkers have been analyzed in *in vitro* model systems exposed to PAHs and extractable organic matter (EOM) from PM, as well as in human populations originating from localities of the Czech Republic with various sources and concentrations of air pollutants with the aim to elucidate mechanisms of toxic effects of these compounds. In humans, the presented data focused not only on adults, but also on pregnant women and their newborns, as well as young children, i.e. the population groups that are particularly sensitive to deleterious effects of environmental toxicants. Apart from increased oxidative damage to

macromolecules associated with exposure to air pollution, a possible adaptation to toxic compounds in the environment has been observed in populations living in a locality with consistently increased levels of air pollution. As for reliable detection of analyzed biomarkers well-established, standardized laboratory techniques are required, a section of the thesis focuses on modification and improvement of 8-oxodG detection by enzyme-linked immunosorbent assay (ELISA), a high-throughput method commonly used for this biomarker detection, particularly in human studies.

The research data presented in this thesis contribute to our understanding how polluted environment affects molecular processes in the organism and how the organism may protect itself if chronically exposed to air pollutants over an extended period of time.

1. Introduction

Concentrations and chemical nature of ambient air contaminants have been changing over the centuries. While in the pre-industrial era they were mostly of natural origin (e.g., forest fires, volcanoes eruptions, particles from soil erosion), with the onset of industrial production the sources and amounts of toxic compounds released into the environment became significantly different. Burning wood, coal and other organic material, road traffic (mostly passenger cars and trucks), as well as growing human population and its concentration in towns/cities, resulted in significant increase in environmental air pollution that became a factor negatively impacting human health and contributing to the increased incidence of cardiovascular, pulmonary and neurodegenerative disorders, as well as cancer [1]. Currently, it is generally accepted that there are three dominant sources of air pollutants: industry, heating, and traffic.

Environmental air pollutants toxicity research has become an important field in which more than 26,000 peer-review articles were published in the last 50 years. The need for such research direction is further supported by the fact that outdoor air pollution was classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans [2]; in addition, diesel engine exhaust was evaluated to be carcinogenic, while gasoline engine exhaust is possibly carcinogenic to humans [3]. It should be further noted that human health is also impacted by pollutants present in other components of the environment and/or products of human activity (e.g., water or soil pollution, food contaminants); however, this thesis strictly focuses on the effects of ambient air pollutants.

Air pollution is a complex mixture of particles of various size and composition [particulate matter (PM)] and gaseous components (e.g. volatile organic compounds, ozone, CO, SO_2, NOx) [4]. To PM, organic and/or inorganic chemicals are bound and their properties significantly affect toxicity of PM. Health impacts of PM are determined by its size [PM of aerodynamic diameter < 2.5 μm (PM2.5) or smaller is of particular concern], chemical characteristics of compounds bound to it [the presence of polycyclic aromatic hydrocarbons (PAHs), including benzo[a]pyrene (BaP, a model PAH), reactive metals, dioxins] and reactivity/toxicity of the gaseous fraction [5]. The following text will mostly focus on toxicity of PM [alternatively extractable organic matter (EOM) obtained from PM] and PAHs, as widespread pollutants, commonly monitored by meteorological services and health

authorities in many countries. These components of air pollution have known deleterious health effects and thus for many of them concentration limits in the ambient air are set.

Overall impacts of environmental pollutants on human health represent a complex interplay between external and internal factors whose interactions drive the resulting health outcome. As illustrated in **Figure 1**, effects of environmental pollution are further modulated by lifestyle factors (e.g., smoking, alcohol drinking, physical activity) and genetic and epigenetic settings of the organism, including the effectivity of repair mechanisms. These interactions will determine the extent of damage to cellular macromolecules and the resulting response of the organism: increased disease risk (e.g., cancer), or adaptation to negative environmental conditions.

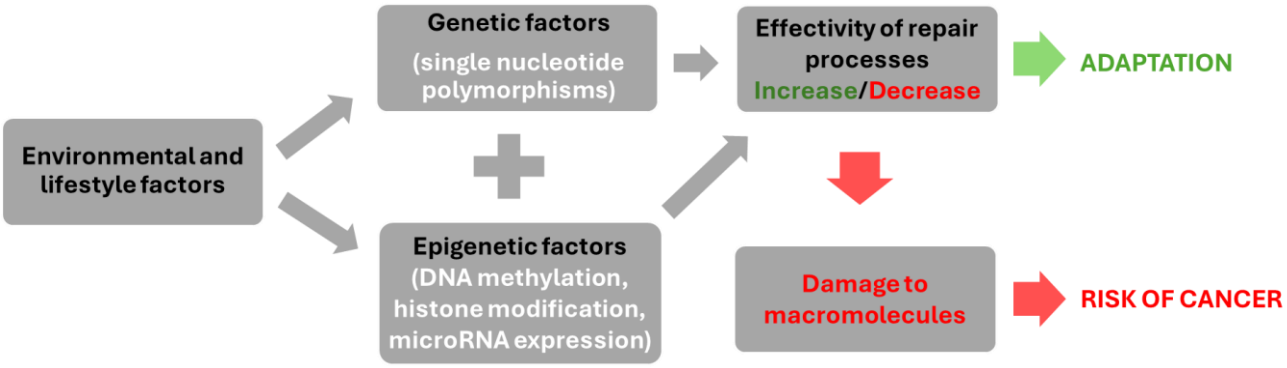

Figure 1. The interplay between environmental and cellular processes impacting the disease risk.

Upon inhalation, PM is either deposited in the upper airways, or, if smaller, it penetrates to the lungs, potentially reaching alveoli. Ultrafine particles (i.e., particles of aerodynamic diameter < 100 nm; nanoparticles/ultrafine particles) may even enter the blood stream, affect distant organs, enter the cells, including nuclei. In the organism the air pollutants exert their effects either directly, or after metabolic activation by relevant enzymes. Given its physicochemical properties, PM mostly causes reactive oxygen species (ROS) formation [6]. This process depends on chemical composition of PM-bound compounds (external sources of ROS: the presence of transition metals or other components with pro-oxidant properties). In addition, it is initiated as a secondary response of the organism after PM has been phagocyted by macrophages (cellular sources of ROS). ROS, including e.g., the hydroxyl radical, superoxide anion, or hydrogen peroxide, attack macromolecules and cause oxidative damage to DNA,

lipids and proteins (**Figure 2**). The presence of oxidatively damaged DNA may result in the induction of mutations, a serious consequence potentially leading to cancer [7]. However, the organism possesses efficient repair mechanisms that remove the damaged DNA components. In contrast, no such processes exist for lipids and proteins, that become dysfunctional and/or converted into reactive intermediates (peroxidized lipids) that attack other cellular structures [8]. Apart from direct damage to macromolecules, ROS may act as second messengers that contribute to gene expression regulation, potentially activating oncogenes or deactivating tumor-suppressor genes. In should be noted that the organism possesses efficient antioxidant mechanisms (both enzymes and small molecules of non-enzymatic character) that eliminate excessive ROS levels and keep redox balance in the cells. **Oxidative stress** is induced only if the antioxidant systems are overwhelmed by high levels of pro-oxidant factors. The details on these mechanisms, however, are beyond the scope of this thesis.

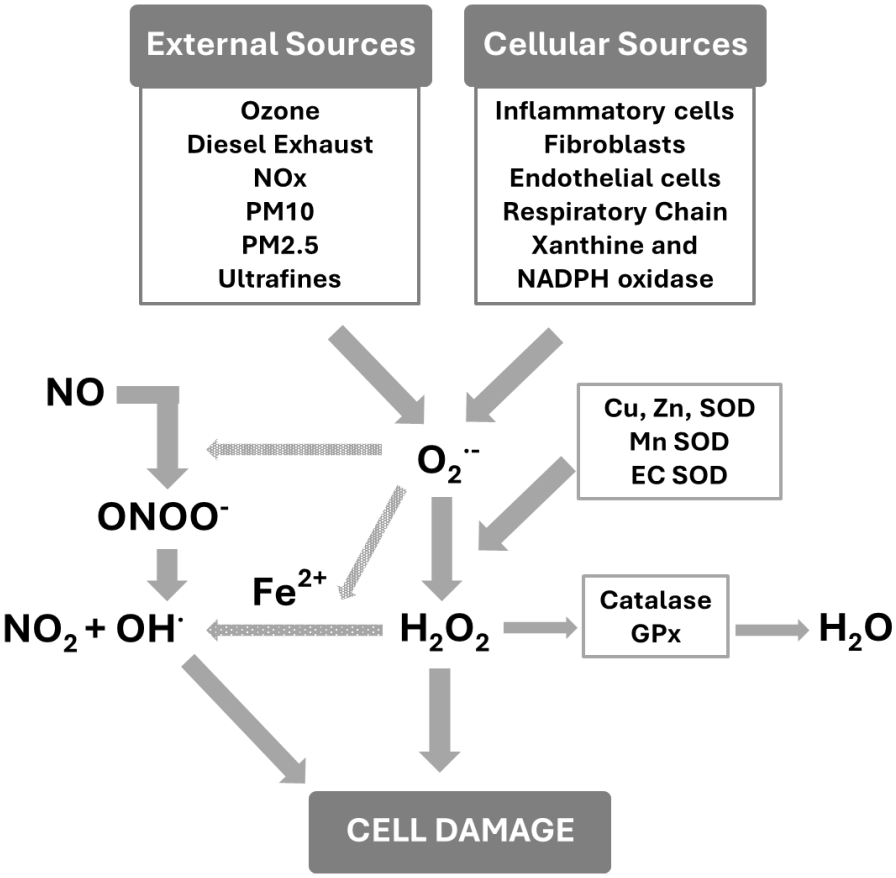

Figure 2. Sources and fate of reactive oxygen species in a cell.

Many PM-bound chemicals are genotoxic, causing formation of (bulky) DNA adducts and/or chromosome breaks/rearrangements [6]. Among these compounds, PAHs raise most health concerns, as some of them are possible human carcinogens. Three principal pathways of PAHs metabolic activation have been identified [9]: (i) dihydrodiol epoxides pathway, (ii) radical cation pathway and (iii) PAH-*o*-quinones generation pathway (**Figure 3**). The latter process is particularly associated with ROS generation and thus links PAHs exposure with oxidative damage induction.

In summary, environmental air pollution contributes to generation of ROS (both endogenous and endogenous), induction of oxidative damage/oxidative stress and thus to increased risk of many diseases in exposed humans. Understanding the processes that contribute to oxidative damage of macromolecules induced by polluted ambient air and identifying mechanisms of impacts of environmental toxicants on the organism may help to prevent/reduce resulting negative health consequences of exposure in human populations.

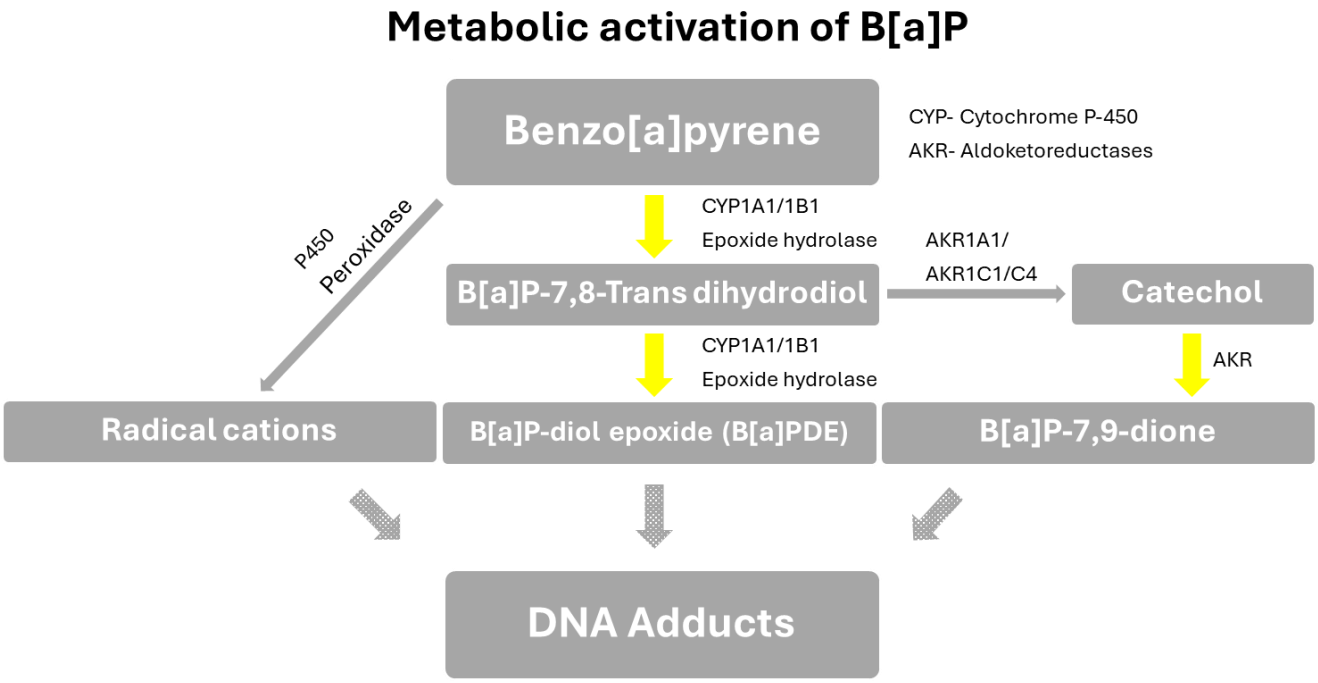

Figure 3. Metabolic activation pathways of BaP, a model PAH. The ROS-generating processes are highlighted.

2. Biomarkers

The impact of potentially toxic compounds on the organism is assessed using the analysis of biomarkers, i.e. parameters that reflect the effect of the factor of interest in the biological system [10]. The **biomarkers** should meet certain criteria that include:

(i) sensitivity; the biomarkers can be detected even at low levels of exposure;
(ii) specificity; the biomarkers reflect exposure to the compound of interest;
(iii) standardization/validation; the analysis should be reproducible;
(iv) cost-effectiveness; the analysis should not be expensive;
(v) accessibility of matrices; the samples should be easy to collect;
(vi) speed of analytical methods; ideally the analyses should be high throughput.

Traditionally, the following biomarkers are recognized (**Figure 4**): (i) **exposure** (reflect concentrations of xenobiotics, their metabolites or levels of modified macromolecules resulting from the interactions between xenobiotics and target structures; e.g., oxidative stress biomarkers), (ii) **effect** (represent measurable biochemical modifications in the organism that are known to be deleterious to health; e.g., chromosomal aberrations) and (iii) **susceptibility** (take into account genetic background of the organism; e.g., SNPs). With the development of omics-techniques (i.e., whole genome mRNA/miRNA expression analyses, SNPs detection using BeadArrays, DNA methylation), a class of **omics- biomarkers of effect**, that allow in-depth molecular analyses of cellular processes affected by the exposure, has been introduced.

The principal aim of biomarker analyses is to detect negative changes in the organism before they become clinically manifested (i.e., before the disease onset). In human epidemiological studies, the most commonly analyzed **oxidative stress biomarkers** include **8-oxo-7,8-dihydro-2'deoxyguanosine** (8-oxodG; a parameter reflecting DNA damage), **15-F_{2t}-isoprostane** (IsoP; a lipid peroxidation product), and **protein carbonyl groups** (a marker of protein oxidation). The following text focuses on description of these biomarkers and their application both in *in vitro* and *in vivo* (human) studies.

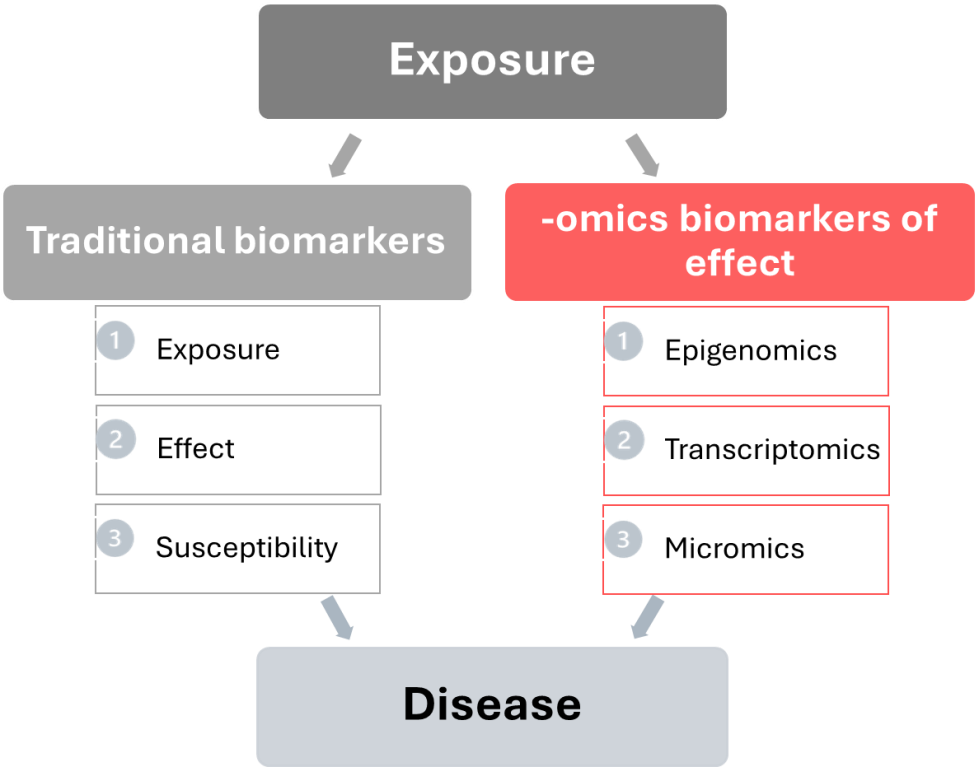

Figure 4. The overview of biomarkers used to evaluate negative biological effects of (environmental) toxicants.

2.1. Oxidative DNA damage

ROS attack on DNA causes nucleobases oxidation, of which 8-oxo-7,8-dihydroguanine (8-oxoGua) is regarded as the most common. However, in total over 70 lesions have been identified as a result of oxidative damage to DNA [11]. While 8-oxoGua can be easily detected in urine using standard analytical methodology, it is more prone to artefactual oxidation than the corresponding nucleoside, **8-oxodG** (**Figure 5**), that is relatively stable, particularly if assessed in urine [12]. The most important biological consequence of 8-oxoGua/8-oxodG formation is the induction of mutations (GC-TA transversions), a potential cancer initiation step. Thus, 8-oxodG is commonly assessed in humans exposed to possibly toxic factors, as well as in diseased individuals. Currently, there is a consensus that the detection of 8-oxodG in urine is methodologically the most reliable approach for determination of whole body oxidative stress [13]. Oxidized nucleobases are removed from DNA by the activity of enzymes of base excision repair (BER), predominantly 8-oxoguanine DNA glycosylase (OGG1). It is important to note that 8-oxodG in urine does not originate from DNA repair. Also, it is believed that the contribution of diet to changes in urinary 8-oxodG levels is not probable.

Currently, sanitization of the GTP pools by the activity of Nudix hydrolases seems to be the most probable source of urinary 8-oxodG; however, the precise biological meaning of the presence of this nucleoside in urine is not clear which complicates the interpretation of changes of this biomarker levels [11,14].

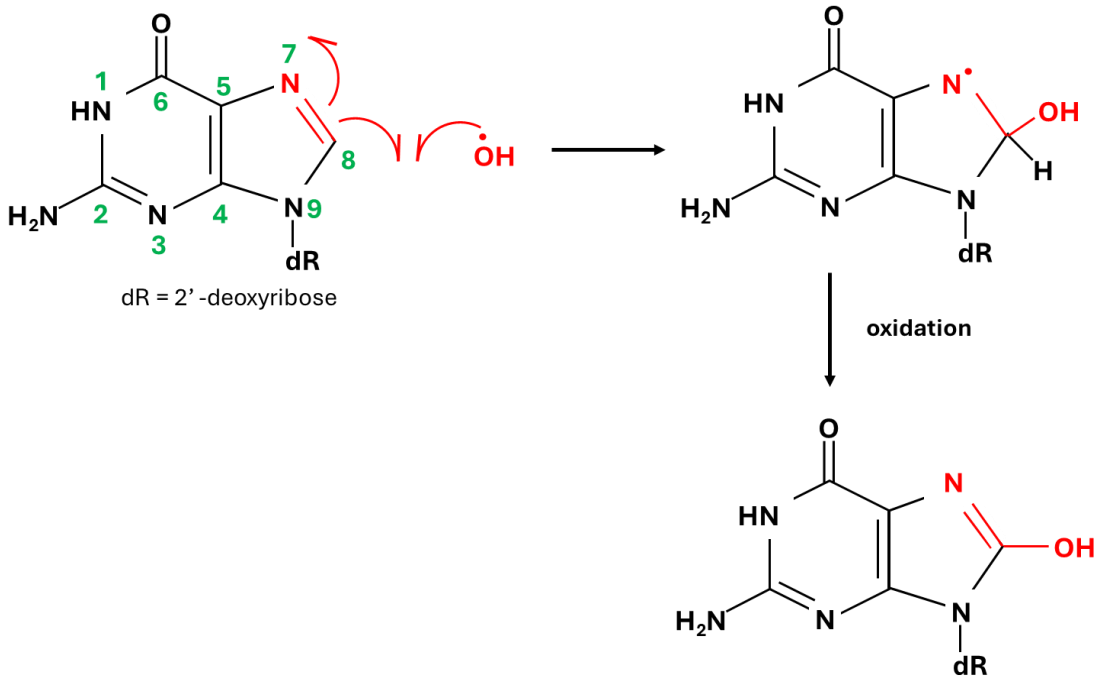

Figure 5. The formation and structure of 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodG).

2.2. Lipid peroxidation

Lipid peroxidation (LPO) is initiated by ROS attack on lipids containing unsaturated bonds, such as polyunsaturated fatty acids [15]. Three mechanisms of LPO generation are recognized: (i) free-radical-mediated oxidation, (ii) free-radical-independent non-enzymatic oxidation and (iii) enzymatic oxidation [16]. The latter process is based on lipoxygenase and prostaglandin-endoperoxide-synthase (PTGS)-catalyzed arachidonic acid (AA) oxidation. Its result is a number of LPO products, including prostaglandins, thromboxane, or leukotrienes. Expression of PTGS2, an inducible form of the PTGS enzyme, is associated, among other reactions, with the activation of NF-kB transcription factor. This molecule, activated by various stimuli, including e.g., ROS, regulates expression of many target genes, such as aryl hydrocarbon receptor (AhR) and CYP enzymes [17,18]. AA may also serve as a substrate to formation of F_2-isoprostanes, generated by a free-radical-mediated peroxidation of the molecule. F_2-isoprostanes are a complex group of compounds, classified into four regioisomer

classes (5-, 12, 8- and 15-series). **IsoP**, belonging to the 15-series of F_2-isoprostanes (**Figure 6**), became a reliable marker of lipid peroxidation [19,20] implicated in various pathological conditions. Thus, AA may serve as a substrate for non-enzymatic IsoP formation, as well as for PTGS-mediated prostaglandins and other LPO products generation. If active enough, the latter step may reduce the amount of AA available for IsoP production, thus effectively decreasing the concentration of IsoP, questioning the universal suitability of this molecule as a marker of lipid peroxidation. Importantly, many peroxidized lipids are reactive, interacting with other cellular molecules and causing damage.

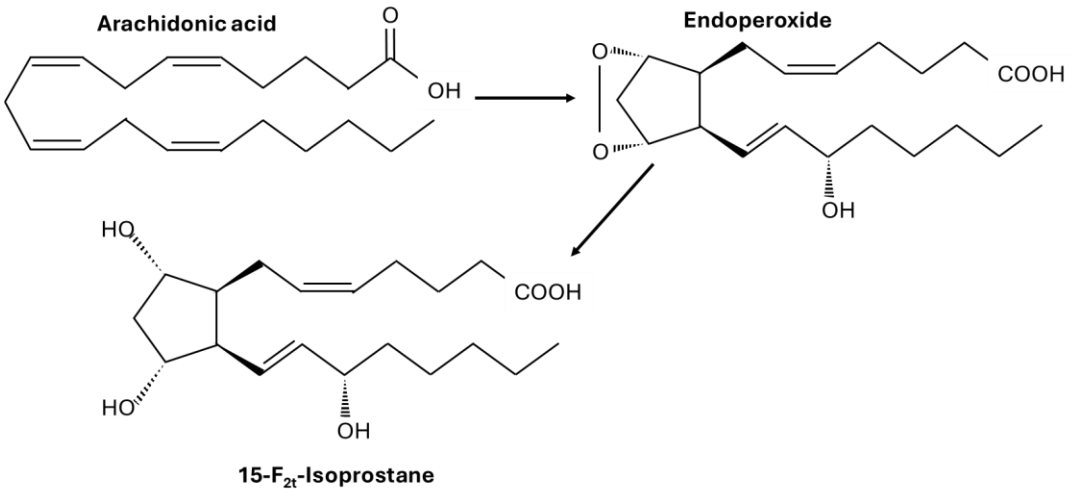

Figure 6. The formation and structure of 15-F2t-isoprostane (IsoP).

2.3. Protein oxidation

Non-enzymatic oxidation of proteins is a common biological process that results in formation of **carbonyl groups** (**Figure 7**). Mostly, such modifications cause protein dysfunction and/or changes in its structure, causing molecule degradation. Although the type of protein modification depends on radical species that induced oxidation, it has been shown that both aliphatic and aromatic amino acids are commonly affected [21]. In general, processes involved in protein oxidation are very complex, mostly due to the differences in physicochemical characteristics of amino acids and the number of redox-sensitive residues and their response to the oxidant [22]. The most sensitive amino acids include cysteine (thiol group), methionine (sulfur) and tyrosine, tryptophan and histidine (aromatic rings). It should be noted that some reactive products of lipid peroxidation (e.g., 4-hydroxy-nonenal) are also involved in protein carbonylation.

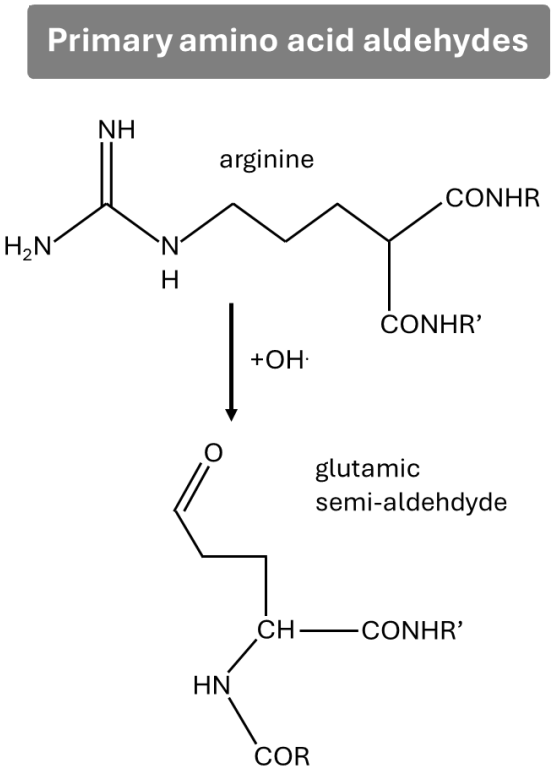

Figure 7. The formation and structure of molecules containing carbonyl groups.

3. Biomarkers analyses: *in vitro* vs. human studies

When investigating toxicity of environmental air pollutants, the most important consideration is if analyses will be performed in model systems *in vitro*, or if conditions mimicking real-world scenarios (exposure of human populations) will be used. Application of each of these approaches has its positive and negative consequences.

3.1. *In vitro* tests

In *in vitro* studies either **DNA alone** (usually calf thymus DNA, CT-DNA; an acellular system), or model cell lines are used. The acellular system eliminates the role of cellular processes that may affect delivery of the tested compound to macromolecules of interest. Also, it allows the researcher to concentrate specifically on a selected target molecule, e.g. DNA. As for some chemicals metabolic activation is needed so that they can bind to DNA, the acellular system may be supplemented by a mixture of microsomal fraction enzymes (S9 fraction), obtained from liver, that include important drug-metabolizing enzymes, as cytochrome P450 (CYP) and UDP-glucuronosyltransferases (UGT) [23]. The acellular system is suitable for evaluation of (geno)toxicity of chemicals of interest, however, it is rather artificial

as it omits interactions of the chemicals with the cellular components, as well as cell-to-cell interactions that may be affected by the tested compounds. These facts should be considered when planning the acellular tests and evaluating the data of such experiments.

If **model cell lines** are to be used, they should be carefully selected so that they correspond to the cells in the target organ/tissue of the tested compound. Thus, for air pollutants, cell lines of lung origin, such as A549 (adenocarcinoma of alveolar basal epithelial cells), BEAS-2B (immortalized lung epithelial cells) or HEL (human embryonic lung fibroblasts) are often applied. Tumor/immortalized cell lines can be easily propagated in cell cultures, but they suffer from genomic instability (particularly A549 cells) so they are not suitable for experiments where chromosome damage and/or gene expression changes are analyzed. Cells of normal origin (e.g., HEL cells) are characterized by a limited number of divisions which complicates their application in long-term experiments. In addition, monolayers in which the cells grow represent another limitation which makes these models (and results of the experiments) rather artificial. In this regards, application of advanced 3D models seems to be a better approach [24]. However, this topic is beyond the scope of this thesis.

The methods of application/distribution of toxic compounds to the cell models are also to be considered when evaluating biological impacts of air pollutants. As mentioned above, air pollution is a complex mixture of solid, liquid, and gaseous components. In standard cell cultivation settings, the complex mixtures cannot be delivered to the cells in their natural form, as tested compounds need to be added to the cultivation media in a liquid form (solution/suspension). Thus, toxicity of PM and compounds bound to it can be evaluated only after performing extraction of chemicals of interest using a suitable solvent. Specifically, water-based extractions are used to eluate inorganic metal-containing compounds (often responsible for oxidative stress induction), while organic solvents (e.g., dichloromethane) are applied to extract organic chemicals. This particular type of EOM contains compounds responsible for most of the genotoxic activity (e.g., PAHs) [25]. Selection of the solvent thus determines which processes can be studied with the extracted chemicals.

Concentrations of compounds used for treatment in the experiments and exposure times are other parameters that modulate biological response of the model system and consequently affect conclusions on toxicity/biological activities of EOM. Unrealistic exposure

conditions (high tested concentrations; long/short exposure times) should be avoided to get reliable data.

In summary, while *in vitro* model systems are very important in evaluation of air pollutants toxicity (or toxic of xenobiotics in general), the tests should be performed after thorough evaluation of exposure protocols and the data interpreted with caution. These studies play a key role when investigating mechanisms of action of the tested compounds, changes on molecular level and/or regulation of key biological processes/pathways [26]. However, to evaluate effects of ambient air pollution on the level of the entire organism, when cell-cell interactions are considered and impacts on function of organs/tissues are assessed, the *in vitro* tests are not sufficient. For these purposes, studies in exposed human populations are more valuable and bring realistic information on impacts of environmental pollutants on the organism.

3.2. Evaluation of impacts of environmental pollutants in human populations

Investigations in human populations allow the researchers to gather the most relevant information on the health effects of a polluted environment. However, when planning this type of studies, relatively high cost, demanding logistics, as well as ethical considerations should be taken into account. Several key points need to be met when a human study is to be conducted:

(i) the study group should be large enough to allow sufficient statistical power;

(ii) a relevant control group should be included (matched on gender, age, body mass index, lifestyle, or other parameters that may affect the biomarkers of interest);

(iii) the exposure to the investigated factor(s) should be well characterized/assessed, ideally on the personal level;

(iv) the study groups should be followed long enough to be able to see biological response of interest and its change over time;

(v) a relevant battery of biomarkers should be analyzed, and thorough multivariate statistical analyses adjusted to appropriate confounders performed.

4. Investigation of environmental air pollutants toxicity using oxidative stress biomarkers in *in vitro* and human studies

The following text summarizes the key results of the studies included in the thesis with the aim to provide information on toxicity of ambient air pollution on biological systems mediated by oxidative damage to macromolecules. In addition, to complement the interpretation of the data on oxidative stress, information on changes of other biomarkers, relevant to processes of oxidation of macromolecules (e.g., gene expression modification, DNA repair processes), is also reported. As these parameters were reported in a minority of the studies included here and are not the main topic of the thesis, they are not discussed in further detail in this text. However, thorough explanation on their biological significance can be found in the respective articles.

As illustrated in Figure 8, the thesis summarizes results from three individual topics:

(i) **Studies *in vitro***, including acellular tests and experiments in model cell lines

(ii) **Human studies**, involving mothers/their newborns and children, as well as adult populations

(iii) **Methodological aspects of biomarkers analysis**, specifically focused on urinary 8-oxodG detection

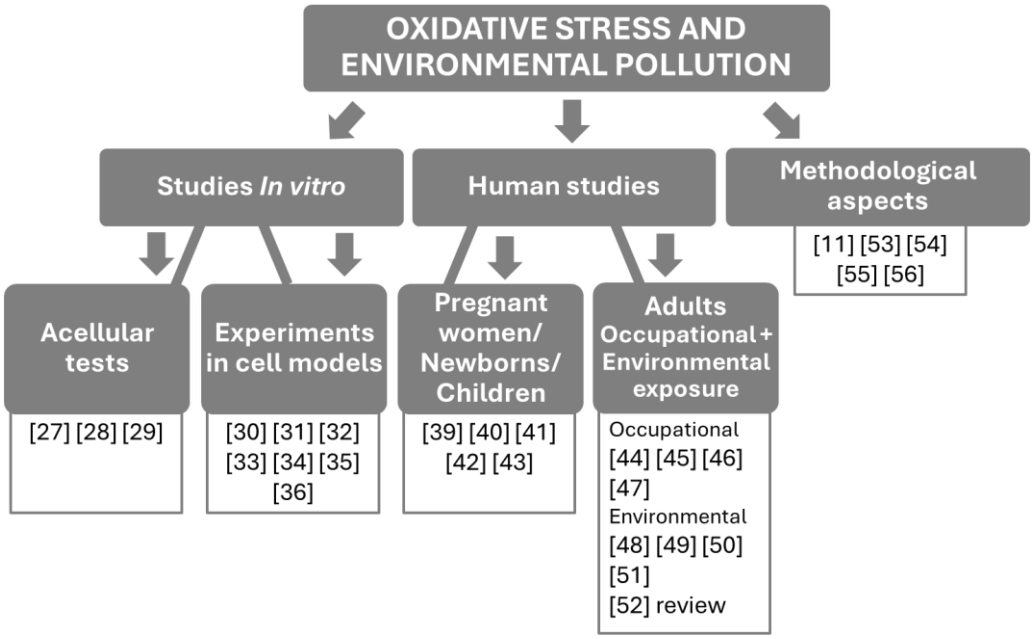

Figure 8. The structure of the topics presented in the thesis (references of corresponding articles in parentheses).

Human populations that were followed, and PM samples that collected in several regions of the Czech Republic, are described further. These localities, along with BaP concentrations in the year of 2010 (selected to be relevant to the time period when the reported studies were performed), are depicted in Figure 9. Ostrava region and Northern Bohemia served as polluted localities; Southern Bohemia was used as a control region; depending on the experimental design, Prague was regarded as polluted/control region.

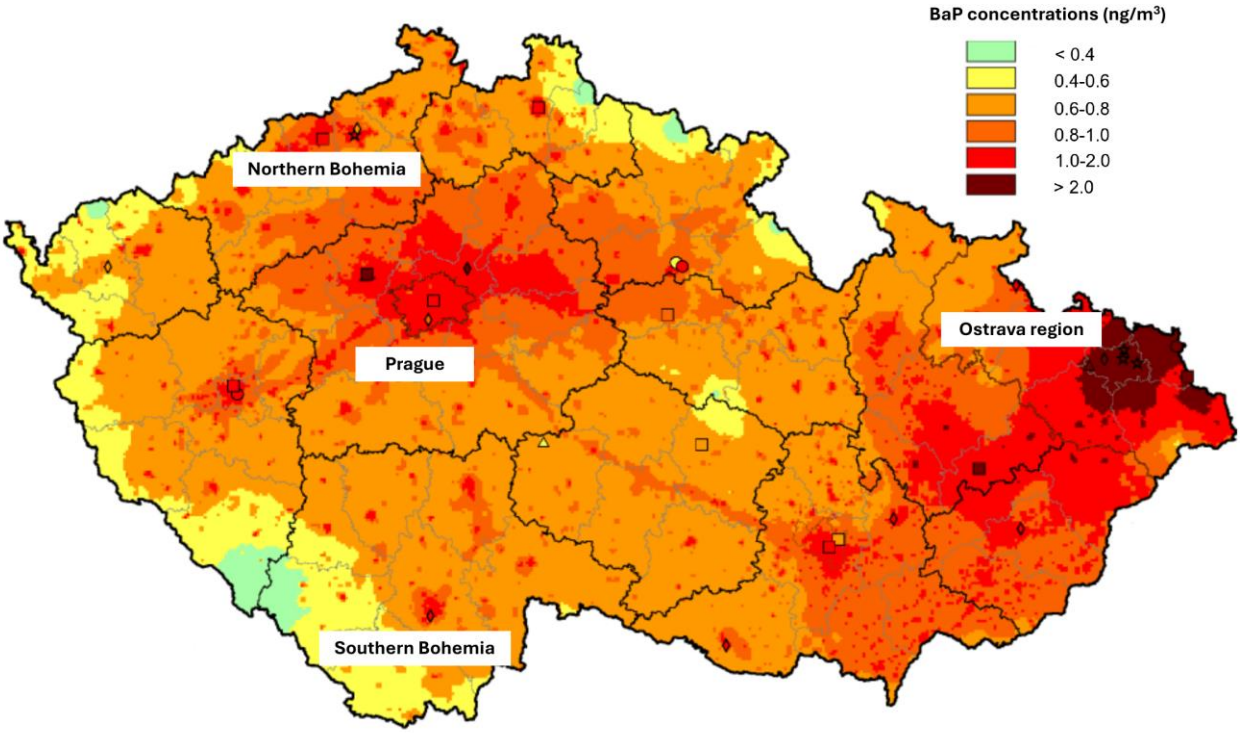

Figure 9. The overview of the localities involved in the studies included in the thesis. BaP concentrations in 2010 (www.chmi.cz, modified).

4.1. Studies *in vitro*

The first set of studies presented in this thesis used **acellular tests** in which calf thymus DNA was treated with EOMs from PM of various aerodynamic diameter and oxidative DNA damage was evaluated [27–29].

As the PM size plays an important role in biological effects of environmental pollutants, the ability of EOM from various fractions of PM (1–10 µm, 0.5–1 µm and 0.17–0.5 µm) collected at four localities of the Czech Republic differing in air pollution levels to induce oxidative DNA damage was investigated in the presence and absence of S9 fraction [27]. Although in the given experimental settings the physical size of PM did not directly affect

biological activities of the particles, oxidative DNA damage (8-oxodG) still tended to increase with decreasing size of PM. In addition, S9 metabolic activation increased the oxidative capacity of PM suggesting induction of metabolizing enzymes-related ROS production. This was further confirmed by a significant positive association between concentrations of c-PAHs in EOM and DNA oxidation.

The role of sampling collection locality was observed in another study involving EOM from PM2.5 collected from the polluted industrial Ostrava region and the control Southern Bohemia locality [28]. Higher pro-oxidant potential was found in samples from the region with elevated air pollution. In contrast, in further report the pro-oxidant effects of c-PAHs in the acellular system were not confirmed when toxicity of EOM from coarse (1 – 10 μm), upper- (0.5 – 1 μm), lower-accumulation (0.5 - 0.17 μm) and ultrafine (<0.17 μm) fractions collected in the polluted Ostrava locality were evaluated [29]. The article suggested that genotoxicity and dioxin-like activity are the major toxic effects of organic compounds bound to size segregated aerosol, while DNA oxidation seemingly plays a less significant role. This unexpected finding was explained by the presence of compounds with antioxidant properties in EOM that may have affected the redox potential of EOM resulting in lower levels of DNA damage markers.

Overall, data presented in the thesis suggest that **in the acellular system EOM induces oxidative DNA damage, underlining pro-oxidant properties of organic contaminants (PAHs) of ambient air.**

The second set of studies summarizes results of experiments in **model cell lines**, including those of lung (human diploid lung fibroblasts, HEL; human adenocarcinoma alveolar basal epithelial cells, A549) and liver (human hepatoma cells, HepG2) origin. The impacts of the model PAHs and their derivatives, as well as EOMs were studied.

The importance of metabolic activation of PAHs and selection of a suitable cell model has been shown in a study where HEL and HepG2 cells were treated with the tested compounds and induction of 8-oxodG, IsoP and protein carbonyl groups was evaluated [30]. While in HepG2 cells, EOM induced DNA oxidation, no such effect was detected in the lung cell model. Importantly, IsoP levels increased in HepG2 cells upon treatment with PAHs, while in HEL cells these compounds caused **a decrease of IsoP formation**. Protein oxidation was induced only

after extended exposure, mostly in HepG2 cells. Apart from results of toxicological tests, in this study methodological aspects of oxidative stress markers detection were optimized for samples from *in vitro* experiments and these protocols were used in the following studies in model cell lines. In another report, response in A549 cells was studied and oxidative stress biomarkers were analyzed after exposure to PAHs and their derivatives [BaP, 1-nitropyrene (NP) and 3-nitrobenzanthrone (NBA)] [31]. In general, the tested compounds had very weak pro-oxidant properties in this cell model; the strongest pro-oxidant properties were detected for NP.

In summary, these reports showed **the ability of EOM to induce oxidative damage to DNA, lipids, and proteins in HepG2 cells**, while **the effect of c-PAHs and their derivatives was weak, particularly in HEL and A549 cells**, mostly limited to oxidation induced by NP. These results underline the importance of a suitable cell model selection with sufficient metabolic activity that allows ROS generation during PAH conversion to secondary metabolites.

To further investigate **mechanisms of lipid peroxidation** induction upon PAHs and EOM treatment, HEL12469 and A549 cells were exposed to the selected compounds followed by evaluation of ROS production and detailed analyses of molecular processes involved in IsoP production from arachidonic acid [32–34]. This research was initiated with the aim to explain decreased IsoP levels after cell treatment with chemicals (PAHs) with potentially pro-oxidant properties observed in the previous studies. First, a time-dependent investigation of ROS production showed the ability of **EOM to induce ROS production after short exposure times; longer treatment periods were characterized by a marked decrease in ROS production** suggesting **the activation of antioxidant response**. The effects of PAHs were generally weak. Next, the in-depth investigation of processes related to lipid peroxidation involved in response to oxidative stress [including analyses of expression of PAH metabolic activation and antioxidant genes, the gene encoding prostaglandin-endoperoxide synthase 2 (PTGS2), activity of the NF-kB transcription factor, levels of arachidonic acid, prostaglandin E_2 and IsoP; for details, see Chapter 2.2] revealed that **exposure of HEL12469 cells to PAHs with low pro-oxidant properties results in a decrease of IsoP levels seemingly implying PAH "antioxidant" properties**. **For such compounds, IsoP may not be a suitable marker of lipid peroxidation.** In contrast, in A549 cells, analogical research showed that **EOM, and partly BaP, reduce lipid peroxidation by a mechanism that involves AhR-dependent inhibition of PTGS-2 expression**.

This, again, underlined the importance of suitable cell line selection and underscored the need for careful data interpretation.

DNA repair is a key process that keeps DNA integrity and decreases the risk of induction of mutations. As PAHs are known to be genotoxic and ROS may contribute to nucleobases modifications and/or DNA strand breaks, the thesis also includes studies that focused on DNA repair processes associated with DNA damage induced by these compounds and EOM [35,36]. In HEL12469 cells, molecular changes associated with nucleotide excision (NER) and nonhomologous end-joining (NHEJ) repair were assessed. While NER is initiated to remove bulky DNA adducts formed by binding of metabolically activated PAHs to DNA, NHEJ repairs double strand DNA breaks induced, e.g., as a result of ROS attack to DNA strands. The results obtained in the referenced studies suggest that **the tested compounds induce DNA damage and affect the expression of NER genes; however, NER itself was not induced in the selected experimental model**. In contrast, **DSBs were created** (chromosomal aberrations mostly affecting chromosome 7) **and NHEJ was induced**. Expression of the Ku70/Ku80 (XRCC6/XRCC5) protein involved in NHEJ and ligation activity was weak in this cell model.

4.2. Human studies

As evident from the text above, when considering impacts of air pollution on oxidative stress biomarkers in humans, the concentrations of PM (particularly PM2.5 or smaller) in the ambient air are the key factor driving biochemical changes in the organism. There are other parameters to be considered, including the duration of exposure, lifestyle parameters (e.g. physical activity, cigarette smoking, nutrition…), or, importantly, the age group (children, adults) of exposed subjects. While the information on exposure to environmental pollutants is usually obtained from the monitoring facilities of hydrometeorological service or ambient air samplers provided by the researchers, for lifestyle parameters questionnaires distributed to the study subjects must be used. Generally, the questionnaire data should be regarded with caution as some individual bias may be involved.

This thesis covers studies in **subjects from various age groups** so that comprehensive information of air pollution impacts on oxidative stress markers is obtained. **Newborns and young children** represent a specific group, as their organism is particularly sensitive to deleterious effects of toxic compounds. In addition, the changes induced in these population

groups may affect the health of these individuals later in life and may contribute to increased incidence of pulmonary, cardiovascular, or neurodegenerative disorders, diabetes, cancer, and other diseases. Asthma was identified as a common disorder resulting from exposure to air pollutants in young children [37]. Thus, studies in these subjects require particular attention. **Pregnant women** are at increased risk as well, as pregnancy itself is characterized by elevated oxidative stress levels and the health impacts affecting the woman are subsequently mirrored in the organism of the fetus/newborn. Indeed, it has been repeatedly shown that environmental air pollution exposure is associated with intrauterine growth restriction (IUGR) and low birth weight (LBW; weight < 2500 g) [38]. Although the antioxidant mechanisms in the fetus are rather effective, if they are overwhelmed by pro-oxidant factors, oxidative damage is induced.

4.2.1. Studies in newborns/pregnant women and young children

First, possible **transplacental effects** of environmental pollutants were investigated in a study of 80 newborns and 79 mothers from Prague [39]. The placenta plays a key role in numerous physiological functions, including e.g. exchange of gases, metabolites, nutrients and waste products between the mother and fetus circulation, production of hormones and the metabolism of toxic compounds to which maternal organism is exposed. It could be thus expected that it will reduce/modulate negative impacts of toxic compounds to which a mother is exposed in the organism of the fetus. Smoking during pregnancy and its effects on the fetus served as a parameter helping to determine the role of the placenta in the protection of fetus against environmental toxicants. Although oxidative DNA damage in placentas of smoking and non-smoking mothers was comparable, as were protein oxidation and lipid peroxidation levels indicating **no effect of tobacco smoke exposure** on oxidative damage biomarkers, a significant **correlation between oxidative stress markers in newborns and mothers was observed**. This result underscores a close relationship between the fetus and mother and emphasizes the importance of a healthy lifestyle during pregnancy.

In another study, environmental pollution in two localities of the Czech Republic (the Ostrava region, Southern Bohemia) was investigated and its **impacts in newborns and their mothers were compared** [40]. Apart from different concentrations of air pollutants in the localities, seasonal variability was also studied (the samples were collected in winter and summer). **In newborns, lipid peroxidation and DNA oxidation was increased along with**

higher levels of air pollutants (polluted locality, winter season). In mothers, different effects were observed, including the impact of age on lipid peroxidation levels: **in younger mothers a higher probability of increased IsoP levels was found** suggesting the **adaptation of the adult organism to deleterious effects of air pollution**.

The three large studies were focused specifically **on environmental air pollution impacts on young children/newborns**. A total of 894 urine samples from children living in two regions with various levels of air pollutants (Northern Bohemia, Southern Bohemia) were used to assess oxidative damage to DNA (8-oxodG) [41]. Concentrations of PM10, PM2.5 and c-PAHs assessed by stationary monitors were applied as parameters of environmental air pollution; individual lifestyle [environmental tobacco smoke (ETS) exposure], health and pregnancy outcomes were included as potential co-factors. Among other parameters, **DNA oxidation was affected by both PM and c-PAHs concentrations**. Given the large sample size, this study serves as a good indication of suitability of urinary 8-oxodG as a marker of environmental air pollutants exposure.

Both IUGR and LBW, and their link with oxidative stress detected as 8-oxodG levels in placental DNA were addressed in another study included in this thesis [42]. As the pregnancy outcomes might be further modulated by genetic polymorphisms, frequencies of SNPs in 94 selected genes were also assessed. A total of 891 samples was then investigated for the associations between pregnant mothers' exposure to air pollution, oxidative damage to placental DNA, SNPs and IUGR/LBW. The results showed that **above-median levels of 8-oxodG were positively associated with IUGR, as well as LBW**, suggesting a causal link between DNA oxidation and adverse pregnancy outcomes. In addition, LBW was linked with other factors including haplotypes in the promoter region of the gene encoding mannose-binding lectin 2 (MBL2), involved in innate immunity. Unexpectedly, 8-oxodG in placental DNA was not found to be associated with air pollutants concentrations. **Thus, this study did not show a direct link between air pollution and oxidative damage to DNA in placenta.**

The state-of-the-art approaches were used to investigate molecular mechanisms of response to environmental pollution on the developing organism in another study that involved 202 newborns from two localities of the Czech Republic (the Ostrava region, Southern Bohemia) [43]. In cord blood lymphocytes global gene expression changes were assessed using BeadChip arrays and pathway analyses were performed. The data revealed

locality-specific differences, with the higher number of deregulated genes found in subjects from the Ostrava region. Although this locality was characterized by high concentrations of air pollutants, these factors did not show any direct effect on gene expression changes. Rather, the **locality itself was responsible for differences in gene expression profiles suggesting that a complex set of region-specific factors** (not identified here) **modulated the molecular response of the newborns**. The affected processes included those involved in cell growth, apoptosis or cellular homeostasis, immune response-related processes, or **oxidative stress response**.

4.2.2. Studies in adult populations

When investigating health effects of air pollutants in human populations, the researcher needs to decide if target subjects will be those working in a specific environment with high concentrations of pollutants (**occupational exposure**), or if a general population representing real-life air pollution exposure scenarios will be involved (**environmental exposure**). For occupational exposure, higher toxic compounds concentrations and regular, repeated exposure (i.e., on weekdays/working shifts) are expected and thus changes in biomarker levels are more likely to be significant when compared with a control group. However, these studies do not reflect scenarios to which the majority of human population is exposed. It is thus important to perform both types of studies: occupational exposures and investigations in general population exposed to ambient air pollutants.

4.2.2.1. Occupational exposure

This thesis summarizes the data of investigation of oxidative stress markers in a group of Prague **bus drivers**. As these subjects spent their entire working shifts outdoors, in busy city streets, they were regarded occupationally exposed workers with high risks of negative health consequences. To account for seasonal variability of air pollution, repeated sampling was conducted. The results reported in four articles [44–47] focused not only on parameters of DNA, lipid and protein oxidation, but also on assessment of nitrosative stress, detection of DNA breaks in peripheral blood lymphocytes (PBL) and the role of SNPs in modulation of the biological response of the exposed subjects. In one study, a group of garagemen, with potentially higher risk of exposure to air pollutants, was also included [47].

Overall, the data indicated **elevated oxidative and nitrosative stress in bus drivers when compared with the control group**. When any sampling season was considered, 8-oxodG levels were increased in bus drivers in all seasons, while lipid peroxidation and protein oxidation markers showed elevated levels in bus drivers in winter seasons only. In PBL DNA, increased level of DNA breaks and elevated DNA oxidation was observed in both the bus drivers and garagemen. The presence of a variant allele in *hOGG1* was associated with increased oxidative damage in PBL DNA, while the Gln/Gln homozygotes in the DNA repair gene *XPD23* tended to be more susceptible to DNA strand breaks induction.

In summary, the research indicates **possible health risks in bus drivers**, caused by increased levels of oxidative damage to macromolecules, as well as induction of DNA breaks. These effects might be more pronounced in subjects with certain SNPs in DNA repair genes.

4.2.2.2. Environmental exposure

To address the impacts of environmental exposure, studies involving city policemen from Prague and city policemen and office workers from the Ostrava region were performed [48–51]. Again, sampling was done repeatedly to address the effect of seasonal variability, particularly inversion episodes in winter that significantly increase the concentrations of pollutants in the ambient air. Although city policemen can be regarded as occupationally exposed workers, the enrolled subjects worked not only in the streets, but also in the offices. In addition, a group of office workers was included in the City of Ostrava. Thus, the study can be considered environmental exposure investigation, rather than occupational exposure setting.

This comprehensive investigation focused not only on the analysis of oxidative stress biomarkers, but also on the detection of bulky DNA adducts, stable and unstable chromosomal aberrations and changes in gene expression. To assess the exposure to air pollutants, personal and stationary monitors of BaP, PM2.5 and benzene were used. Considering the high levels of air pollution in the Ostrava region and the previous data obtained in bus drivers, significant damage to macromolecules was expected among the Ostrava region subjects. Unexpectedly, the levels of stable chromosomal aberrations were mostly comparable in samples from both localities; in addition, the frequency of micronuclei, a marker of unstable chromosomal aberrations, was decreased in the Ostrava region subjects.

Urinary 8-oxodG excretion did not differ between localities. For lipid peroxidation and protein oxidation, parameters not influenced by possible repair processes, inconsistent differences between localities were noted. Interestingly, among all subjects, lipid peroxidation levels were significantly positively correlated with air pollution levels. In a subset of the samples, additional analyses focused on the expression of DNA repair genes involved in base excision repair and non-homologous end-joining repair were performed. Apart from the lack of air pollution impacts on DNA damage parameters, increased odds of having above-median expression levels of *XRCC5* were noted among the Ostrava region subjects [48] indicating the induction of processes related to NHEJ repair. To get more detailed, in-depth information on the response of the study subjects on impacts of air pollutants, global gene expression analysis using BeadChips microarrays was conducted [51]. This research showed significant differences in gene expression profiles between seasons and localities, as well as variability in numbers of deregulated genes. Importantly, in the Ostrava region subjects, a decreased expression was observed for numerous genes, including e.g. *APEX*, *ATM*, *FAS*, *GSTM1*, *IL1B* and *RAD21*. The study suggested that **chronic exposure to high levels of air pollutants may result in modified gene expression response, manifested by unexpected findings in biomarker levels, but potentially causing negative health consequences later in life** [51].

The inconsistent data from the human studies reported above were discussed, compared with the results of others and summarized in the review article in which **the role of adaptation to negative effects of the environment was suggested** [52]. Although adaptive response was described several decades ago and observed in various organisms, including bacteria, plants and animals, its existence in humans is still the source of controversy. However, the lack of consistent effects of air pollutants on biomarkers in populations from the Ostrava region exposed for many years to elevated levels of toxic compounds in the ambient air suggests that such response was induced. In follow-up studies the possible mechanisms of adaptation were further investigated, however, they are not included in this thesis.

4.3. Methodological aspects: how to detect oxidatively damaged DNA in human samples

The application of reliable and reproducible methods for biomarkers detection is a key requirement when performing any laboratory test. For detection of DNA oxidation either

instrumental techniques [e.g., high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS), liquid/gas chromatography coupled with mass spectrometry (LC/GC-MS), gas chromatography/mass spectrometry (GC/MS)], or antibody-based methods (e.g., ELISA) are most commonly used. While chromatography/spectrometry-based analyses allow precise biomarker detection, they are time-consuming and expensive. In contrast, ELISA is often a method of choice if a large sample set is to be analyzed and/or the required instrumentation is not available in the laboratory. As ELISA uses anti-8-oxodG antibodies, it suffers from lower sensitivity when compared with the instrumental approaches and potential cross-reactivity with other urine components. Thus, it has been often regarded as less reliable method of 8-oxodG detection. Despite that, numerous studies have been published using this analytical approach [11]. Thus, it had been of utmost importance to compare ELISA 8-oxodG detection with instrumental techniques and, if discrepancies appeared, to find a modification that would increase ELISA specificity/reliability.

Additionally, suitable biological material should be selected for the evaluation of oxidative DNA damage levels. Theoretically, genomic DNA (extracted e.g. from PBL or other tissue of choice) could provide most direct information on potential genetic damage induced by ROS and the role of repair processes; however, there are methodological limitations when using this material. First, obtaining blood is an invasive approach, not suitable for some population groups (particularly children). Also, skilled personnel are required to draw blood samples. Additionally, for further analyses, DNA is usually extracted from PBL, and samples handling may result in artefactual DNA oxidation. For these reasons, in human studies this biomarker is commonly assessed in urine, as this matrix is easily accessible and 8-oxodG is stable for a long period of time, even if the sample is kept at room temperature [12]. However, the origin of 8-oxodG in urine has been repeatedly discussed and it is generally agreed that DNA repair does not play any role in accumulation of 8-oxodG in this biological fluid. Rather, the nucleotide pool is the most probable source of this oxidized nucleoside (discussed in Chapter 2.1).

With these points in mind, an initiative had been established to standardize the method of 8-oxodG detection in urine, in which P.R. had also been involved [11,53,54]. The compared approaches were those most commonly used for 8-oxodG assessment (HPLC-MS/MS, LC-GC/MS, GC-MS, ELISA). Initially, ELISA had been identified as the method that suffers from

higher within-technique variation than chromatographic techniques and 8-oxodG values obtained by this method were generally higher than instrumental approaches indicated [53,54]. The concerns over ELISA results led to a series of optimization experiments that resulted in publication of an improved ELISA protocol [55]. **The optimization steps included urine purification by solid-phase extraction, incubation of the purified samples with anti-8-oxodG antibody at 4 °C overnight and normalization of 8-oxodG levels per urinary creatinine. These steps resulted in a near-perfect correlation of ELISA data with chromatography.**

The modified technique was further used in an inter-laboratory comparison of 8-oxodG detection by ELISA and correlation of these data with HPLC-MS/MS analysis [56]. In most of the laboratories involved in the study a significant correlation of 8-oxodG data was observed. In addition, for the majority of the analyzed samples a correlation with chromatography was found. However, several outlier samples were identified for which a correlation between ELISA and chromatography was poor. In these samples, the presence of saccharides, aromatic and heterocyclic compounds was identified. These compounds probably interfered with anti-8-oxodG antibody binding thus affecting the reliability of ELISA. In summary, while **improved ELISA gives better 8-oxodG estimates than a standard approach, there are still samples for which the antibody-based detection may not yield reliable results.**

An update on biomarkers of nucleic acid oxidation was later published in a collaborative paper, in which the application of improved ELISA for urinary 8-oxodG detection is further discussed, including its potential limitations [11].

5. Conclusions

The results presented in this thesis suggest that PM and compounds bound to it, rather than PAHs alone are responsible for oxidative damage induction by environmental air pollution.

(i) *In vitro* tests showed the ability of EOM to induce ROS generation and oxidative damage induction, while PAHs alone, or in a mixture had generally weaker effects, or even caused a decrease in lipid peroxidation levels. These observations are in

agreement with the fact that ROS formation does not represent a dominant mechanism of PAHs metabolism

(ii) In human studies the response to PAHs and PM exposure depended on the investigated group and locality:

a. 8-oxodG levels in placenta DNA were positively associated with IUGR and LBW, but did not reflect levels of air pollutants
b. a significant correlation between oxidative stress markers, modulated by environmental pollutants, in newborns and mothers was observed
c. DNA oxidation in young children was associated with ambient air concentrations of both PM and c-PAHs
d. elevated oxidative and nitrosative stress was found in Prague bus drivers exposed to moderate levels of air pollutants
e. chronic exposure to high levels of air pollutants results in modified gene expression response, manifested by unexpected findings in biomarker levels and adaptation to deleterious impacts of environmental toxicants

Overall, the data confirms generally accepted conclusions of research studies on oxidative stress induction by environmental air pollutants, modulated rather by PM, than PAHs alone. In addition, exposure to high concentrations of ambient air toxicants induces adaptation processes rendering the analyzed biomarkers less reliable in their application as parameters reflecting deleterious impacts of the environment. Thus, careful evaluation of the results from the comprehensive set of biomarkers, accounting for population specifics and exposure conditions, is needed before drawing conclusions on impacts of the environment of oxidative stress markers.

6. References (the publications that are a part of the thesis are highlighted in red)

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7. The list of publications included in the thesis (in chronological order)

In vitro studies (10 publications)

P. Rossner Jr., J. Topinka, J. Hovorka, A. Milcova, J. Schmuczerova, J. Krouzek, R.J. Sram, An acellular assay to assess the genotoxicity of complex mixtures of organic pollutants bound on size segregated aerosol. Part II: oxidative damage to DNA, Toxicol Lett 198 (2010) 312–6. https://doi.org/10.1016/j.toxlet.2010.06.021.

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Human studies (newborns/pregnant women and young children) (5 publications)

P. Rossner Jr., A. Milcova, H. Libalova, Z. Novakova, J. Topinka, I. Balascak, R.J. Sram, Biomarkers of exposure to tobacco smoke and environmental pollutants in mothers and their transplacental transfer to the foetus. Part II. Oxidative damage, Mutat Res 669 (2009) 20–26.

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Human studies (adult populations) (9 publications)

P. Rossner Jr., V. Svecova, A. Milcova, Z. Lnenickova, I. Solansky, R.M. Santella, R.J. Sram, Oxidative and nitrosative stress markers in bus drivers, Mutat Res 617 (2007) 23–32.

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Methodological aspects (5 publications)

M.D. Evans, R. Olinski, S. Loft, M.S. Cooke et al. (**P. Rossner**), Toward consensus in the analysis of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine as a noninvasive biomarker of oxidative stress, FASEB J 24 (2010) 1249–60. https://doi.org/10.1096/fj.09-147124.

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