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Molecular mechanisms involved in genotoxicity of industrially important monomers (styrene, 1,3-butadiene)

Mgr. Miroslava Kuricová

Praha 2009

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DISSERTATION THESIS

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Statutory Declaration

I hereby formally declare that I have not submitted this work or it‘s substantial part to obtain another or the same academic degree.

ABSTRACT

The evaluation of individual health risk in workers occupationally exposed to industrial xenobiotics requires the use of a large number of parameters reflecting external exposure, internal exposure, biological effects and individual susceptibility. Environmental, occupational and life style-related exposure to mutagenic agents may contribute to cancer risk in humans. To prevent the potentially hazardous effects of such agents it is important to understand their mechanisms of action. Styrene is one of the most important monomer for producing polymers and copolymers in plastics, latex paints and together with 1,3-butadiene (BD) in the manufacture of synthetic rubbers. In this thesis, a large set of parameters, including markers of external and internal exposure and biomarkers of biological effects and susceptibility have been studied in relation to the occupational exposure to both styrene and BD.

First part of the present study was focused on evaluating the role of various biomarkers to assess genotoxic effects of above mentioned xenobiotics. Biomarkers reflecting styrene- and BD-induced genotoxicity and mutagenicity: O^6-styrene guanine DNA adducts, haemoglobin adducts, single-strand breaks (SSBs), SSB Endo III sites, chromosomal aberrations (CA), hypoxanthine-guanine phosphoribosyltransferase gene mutation freguencies (*HPRT* MF), from the aspects of their accumulation over time and of the role of adaptation and/or selection in the genotoxic risk of styrene exposure have been analyzed (Publications No. II, III, V, VI).

Second topic of the study was to investigate the possible modulating role of genetic polymorphisms of genes encoding for metabolizing and detoxifying enzymes in individuals occupationally exposed to styrene and BD (Publications No. II, III, VI). DNA samples of exposed workers and controls were subjected to genotype analysis for *EPHX1* (Tyr113His and His139Arg), *GSTM1* (deletion), *GSTP1* (Ile105Val) and *GSTT1* (deletion) polymorphisms. Hand-lamination workers exhibited a significantly higher proportion of low *EPHX1* activity genotype. Styrene-exposed individuals with *GSTP1* genotype Ile/Ile exhibit significantly lower MF at the *HPRT* locus as compared to those with heterozygous *GSTP1* genotype.

Third part of the study was focused on assessing the role of DNA repair capacities (DRC) in styrene- and BD-exposed workers (Publications No. II-VII). Individual DRC in styrene-exposed workers was significantly higher in comparison with controls. The stimulation of DNA repair in laminators could explain their enhanced capacity to repair DNA damage, which is assumed to be repaired mainly by base excision repair pathway. Possible relationships between the capacity to repair oxidative DNA damage, parameters of exposure and parameters of genotoxic effects have been analyzed. The only positive correlation was found between DRC and DNA damage in females. An increased capacity to incise 8-oxoguanine, which represents oxidative damage in lymphocytes, was recorded among highly exposed workers. Significant association between both internal and external exposure parameters and repair capacity to remove oxidative DNA damage suggests a possible role of oxidative stress in styrene-related genotoxicity.

In the next part of the study, modulating effect of DNA repair gene polymorphisms in the context of styrene and BD exposure has been investigated. Genetic polymorphisms in DNA repair genes and possible links with DNA repair rates, CAs and SSBs in DNA are summarized in Publications No. IV, VI, VII. Among all analyzed polymorphisms, *XPD* Lys751Gln polymorphism was a major factor influencing the frequencies of CAs. SSBs and CAs frequencies were the highest in individuals with common *AA* genotype and the lowest in those with variant *CC* genotype for this polymorphism. Tire workers with a combination of low *EPHX1* activity genotypes and the *AA* (wild type) and *AC* (heterozygous) *XPD* alleles exhibited higher levels of CAs than individuals with combined high *EPHX1* activity genotypes and variant allele *CC* genotype for *XPD*. This observation suggests an increased risk of genotoxic effects in individuals with particular genotype combinations.

Finally, analysis of immune markers and their relationship with various genetic polymorphisms has been performed for the first time in the present study (Publications No. I, IV). An increase number of leukocytes and lymphocyte was observed in individuals with *GA* and *AA* genotypes of *Cyclin D1* Pro242Pro polymorphism as compared with those with common *GG* genotype. The number of eosinophiles was positively associated with variant *C* allele for *XPD* Lys751Gln. Immunoglobulin IgA was positively associated with variant *T* allele *XRCC3* Thr241Met and negatively with *AC* and *CC* genotypes of *XPC* Lys939Gln. The relationships between various DNA repair polymorphisms and immune parameters are even more difficult to explain at the moment, due to the lack of knowledge on functional aspects of the genetic polymorphisms analyzed and due to the complexity of the immune system.

It is important to use many biomarkers in large population and consider altogether all aspects of genotxicity. A comprehensive approach may provide fundamental information about the suitability of the biomarkers and may contribute to the understanding of the mechanisms of genotoxic effects of industrial xenobiotics and their metabolites in humans.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be refered to by their Roman numerals:

I

Kuricová M, Jahnová E., Dušinská M., Líšková A., Tulinská J. Vodička P, Šulcová M and Fuortes L. (2001) Immune markers in biological monitoring of occupationally exposed workers. *BIOLOGIA* 56 (3):293-296.

II

Vodicka P, Stetina R, Koskinen M, Soucek P, Vodickova L, Hlavac P, Kuricova M, Necasova R, Hemminki K. (2002) New aspects in the biomonitoring of occupational exposure to styrene. *Int. Arch. Occup. Environ. Health* 75:75–85.

III

Vodicka P, Koskinen M, Stetina R, Soucek P, Vodickova L, Matousu Z, Kuricova M, Hemminki K. (2003) The role of various biomarkers in the evaluation of styrene genotoxicity. *Cancer Detect. Prevent*. 27:275–284.

IV

Vodicka P, Kumar R, Stetina R, Sanyal S, Soucek P, Haufroid V, Dusinska M, Kuricova M, Zamecnikova M, Musak L, Buchancova J, Norppa H, Hirvonen A, Vodickova L, Naccarati A, Matousu Z, Hemminki K. (2004a) Genetic polymorphisms in DNA repair genes and possible links with DNA repair rates, chromosomal aberrations and single-strand breaks in DNA. *Carcinogenesis* 25:757–763.

V

Vodicka P, Tuimala J, Stetina R, Kumar R, Manini P, Naccarati A, Maestri L, Vodickova L, Kuricova M, Järventaus H, Majvaldova Z, Hirvonen A, Imbriani M, Mutti A, Migliore L, Norppa H, Hemminki K. (2004b) Cytogenetic markers, DNA single-strand breaks, urinary metabolites and DNA repair rates in styrene-exposed lamination workers. *Environ. Health Perspect.* 112:867–871.

VI

Vodicka P, Kumar R, Stetina R, Musak L, Soucek P, Haufroid V, Sasiadek M, Vodickova L, Naccarati A, Sedikova J, Sanyal S, Kuricova M, Brsiak V, Norppa H, Buchancova J, Hemminki K. (2004c) Markers of individual susceptibility and DNA repair rate in workers exposed to xenobiotics in a tire plant. *Environ. mol. Mutag.*, 44:283–292.

VII

Kuricova M, Naccarati A, Kumar R, Koskinen M, Dusinska M, Tulinska,J, Vodickova L, Liskova A, Jahnova E, Fuortes L, Haufroid V, Hemminki K, Vodicka P. (2005) DNA repair and cyclin D1 polymorphisms and styrene-induced genotoxicity and immunotoxicity. *Toxicol. Appl. Pharmacol*, 207:S302–S309.

VIII

Slyskova J, Dusinska M, Kuricova M, Soucek P, Vodickova L, Susova S, Naccarati A, Tulupova E, Vodicka P. (2007) Relationship between the capacity to repair 8-oxoguanine, biomarkers of genotoxicity and individual susceptibility in styrene-exposed workers. *Mutat Res.*;634(1-2):101-11.

ABBREVIATIONS

4-VPT	4-vinylphenol
6-4PP	Pyrimidine-(6-4)-pyrimidone photoproduct
8-OhdG	8-hydroxy-2'-deoxyguanosine
ADH	Alcohol dehydrogenase
ADLH	Aldehyd dehydrogenase
AP	Apurinic/apyrimidinic site
BD	1,3-butadiene
BER	Base excision repair
CA	Chromosomal abberations
Con A	Mitogen concanavalin A
Cis-Pt	Cisplatin, anti-tumor agent
CPD	Cyclobutane pyrimidine dimer
CYP	Cytochrome P-450 enzyme
DEB	Diepoxybutane
DNA	Deoxyribonucleic acid
DRC	DNA repair capacity
EB	Epoxybutene
EBD	Epoxybutanediol
EH	Epoxide hydrolase
EPA	US Environmental Protection Agency
EPHX1	Epoxide hydrolase 1 gene
GSTM1	Glutathione S-transferase *M1* gene
GSTP1	Glutathione S-transferase *P1* gene
GST	Glutathione S-transferase
GSTT1	Glutathione S-transferase *T1* gene
Hb	Haemoglobin
hOGG1	8-oxoguanine DNA glycosylase gene
HPRT MF	Hypoxanthine-guanine phosphoribosyltransferase mutation frequency
HPRT	Hypoxanthine-guanine phosphoribosyltransferase gene
HR	Homologous recombination

IARC	International Agency for the Research on Cancer
IgG, IgA	Imunoglobulines G, A
IgM, IgE	Imunoglobulines M, E
MA	Mandelic acid
MMC	Mitomycin, anti-tumor agent
MMR	DNA mismatch repair
MN	Micronuclei
mRNA	Messenger ribonucleic acid
NER	Nucleotide excision repair
NHEJ	Nonhomologous end join
NK	Natural killer cells
PAH	Polycyclic aromatic hydrocarbons
OGG1	8-oxoguanine glycosylase
PBLs	Peripheral blood lymphocytes
PCR	Polymerase chain reaction
PEG	Phenyletylene glycol
PGA	Phenylglyoxilic acid
PHEMA	Phenylhydroxyethyl mercapturic acid
RFLP	Restriction fragment length polymorphism
SBR	Styrene-butadiene rubber
SB	Strand break
SCE	Sister chromatid exchange
sICAM-1	Solubile intercellular adhesion molecule 1
SNP	Single-nucleotide polymorphism
SO	Styrene-7,8-oxide
SSB	Single strand breaks
SSB Endo III	Single strand breaks produced by endonuclease-III
SULT	Sulfotransferase
UGT	Uridine diphosphate (UDP)-glucuronosyltransferase
XME	Xenobiotic-metabolizing enzyme
XPC	Xeroderma pigmentosum, complementation group C gene
XPD	Xeroderma pigmentosum, complementation group D gene
XPG	Xeroderma pigmentosum, complementation group G gene

XRCC1	X-ray repair cross complementation group 1 gene
XRCC3	X-ray repair cross complementation group 3 gene

GENERAL BACKGROUND

Exposure to industrial chemicals, biotransformation and DNA repair

Xenobiotics (from the Greek *xenox* = foreign, *bios* = life) are chemical compounds that are alien to the living organisms. Principal xenobiotics include drugs, carcinogens and various compounds that have been introduced into the environment by artificial means.

It has been assumed for long that environmental and occupational exposure to chemicals may result in the development of cancer. The first example of a specific causal relationship between occupational exposure and cancer has been reported already in the 18th century, where several articles were published reporting that miners, smelters, dyers and chimney sweepers were affected by specific cancers.

The persistence of xenobiotics in the environment as well as their chemical property and toxicity determine the potential health hazard. Persistence is a function of the biotransformation rate in living organism, which influences the compound's form, time, and mobility in the environment (Wilson *et al.* 1985). Recent reviews on the toxic properties of chemicals and their classifications with respect to the carcinogenic potency have been published by international agencies like IARC (International Agency for the Research on Cancer, France) or EPA (US Environmental Protection Agency, USA) on a regular basis and summarizing the most recent scientific achievements. These data are frequently used for decision making by the civil authorities to impose occupational, environmental and dietary exposure limits. However, a complete understranding of genotoxic/carcinogenic potential of industrial chemicals has yet to be accomplished.

Biotransfromation of xenobiotics represents a key step in their elimination from the body. This process consists of the deactivation and the excretion of metabolites, and occurs mostly in the liver. Several major enzymes and pathways are involved in drug metabolism, and are divided into Phase I and Phase II reactions. Phase I reaction comprises oxidation, reduction, hydrolysis and/or hydration of the xenobiotics, followed by conjugation the active secondary metabolites with glucuronic

or sulphuric acid, or glutathione (Phase II) with subsequent excretion in bile or urine (Jakoby *et al.* 1990).

The metabolic conversion of environmental compounds may result in the formation of reactive metabolites, capable to attack directly the nucleophilic centers of biological macromolecules (DNA, proteins) and thus exerting the genotoxic effects (reviewed in Pfohl-Leszkowicz 2008).

DNA is constantly damaged by a variety of factors both physical (UV or ionising radiation) and chemical (reactive oxygen species, alkylating agents, bulky adducts). More than 10 000 various kinds of such lesions per cell and day can result in mutations, genomic instability, or cell death. Such damages must be repaired quickly and efficiently to keep the integrity of the genome. Therefore, the cells have developed a very complex mechanism of more than 150 genes involved in 6 major repair pathways in which different kinds of DNA damage can be detected and repaired: (1) the direct reversal pathway, (2) Homologous Recombination (HR), (3) Nonhomologous End Joining (NHEJ), (4) Nucleotide Excision Repair (NER), (5) Base Excision Repair (BER) and (6) Mismatch Repair (MMR) (Wood *et al.* 2001; Ataian *et al.* 2006). The BER proteins excise and replace damaged DNA bases, mainly those arising from oxidative damage or alkylated bases. NER mainly removes bulky adducts or UV photoproducts caused by environmental agents. MMR corrects occasional errors of DNA replication as well as heterologies formed during recombination. Two additional types of DNA repair, HR and NHEJ, are employd when the most serious type of DNA damage, a doublestrand break, occurs (Lindahl and Wood 1999; Wood *et al.* 2001; Hakem 2008; Figure 1).

Apparently, DNA repair seems to remove DNA damage induced by simple alkyl epoxides by different mechanisms (base excision repair, BER: short-patch repair) in comparison to methylating agents (BER: long-patch repair) and polycyclic aromatic hydrocarbons (NER) (Lindahl and Wood 1999; Mishina *et al.* 2006; Braithwaite *et al.* 1998; Wyatt and Pittman 2006).

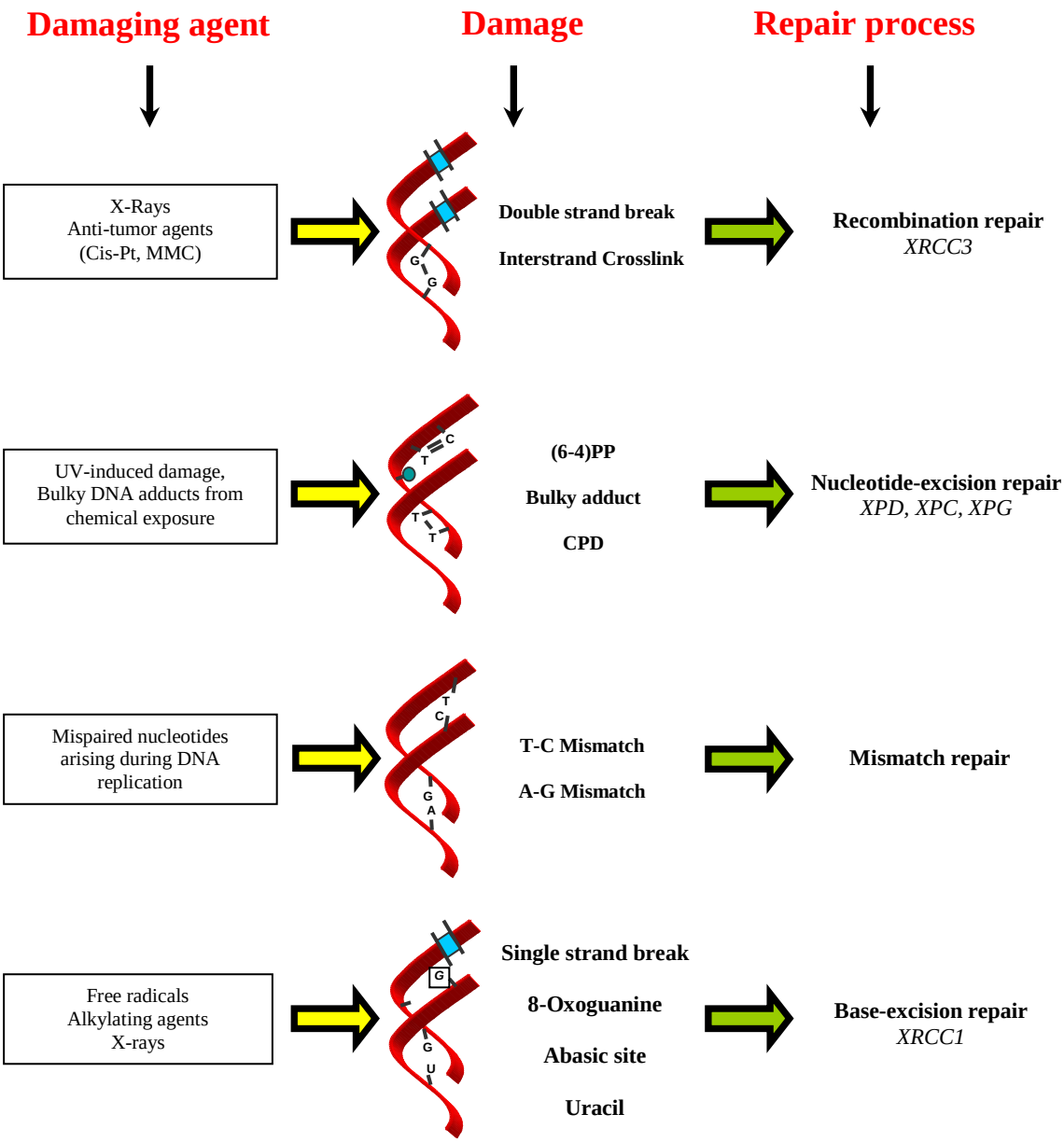

Figure 1: A simplified scheme of the most important DNA repair pathways (modified from Hoeijmakers *2001*).

The cell cycle and mitotic spindle checkpoints are also critical in this process to ensure that cell proliferation only follows correct replication and organization of genetic material, respectively. However, if the genetic material is altered, it can be repaired at the DNA level, enabling the cell to replicate. If the genetic damage is too excessive for repair, the cell avoids propagating the damaged DNA by undergoing apoptosis (Friedberg 2003; Hakem 2008).

Markers assessing the genotoxic effects

Humans are continually exposed to numerous environmental and/or occupational xenobiotics and if the metabolic system is less efficient this may lead to their accumulation in the body. Exposure to a potential chemical carcinogen involves a continuum of events starting from absorption, continuing through activation to reactive metabolites and binding to DNA and resulting into mutations. In the worst case, the above process may result in cancer development (Perera *et al.* 2000; Au and Salama 2005; Figure 2).

In the past years there is increasing interest in using biological markers to monitor populations for identification oversized exposure to environmental xenobiotics. The basic aim is to use these findings to predict increased risk for development long-term health consequences. With improved occupational conditions, current workers are usually exposed to lower concentrations of xenobiotics then in the past. In addition, the automation of industrial processes leads to decrease of number of employed workers. This may cause a problems when biomonitoring studies recquired extensive population size for sophisticated statistical analyses (Au *et al.* 1996).

Various biomarkers can be used to elucidate the mechanisms of the genotoxic/carcinogenic process as well as the individual response to carcinogens. Any measurable alteration of the DNA is regarded as a potential marker of exposure. As a biomarker, DNA damage in lymphocytes probably reflects an exposure over the previous few weeks, but if some damage is resistant to repair, a cumulative increase in the steady state level might appear over time. Moreover, the level of DNA damage represents a steady state between induction of damage and its repair (Somorovska *et al.* 1999).

DNA adducts, DNA and chromosomal damage

DNA adducts are, among various other biomarkers, particularly appropriate as biomarkers of DNA damage. This important integral measure of DNA damage reflects the biological effect of a potential mutagen and may trigger a cascade of events that

can be directed to carcinogenesis (Perera and Weinstein 2000; Hemminki *et al.* 2000; Veglia *et al.* 2008).

DNA adducts also represent an individual measure of metabolic (activating or detoxifying) enzymes as well as DRC (Hemminki *et al.* 2000; Perera and Weinstein 2000). These primary DNA lesions cause alterations in the DNA structure, resulting in the formation of DNA strand breaks, chromosomal rearrangements, deletions and mutations. Following ineffective repair, the residual DNA damage may lead to the insertion of an incorrect base, followed by transcription and translation of mutated templates, finally resulting in the synthesis of modified protein. Serious are the mutations in genes controlling the cell cycle, oncogenes or tumor-suppressor genes, resulting in a cell population with a proliferative or survival advantage. (Vodicka *et al.* 2006a; Figure 2). Evidence of a relationship between DNA adducts and cancer has been summarized elsewhere (Poirier 2004; Farmer *et al.* 2008). The possibility to determine specific DNA adducts quantitatively, and their comparison to other genotoxic parameters or biomarkers provides essential information on the mechanisms of potentially genotoxic agents. The sensitivity of the currently available methods for adduct measurement has been shown to be sufficient for detecting the background levels of DNA and protein damage (Vodicka *et al.* 2006c).

Persistant DNA adduct lesions lead to several types of genomic alterations. Among them, there are macrolesions corresponding to changes in the structure of chromosome. Structural CAs represent microscopically recognizable changes in the morphology of the chromosome, primarily breakage and exchanges between and within chromosomes. Recent prospective studies have indicated a positive correlation between the frequencies of spontaneous CA in peripheral lymphocytes and a later onset of cancer (Natarajan 2002; Hagmar *et al.* 2004; Boffetta *et al.* 2007). The predictive value of CA frequencies was particularly strong for stomach and colorectal cancer, but somewhat weaker for lung cancer (Rossner *et al.* 2005).

Other types of cytogenetic damage observable in microscope include sister chromatid exchanges (SCEs) and micronuclei (MN), which are formed either from acentric chromosomal fragments or whole chromosomes lagging in cell division.

In human lymphocytes *in vivo*, tobacco smoking, alkylating cytostatics, and ethylene oxide are well-documented SCE inducers (Tucker *et al.* 1993). Moreower,

increased MN frequencies have been associated with exposure to ionizing radiation, age, and gender (Norppa *et al.* 1993).

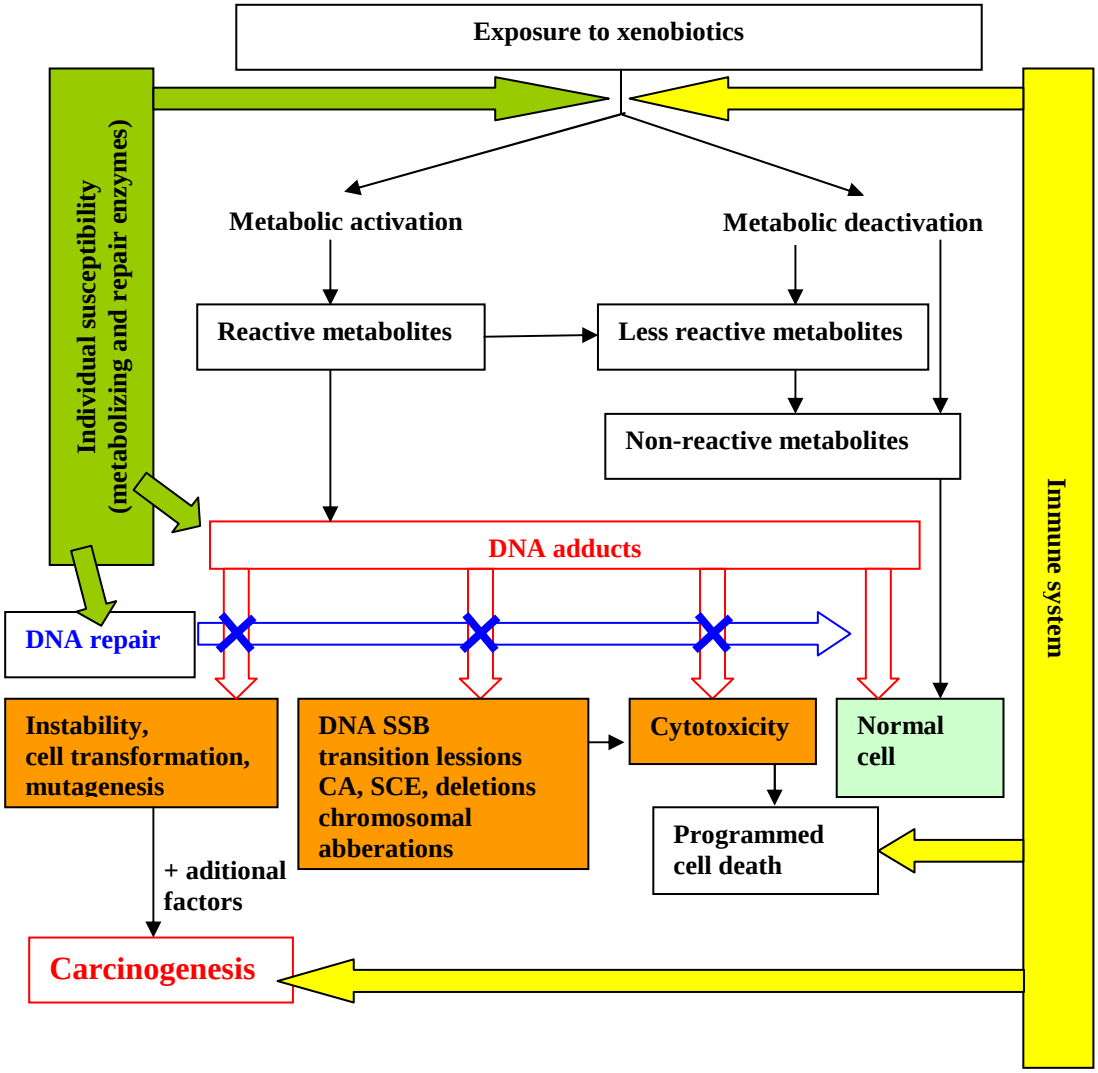

Figure 2: Events involved in the cascade of genotoxic effects following exposure to xenobiotics

The most commonly analysed DNA damage is represented by SSBs in DNA. These are transient promutagenic lesions, representing direct effects of damaging agents. They may also be related to apurinic or apyrimidinic sites and also represent intermediates in cellular repair, since both NER and BER cut out the damage and replace it with undamaged nucleotides (Collins *et al.* 1997).

Markers of individual susceptibility

Optimally, various biomarkers should be employed to understand genotoxic effects of xenobiotics. Simultaneously, it should also be considered the role of interindividual variability which may markedly modulate the observed results by affecting metabolism, cellular response to carcinogens and defence mechanisms. In particular, for understanding individual factors modulating genotoxic and/or carcinogenic risks, biomarkers of individual susceptibility and DNA repair are important. Modern standard techniques, such as polymerase chain reaction (PCR), which allows the amplification of small gene regions, can be used for the detection of gene polymorphisms. In particular, single nucleotide polymorphisms (SNPs) can be identified by restriction fragment length polymorphism (RFLP) techniques or using DNA microarray (SNP array).

For genotyping, to assess properly all possible combinations of various relevant genotypes and their modulating effects on biomarkers large cohorts are needed. (Vodicka *et al.* 2003). Ideally, studies on individual susceptibility should enable us to establish various positive and adverse genotypes which should minimize the risks resulting from exposures in sensitive individuals. Unfortunately, this is not possible at the moment, since we have insufficient information on all the relevant genetic variations, and we do not know enough about the functional effects of individual polymorphisms or in combination (gene-gene interactions, SNP-SNP interactions, haplotypes). Because the effects of these polymorphisms are relatively subtle, and some important alleles are relatively rare, much larger study populations are necessary to evaluate their modulating effects on biomarkers, especially when gene-gene interactions are regarded (Vodicka *et al.* 2004c).

A number of studies have addressed the role of genetic polymorphisms for the risk of genotoxic effects and cancer in humans in the past few years. Because carcinogenesis is influenced by a multitude of genes, any single polymorphism affecting cancer risk is, in general, expected to have a relatively small contribution at the individual level. However, the attributable risk to the population of common low-penetrance susceptibility genes may be substantial (Shields and Harris 2000; Hemminki *et al.* 2006).

Associations between various genetic polymorphisms and molecular markers involved in the cascade of genotoxic events may provide useful information on the modulating effects of genetic polymorphisms, on individual susceptibility towards environmental and occupational carcinogens and on the possible links between DNA repair polymorphisms and individual DNA repair rates (Vodicka *et al.* 2004a). Individual susceptibility to exogenous genotoxicants is determined by factors including exposure, nutritional status, genetic and immunological constitution. It seems to be critical to understand the functional effect of genetic polymorphisms in key enzymes and proteins involved in chemical biotransformation and DNA repair to define the role of genetic background in modulating sensitivity towards xenobiotics.

XME polymorphisms

A specific response to exogenous and endogenous genotoxicants may be modulated by the genetic polymorphisms of xenobiotic-metabolizing enzymes (XMEs), resulting in increased or decreased efficiency of biotransformation. Many of the various XMEs enzymes are polymorphic – several common forms of the enzyme coexist in the population, due to a multiple alleles. As the enzyme variants may differ from each other in function, genetic polymorphism may be considered as a marker representing a different individual reaction to environmental exposure. The toxicological importance of a polymorphism in a population depends on the prevalence of the „risk“ genotype (Rothman *et al.* 2001).

Several drugs, xenobiotics and some endogenous substances are metabolised by cytochrome P450 monooxygenases (CYP). The mutations in the CYP genes can cause enzyme products with abolished, reduced, altered or increased enzyme activity. It is attractive to speculate that polymorphism in the *CYP* genes would influence the individual’s capacity to convert different precarcinogenic compounds into their ultimate carcinogens and thus, being a major factor of importance for the individual’s susceptibility for developing chemically induced cancer (Ingelman-Sundberg 2001).

Another source of inter-individual variation in metabolism of xenobiotics are polymorfic genes, coding for the enzymes involved in the metabolic phase II pathway. Phase II enzymes include glutathione S-transferases (GSTs) and microsomal EH. Currently, two polymorphisms of *EPHX1* gene are known: a T to C substitution in exon 3 (Tyr113His) reduces enzyme activity, whereas an A to G transition in exon 4 (His139Arg) is associated with increased activity (Seidegard and Ekström 1997).

GSTs are a complex multigene superfamily of inducible enzymes involved in the metabolism of a wide range of chemicals. In humans, cytoplasmic GSTs comprise eight classes: α (GSTA), μ (GSTM), π (GSTP), θ (GSTT), τ (GSTZ), σ (GSTS), ο (GSTO) and κ (GSTK) with distinctive substrate specificity. The μ glutathione S-transferases (GSTM), for example, are highly active against epoxides and metabolize arene oxides such as styrene-7,8-oxide (SO), benzo[a]pyrene diolepoxide and trans-stilbene oxide (Steinkellner *et al.* 2005). GSTP1 is the most abundant GST isoform in the lungs and, therefore, is particularly important in the inactivation of inhaled toxicants, such as styrene or tobacco-related procarcinogens (Saarikoski *et al.* 1998). It is also over expressed in some tumours and drug resistant cell lines, suggesting a significant role in acquired resistance to certain anticancer drugs (Hayes *et al.* 1995).

DNA repair polymorphisms

Another group of genetic susceptibility factors that could influence the levels of DNA and chromosome alterations are polymorphisms of DNA repair genes, which are involved in the repair of various DNA lesions induced by exogenous or endogenous genotoxic compounds as well as ionizing and UV radiations (Duell *et al.* 2000; Christmann *et al.* 2003). The polymorphisms of different DNA repair genes, mainly SNPs, modulate the individual repair capacity in response to DNA damage. Thus, the presence of a large inter-individual variation in DRC may represent that individuals with repair capacity below the population mean can be at an increased risk of developing a disease, including various kinds of cancer (Vodicka *et al.* 2007). Although the links between some SNPs of DNA repair genes and their phenotypic

consequences have been already investigated (Mayer *et al.* 2002; Vodicka *et al.* 2004a), this crucial topic recquires additional attention.

Few examples illustrating associations among DNA repair polymorphisms and various parameters of genotoxicity are hereby reported. A homozygosity for a variant polymorphism in the DNA ligase subunit XRCC1 is associated with higher sister chromatid exchange frequencies in smokers, suggesting an association of this allele with a higher risk for tobacco- and age-related DNA damage (Duell *et al.* 2000). Ladiges *et al.* (2003) briefly summarizes epidemiological and functional relevance of *XRCC1* SNPs in relation to cancer and other age-related diseases. BER rates seem to be decreased with *XRCC1 Arg399Gln* homozygous variant genotype. Similarly, the capacity to repair oxidative DNA damage appears to be significantly decreased among individuals with *hOGG1 Ser326Cys* homozygous variant genotype (Au *et al.* 2004; Vodicka *et al.* 2007). Statistically significant associations have been found between *XPD* polymorphisms and skin, breast and lung cancers (Manuguerra *et al.* 2006). These studies, as well as many others, provide information for better understanding the mechanisms in the development of disease, for quantitative risk assessment and for development of strategies in disease prevention programs.

Hundreds of polymorphisms in DNA repair genes have been identified so far; however, for many of these polymorphisms the impact on repair phenotype and cancer susceptibility remains uncertain. The epidemiology of DRC and its effect on cancer susceptibility in humans is, therefore, an important area of investigation (Naccarati *et al.* 2007). Several other studies have been conducted to investigate the functional effects of variant DNA repair genes by using different biomarkers (Dybdahl *et al.* 1999; Berwick and Vineis 2000; Au *et al.* 2004; Vodicka *et al.* 2004a).

DNA repair capacity

The polymorphisms of genes involved in different DNA repair pathways may modulate the individual response to DNA damage and may have an impact on individual genetic susceptibility to generally all type of cancers (Goode *et al.* 2002). In addition to heritable polymorphisms in DNA repair genes with largely unknown

functional consequences, DRC represents another potentially important source of interindividual variability. Normally there is a dynamic equilibrium between input of damage and its removal by effective and accurate cellular repair enzymes and it is usually the steady state level of damage that is commonly measured. If the steady state is disturbed – by, for instance, a sudden inflammatory event releasing reactive oxygen and causing a surge of damage – the damage measured will increase, but then increased repair activity (through normal enzyme kinetics, with possibly induction or activation in addition) will tend to restore the equilibrium (Collins *et al.* 2004). The steady state level will depend on the intrinsic repair rate in the individual's cells, which may be partly genetically determined and partly affected by metabolic, nutritional or environmental factors.

DRC measurement represents a complex marker integrating several factors: polymorphisms, gene expression, stability of gene product, effect of inhibitors/stimulators, environmental factors, lifestyle factors (Berwick and Vineis 2005). Functional DNA repair assays provide fundamental information about the capacity of the organism to deal with a chronic exposure to numerous environmental and dietary genotoxicants. For instance, our preliminary studies using the Comet assay indicated that lymphocytes from styrene-exposed workers have an increased capacity to remove γ-ray-induced DNA breaks and to convert 8-oxoguanine in DNA of HeLa cells to SSBs. These findings suggest that occupational exposure to styrene, and probably to other xenobiotics as well, may affect both BER and the repair of oxidative lesions (Vodicka *et al.* 2004b). Several studies demonstrated the importance of DNA repair in the genotoxicity of BD-derived epoxides. For example, mice deficient in NER are more susceptible than wild-type mice to the mutagenic effects of BD and diepoxybutane (DEB) (Wickliffe *et al.* 2007). An earlier study has suggested that epoxybutene induces CAs and DNA damage that is repaired by the excision process in G_0 lymphocytes (Kligerman *et al.* 1999). A role of DNA repair in modulating BD-induced genotoxicity/carcinogenicity was suggested by Hallberg *et al.* (1997), who described significantly decreased DNA repair rates in workers exposed to BD. In order to understand better the mechanisms of BD induced genotoxicity, and finally, carcinogenicity, additional controlled study in experimental animals were performed. The BER capacity increased during the exposure reaching the maximum at the day after the termination of 28 days of BD exposure ($p=0.03$) and then returning to the

control level. A possibe induction of DNA repar capacity was further supported by a significant correlation between BD concentration in blood and BER capacity (R=0.866, p=0.050, Vodicka *et al.* 2006b).

Styrene

Styrene is one of the most important monomer for producing polymers and copolymers, used in an increasingly wide range of applications worldwide. The main industrial uses for styrene are in plastics, latex paints and coatings, synthetic rubbers, polyesters and styrene-alkyd coatings (Collins and Richey 1992). Finally, the occupational exposure in hand-lamination work in the reinforced plastics industry may entail a daily intake of grams of styrene via inhalation (IARC 1994, 2002). Styrene is also an environmental contaminant and is present in small quantities in some food items, tobacco smoke and engine exhausts (Sorsa *et al.* 1993; Tang *et al.* 2000).

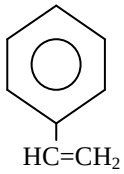

Figure 3: Structural formulae of styrene

IARC has classified styrene as a possible human carcinogen (group 2B) and its principal intermediary metabolite, styrene-7,8-oxide (SO) as a probable human carcinogen (group 2A) (IARC 1994, 2002).

Styrene metabolism has been reviewed (Vodicka *et al.* 2002a, 2006a; Figure 4). Exposure occurs mainly via inhalation of styrene vapour. Inhaled styrene is absorbed into the blood and metabolized in liver and lung to styrene-7,8-oxide (SO) by cytochrome P450-dependent monoxygenases (Hynes *et al.* 1999; Green *et al.* 2001). The role of specific CYP isozymes in the metabolism of styrene has also been examined (reviewed in Vodicka *et al.* 2002a; IARC 2002). In human liver samples, CYP2E1 seems to be the most important CYP for styrene metabolism (Guengerich *et*

al. 1991; Wenker *et al.* 2001; Green *et al.* 2001). Kim *et al.* (1997) identified CYP2E1 and CYP2C8 as being the most important metabolic enzymes at low styrene concentration, while CYP2B6 and CYP2C8 were most prominent at a high concentration of styrene (Nakajima *et al.* 1994). SO is primarily detoxified by microsomal EH to phenylethylene glycol (PEG) which is further metabolized to mandelic acid (MA) and phenylglyoxilic acid (PGA) – the principal urinary metabolites (Sumner and Fennell 1994). To a minor extent, SO is conjugated with glutathione by GSTs, resulting in subsequent formation of phenyl hydroxyethyl mercapturic acids (PHEMAs), excreted in urine (Figure 4). An alternative oxidation on the aromatic ring leads to the formation of 3,4-arene oxide, which may also contribute to the styrene genotoxicity (Pfaffli *et al.* 1981). Potential genotoxic effects of styrene 3,4-oxide are not yet known, but metabolites of the ring oxidation pathway were shown in mice to be much more potent pulmonary cytotoxic than SO (Cruzan *et al.* 2005). This metabolic pathway can be monitored by measuring urinary 4-vinyl phenol conjugates (4-VPT; Manini *et al.* 2003). Another pathway is the conversion of styrene to 1- and 2-phenylethanol, which are further metabolized to phenylacetaldehyde, phenylacetic acid, phenylaceturic acid and hippuric acid.

Styrene by itself exhibits little or no genotoxic effects. The metabolite responsible for genotoxicity is almost exclusively SO. SO have been shown to be genotoxic also in *in vitro* tests (Scott and Preston 1994). The formation of genotoxic SO is slower in humans than in rodents. More sensitive species for development of lung tumors after exposure to high doses of styrene are rats and the most sensitive are mice (Nakajima *et al.* 1994; Carlson *et al.* 2000; Filser *et al.* 2002).

MA, PGA are generally accepted as biological markers of exposure and are the main metabolites of styrene in urine (Figure 4). These two acids represent more than 90% of the styrene metabolites in humans (Bardodej and Bardodejova 1970; Guillemin and Bauer 1979; Johanson *et al.* 2000).

Conjugation of styrene 7,8-oxide with glutathione, a minor metabolic pathway in humans, leads to specific mercapturic acid products (PHEMAs) that can be measured in the urine (Ghittori *et al.* 1997; Figure 4).

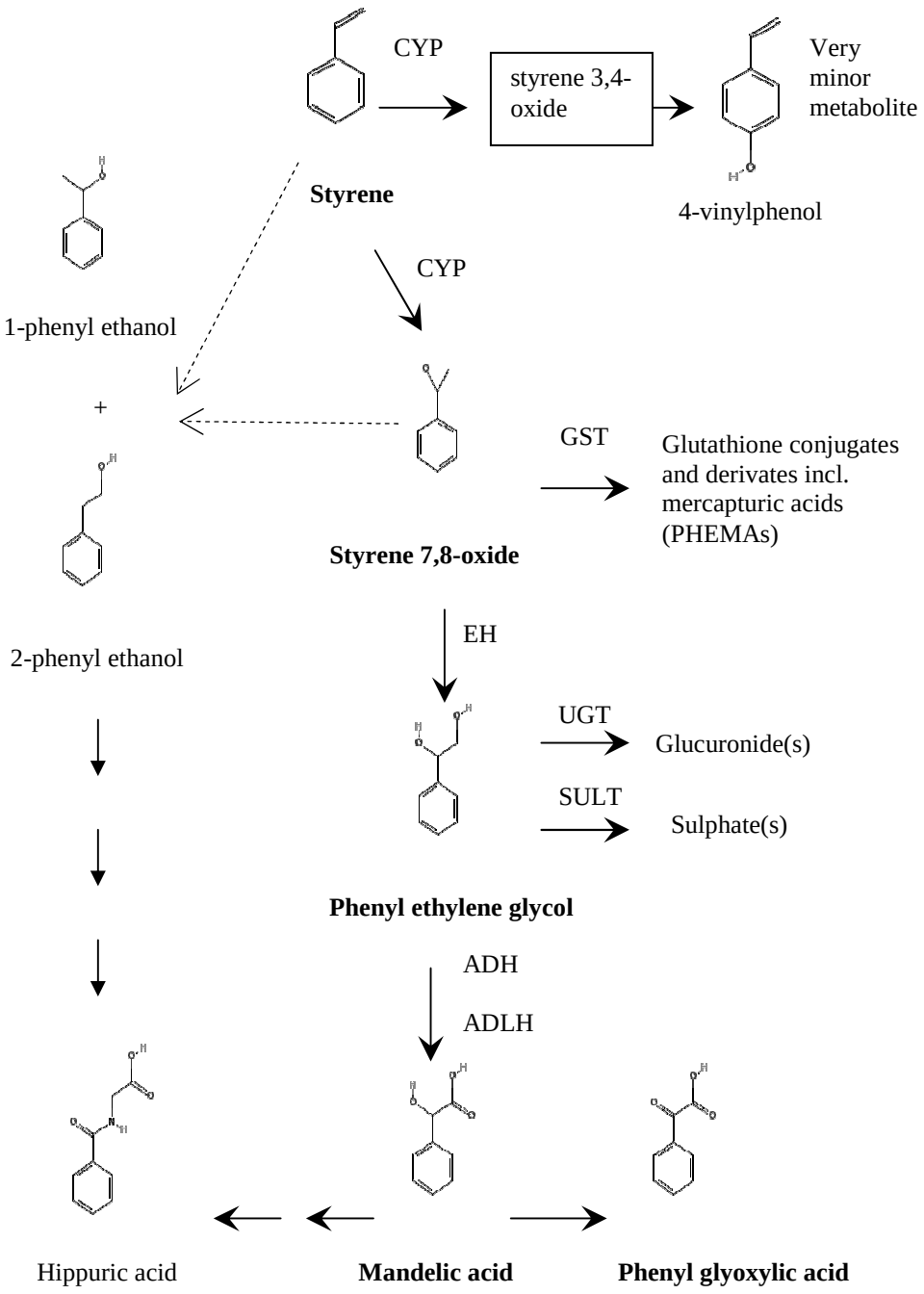

Figure 4: Major routes of styrene metabolism (Vodicka *et al.* 2006a)

The genotoxic effects of styrene and SO and individual susceptibility

Blood protein adducts, reflecting the reactivity of SO with biological macromolecules, represent an excellent marker of internal exposure. *In vitro* incubation of SO with blood from humans, mice and rats, demonstrated that SO reacts with a variety of nucleophilic sites in haemoglobin to form SO–Hb adducts. Measurement of haemoglobin adducts has been proposed to monitor occupational exposure to styrene. (Brenner *et al.* 1991; Phillips *et al.* 1994; Vodicka *et al.* 1995, 1999, 2003; Basile *et al.* 2002; Jagr *et al.* 2007).

Reaction of styrene and SO with nucleic acid constituents and DNA leads to the appearance of various DNA adducts – which represents a direct measure of the biological effect. SO adducts have been detected by several authors *in vivo* and *in vitro*, in mice or rats and in DNA isolated from lymphocytes of workers exposed to styrene (Vodicka *et al.* 2001a, 2002a; Henderson and Speit 2005). Styrene oxide induces 7-guanine alkylation, comprising 93% of total alkylation in double-stranded DNA (Koskinen *et al.* 2000). However, in cultured human lymphocytes the characteristic mutations induced by styrene oxide was AT→GC transition (Bastlova and Podlutsky 1996). This indicates that the mutagenic effects are most likely due to the minor N^6-adenine adducts (1% of total alkylation) (Koskinen *et al.* 2000; Latham *et al.* 1993). *In vitro* data on the effect of SO on cultured human lymphocytes confirmed the relatively long half-life of O^6-guanine DNA adducts and the induction of strand breaks (Bastlova *et al.* 1995). In addition, Horvath *et al.* reported relatively high levels of N^2-styrene guanine DNA adducts in humans (Horvath *et al.* 1994).

Styrene exposure may also result in secondary DNA damage via oxidative stress. White blood cells of styrene-exposed workers showed signifcantly increased levels of 8-hydroxy-deoxyguanosine (8-OHdG), so it has recently been proposed that styrene exposure could create an imbalance between oxidants and anti-oxidants (Marczynski *et al.* 2000; Gamer *et al.* 2002; Collins 2009).

Furthermore, it has been demonstrated that SO induces DNA strand breaks, which are transient promutagenic lesion, representing direct effect of damaging agents. They could be related to AP sites (apurinic and apyrimidinic sites) and also may represent intermediates in BER and NER repair (Collins *et al.* 1997). Various studies showed increased levels of SSBs following exposure to styrene (Somorovska

et al. 1999; Laffon *et al.* 2002; Buschini *et al.* 2003). A negative correlation was found between DNA repair capacities and SSBs in DNA. These data suggest that DNA repair rates may be induced among workers exposed to styrene, and, subsequently, SSBs are efficiently removed (Vodicka *et al.* 2004b).

The possible genotoxic effects of styrene and SO in lymphocytes of exposed workers have also been investigated in some studies via monitoring of chromosome aberration (CA), MN and SCE (Laffon *et al.* 2001; Vodicka *et al.* 2006a). Reviews on cytogenetic markers in workers occupationally exposed to styrene showed mainly negative results for the induction of chromosomal damage. This can be explained by: a) insufficient statistical power of results, caused by small size of studied populations, b) cytogenetic end point is not so specific marker because it can reflect DNA damage induced by a variety of factors and c) there is lack of data which reflect the relationships between DNA repair, cell cycle regulation and individual susceptibility and cytogenetic markers.

In recent years, the large field of individual susceptibility has become of great interest. This includes interindividual differences in genes encoding drug metabolism and DNA repair enzymes. Several studies on xenobiotics indicated that genetic polymorphisms in some XME or DNA repair genes may significantly modulate genotoxic outcomes (Norppa 2003, 2004). Genetic polymorphisms in cytochrome P450 *CYP2E1*, *EPHX1*, and *GSTM1*, *P1*, and *T1* are relevant to styrene biotransformation and modulate the level of SO formed and the extent to which it is detoxified.

Although glutathione conjugation represents a minor detoxification route in metabolism of styrene, it may become important mainly in lungs – the major site of entry of styrene to the human body. Mainly the *GSTM1* genotype, but also the *GSTT1* genotype, play an important role in this particular metabolic pathway in humans exposed to styrene. In styrene-exposed individuals with the *GSTM1* null genotype a lower urinary excretion of PHEMAs was observed (Haufroid *et al.* 2001; De Palma *et al.* 2001). In a study on human volunteers exposed under controlled conditions to 50 mg/m3 of styrene, subjects with *GSTT1* positive genotype excreted four times more PHEMAs in comparison with *GSTT1* null subjects, whereas a 6-fold difference was associated with the *GSTM1* null genotype (Haufroid *et al.* 2002). Godderis *et al.*

(2004) observed a lower level of DNA damage (SSBs) in workers from a fiberglass-reinforced plastic factory exposed to styrene in individuals with *GSTT1* null genotype.

By analyzing an effect of XME polymorphisms on biomarkers in styrene-exposed lamination workers, increased levels of SSBs and increased mutation frequencies (MF) in *HPRT* gene were associated with the heterozygozity in two *CYP2E1* polymorphisms (1053C>T and 7632T>A) (Vodicka *et al.* 2001b). Similarly, higher levels of N1-SO-adenine DNA adducts were found in relation to the heterozygosity in both *CYP2E1* (1053C>T and 7632T>A) polymorphisms (Vodicka *et al.* 2003). The lower level of SCE was found in individuals carrying at least one variant allele in *CYP2E1* (7632T>A) and *GSTP1* (Ile105Val) genotypes (Teixeira *et al.* 2004). The frequency of CA significantly correlated with *EPHX1* activity genotype: individuals with *EPHX1* high activity genotype had the lowest CA frequency, whereas individuals with *EPHX1* low activity genotype exhibited the highest CA frequency (Vodicka *et al.* 2001b).

Interindividual variation in DNA repair may also significantly modulate the measured incidence of markers of genotoxicity. Defects in DNA repair lead to hypersensitivity to DNA-damaging agents, accumulation of mutations in the genome, and, finally, to the development of cancer or various metabolic disorders.

Only few studies investigated the mechanisms of DNA repair of styrene-induced DNA damage. The factors of individual susceptibility modulate styrene-induced genotoxicity and individuals with “disadvantageous“ genotype could be at a higher risk of styrene carcinogenity. Particularly, factors of individual susceptibility and DRC, in combination, may substantially modulate the genotoxic outcome (as recently evidenced for combinations of polymorphisms in XME and DNA repair genes. (Naccarati *et al.* 2006).

1,3-butadiene

BD is a colourless, extremely flamable, chemically reactive gas. It is an important industrial monomer that is used in high volumes in the manufacture of styrene–butadiene rubber (SBR, 85%) and a wide variety of other synthetic rubbers,

resins and polymers. BD-based products are important components of automobiles, construction materials, computers and telecommunications equipments or protective clothings (Jackson *et al.* 2000a; White 2007; IARC 2008). BD is also an environmental contaminant, originating mainly from vehicle emissions or tobacco smoke.

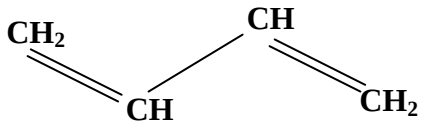

Figure 5.: Structure of 1,3-butadiene

Over the last 30 years, the relationship between exposure to BD and cancer in human populations has been investigated in numerous studies. An IARC working group in 1998 classiffied BD as a probably carcinogenic to humans (Group 2A), based on limited evidence for carcinogenicity (IARC 1999). Recently, the epidemiological studies indicate an increased risk of leukemia and lymphomas among workers occupationally exposed to this chemical (Macaluso *et al.* 1996; Delzell *et al.* 2001; Graff *et al.* 2005; Cheng *et al.* 2007). On the basis of sufficient evidence of an increased risk of leukaemia in humans, sufficient evidence of carcinogenicity in animals and supportive evidence from mechanistic studies, the Working Group reclassified BD as human carcinogen (Group 1) in 2007 (Grosse *et al.* 2007; IARC 2008). Additional studies have established BD to be a rodent carcinogen, with mice being considerably susceptible than rats (Himmelstein 1997; IARC 1999; Boogaard *et al.* 2004). These observations may be due to species differences in the metabolism of BD, particularly the formation of DNA-reactive metabolites EB and DEB. (Filser *et al.* 2007; Wickliffe *et al.* 2007)

The genotoxicity of BD is most probably caused by its metabolic intermediates. BD-induced mutagenicity requires metabolic activation, and the DNA-reactive epoxides formed during BD biotransformation are direct-acting mutagens (Melnick and Kohn 1995; IARC 1999). In the first step of BD metabolism, cytochrome P-450 mono-oxygenases are involved, mainly CYP2E1 at low and CYP2A6 at high BD concentrations. Cytochromes oxidize BD to the epoxybutene (EB) (Duescher and Elfarra 1994; IARC 1999). Two other enzymes which play a

major role in the metabolism of BD are GST and microsomal EH. GST mediates the conjugation of EB with GSH resulting in formation of mono-hydroxylated urinary metabolites. In another way, the EB can be further metabolized to DEB by CYPs (CYP2E1 and CYP3A4) or to 3-butene-1,2-diol via microsomal EHs. This latter product also acts as a substrate for CYPs to form epoxybutanediol (EBD), which can be also alternatively formed from DEB by EH. The latter epoxides are also detoxified by GST or by microsomal EH (Figure 6). EB, DEB and EBD are reactive metabolites which can react with biological macromolecules and may contribute to the mutagenicity and carcinogenicity of BD (Jackson *et al.* 2000a).

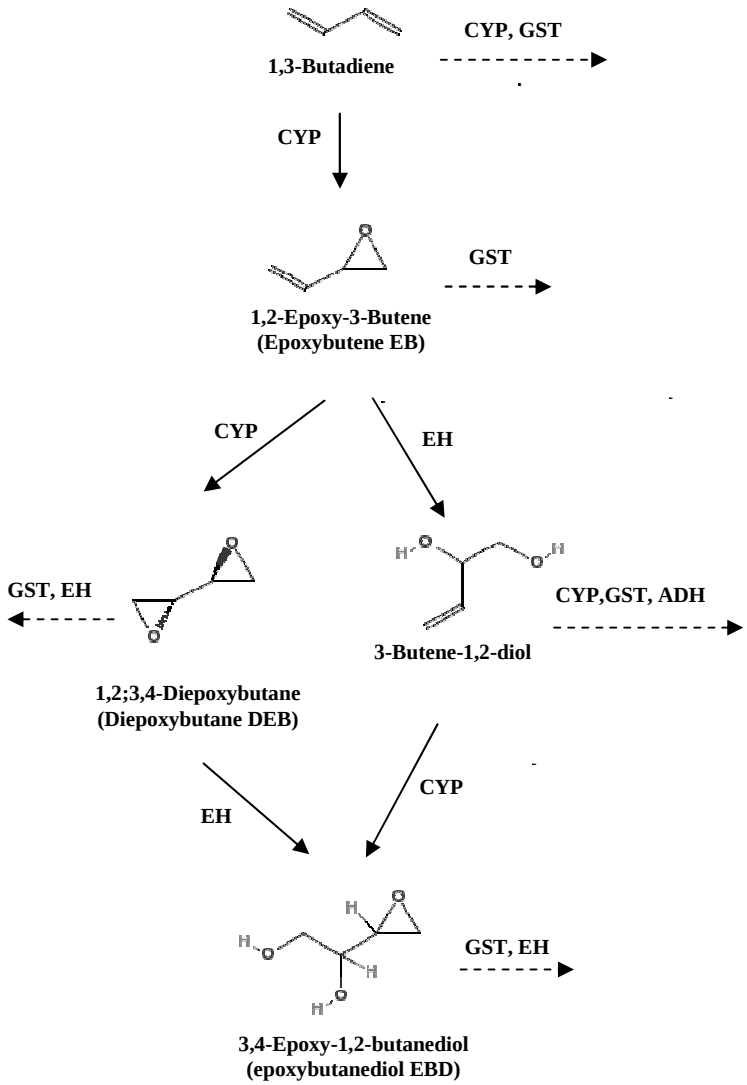

Figure 6. Major steps of metabolic pathways of BD deduced from findings in mammals *in vitro* and *in vivo* (Modified from IARC 2008)

Genotoxic effects of BD and individual susceptibility

All three metabolites – EB, EBD and DEB, are capable of reacting with macromolecules *in vitro*. DNA adducts have been identified in humans exposed to BD and in animals exposed to BD and its metabolites. The major DNA adducts in rats and mice exposed to BD are at the *N*7 position of guanine (Boogaard *et al.* 2001, 2004; Zhang and Elfarra 2003, 2004, 2005, 2006). *N*7-Guanine adducts can undergo spontaneous depurination from DNA, resulting in apurinic sites. The observed DNA adducts are congruent with induction of G-A transition mutations, adenine mutations (A-T and A-G) and deletions (Recio *et al.* 2001; Lee *et al.* 2002).

The similarity in the shape of the dose–response curves for the formation of DNA adducts and the induction of *HPRT* MF in splenic T cells from mice and rats exposed to butenediol suggests that epoxybutanediol (the product of butenediol epoxidation) may play a significant role in the mutagenicity of BD (Powley *et al.* 2005). Several studies in workers exposed to BD have shown significant changes in markers of genotoxicity (*HPRT* MF, chromosomal damage) (Ma *et al.* 2000; Ammenheuser *et al.* 2001; Abdel-Rahman *et al.* 2003) while others have shown no significant differences between exposed workers and control groups (Albertini *et al.* 2003; Šrám *et al.* 2004; Zhang *et al.* 2004; Lovreglio *et al.* 2006).

These inconsistent results may be due to interindividual differences in DNA repair as well as in metabolic capacities of XME enzymes. In workers exposed to BD, levels of haemoglobin adducts were found to be affected by the combined polymorphisms for *CYP2E1*, *GSTM1* and *GSTT1* genes (Fustinoni *et al.* 2002). The main CYP isoenzyme involved in the conversion of BD is CYP2E1 (Jackson *et al.* 2000b; Schlade-Bartusiak *et al.* 2004). The importance of *CYP2E1* polymorphisms may be confirmed by another study showing an association between this SNP and an increased risk for lung cancer among Mexican–American smokers (Wu *et al.* 1997).

Since the absence of GST enzymes could lead to a poor elimination of xenobiotics, *GST* polymorphisms could be expected to influence susceptibility to genotoxic/carcinogenic effects. In studies investigating BD-exposed workers, the *GSTM1* null and *GSTT1* null genotypes were associated with levels of DNA (Zhao *et al.* 2001) and haemoglobin adducts (Fustinoni *et al.* 2002). Several authors have observed higher frequency of SCE, induced by DEB and these results were strongly

associated with the *GSTT1* polymorphism, (Wiencke *et al.* 1995; Schlade-Bartusiak *et al.* 2000). Another report showed an increased sensitivity of *GSTM1* null subjects to the induction of SCE by epoxybutene (Sasiadek *et al.* 1999). The multivariate regression analysis, used to investigate the simultaneous effect of various parameters like occupational exposures, smoking habit, age and different genotypes on logarithmically transformed data of total CAs, revealed that the *GSTT1* polymorphism significantly modulated the total CA frequencies, followed by occupational exposure and smoking habit (Musak *et al.* 2008). Workers with the low *EPHX1* activity genotype were reported to be more susceptible to BD-induced genotoxicity than individuals with the more common *EPHX1* genotype (Abdel-Rahman *et al.* 2001, 2003; Vodicka *et al.* 2004c). In experimental studies, mice that lack the functional *EPHX1* gene were more susceptible than wild-type mice to the mutagenic effects of BD or diepoxybutane (Wickliffe *et al.* 2003).

Several other molecular epidemiological studies have reported no effect of BD on *HPRT* mutations or chromosomal changes at levels of occupational exposures and no significant associations with a particular *GSTT1* or *GSTM1* genotype (Abdel-Rahman *et al.* 2001; Zhang *et al.* 2004). Contradictions among these outcomes may be connected with the impact of exposure to other genotoxic agents from other sources e.g. cigarette smoke, or automobile exhaust (Husgafvel-Pursiainen 2004; Musak *et al.* 2008).

Immunotoxicity and exposure to industrial xenobiotics

The immune system plays an important role in protection of human body from infectious disease, tumor identification and rejection, as well as from various environmental/industrial xenobiotic insults. Adverse effects of xenobiotics on the immune system may be associated with a broad spectrum of short and long-term end-points, including hypersensitivity, autoimmunity, increased susceptibility to infectious agents and even an increased risk for cancer. Therefore immune function parameters are of potential value in the evaluation of individual health risks in workers occupationally exposed to immunotoxic industrial chemicals. Results from

Bergamaschi *et al.* (1995) and Biro *et al.* (2002) suggest immune alterations of cell-mediated immune response of T-lymphocytes and imbalance in leucocyte subsets in peripheral blood of workers exposed to styrene. Moreover, this compound was also found to suppress the activity of mouse splenic T-lymphocyte killer cells *in vitro* (Grayson and Hill 1986)

The expression of adhesion molecules in peripheral blood cells and in serum among workers occupationally exposed to styrene indicated that styrene exposure activates the immune system and altered leukocyte adherence (Jahnova *et al.* 2002) Activation of immune system among styrene workers was also confirmed by increasing of the proportion of natural killer cells (NK) (Mutti *et al.* 1992).

The lymphocyte stimulation responses and phagocytic activity of monocytes in peripheral blood as well as the humoral immunity, evaluated by determining serum IgG. IgA, IgM, IgE, and acute phase reactants C3- and C4-components of complement were assessed in comprehensive study of the biological monitoring of workers occupationally exposed to styrene (Tulinska *et al.* 2000). This study revealed a suppressive effect of styrene on cultured lymphocytes collected from styrene-exposed workers. Decreased proliferative activity was noted in lymphocytes from styrene-exposed workers upon stimulation with Con A (Concanavalin A). Positive correlation of C3-component of complement with duration of the exposure indicates possible association between styrene exposure and these acute phase reactants as indicators of inflammation. Finally, the presented data suggest immune alterations of cell-mediated immune response of lymphocytes and imbalance in leucocyte subsets in peripheral blood in workers occupationally exposed to styrene. Humoral immunity seems to be more resistant to the effect of styrene.

To our knowledge, there are no reports addressing simultaneously the genotoxic and immunotoxic burdens associated with massive occupational exposure to industrial xenobiotics. In this respect our studies represent a pioneering attempt.

STUDIES CONSTITUTING THE PhD THESIS

The aim of this work is summarized into the following points:

- to evaluate the role of various biomarkers to assess genotoxic effects of industrial xenobiotics.

    The use of a comprehensive battery of biomarkers did not reveal simple mechanistic relationships between the exposure and biological effects. The risk assessment of genotoxic styrene in occupationally exposed humans is reviewed in the light of adaptation and or population selection.

- to investigate the role of genetic polymorphisms of metabolising and detoxifying enzymes in styrene and BD exposed individuals:

    Factors of individual susceptibility are important for understanding of mutagenic and carcinogenic effects of xenobiotics.

- to assess the DRC in styrene-exposed workers

    Inter-individual differences in DRC pose an important source of variability in maintenance of genome integrity and thus influencing a predisposition to various diseases, including cancer. A possible role of DRC in modulating the individual susceptibility towards genotoxic effects of environmental and occupational carcinogens is an important field to study.

- to study the possible modulating effect of DNA repair polymorphisms on markers of genotoxicity

    Since clear mechanistic links between various genetic polymorphisms of DNA repair genes and their phenotypic consequences are not known, relationships between transient markers of genotoxic effects (like SSBs and CAs) and DNA repair polymorphisms are of particular interest.

- to evaluate the immune markers in the context of occupational exposure to styrene and to investigate their relationship with various genetic polymorphisms

Markers of genotoxicity play a role in the cascade of carcinogenic events. The same applies for immune parameters, particularly in relation to malignant cell proliferation/metastasis processes.

RESULTS AND DISCUSSION

The populations from all studies included into the present work consisted of: healthy individuals employed 1) in local administration as clerks, employees of regional hygienic stations and research institutes and 2) in various branches of the plastics and rubber industry of central Europe (central and eastern Bohemia, western Slovakia). The study design was approved by the local Ethical Committee and the participants provided their informed consent prior their inclusion into the study. The samplings of biological material were carried out according to the Helsinki Declaration. Confounding factors, like medical drug treatments, dietary (vitamins, particular diets) and lifestyle habits (alcohol, and coffee consumptions, smoking status) and possible exposure-related effects were recorded in detailed questionnaires and considered in the statistical analyses.

Role of various biomarkers in assessment of genotoxic risk of industrial xenobiotics

In the Publication No. II, we have analysed biomarkers reflecting styrene-induced genotoxicity and mutagenicity (O^6-styrene guanine DNA adducts, haemoglobin adducts, SSBs, CA, *HPRT* MF) from the aspects of their accumulation over time and of the role of adaptation and/or selection in the genotoxic risk of styrene exposure.

The level of N-terminal valine adducts in haemoglobin served as a parameter reflecting both internal exposure to styrene and the ability of styrene intermediate, SO, to attack nucleophilic sites. Regression analysis revealed that haemoglobin adducts in styrene-exposed workers strongly correlated only with exposure coefficient, while correlations with styrene workplace concentration, years of employment and age were not significant.

Styrene-induced DNA adducts are considered as potential powerful biomarkers of styrene exposure, representing important integral measures of DNA damage and reflecting the DNA repair activity to remove them. In our study, the levels of O^6-

styrene guanine DNA adducts significantly correlated with years of employment, styrene exposure and, particularly, with exposure coefficient. Finally, only O^6-styrene guanine DNA adducts and CA correlated with workplace styrene concentrations, as well as with the duration of employment. Interestingly, all markers (DNA and haemoglobin adducts, SSBs, CA and *HPRT* MF) correlated significantly with exposure coefficient, illustrating cumulative effect.1-SO-adenine DNA adducts has also been analysed in white blood cells of styrene-exposed workers (Publication No. III). 1-SO-adenine DNA adducts have been detected in 14 out of 19 hand-lamination workers exposed to styrene, while none of seven control subjects showed DNA adducts above the detection limit. Statistical analysis clearly shows a strong effect of both acute styrene and cumulative exposure on the level of 1-SO-adenine adducts in white blood cells.

We did not record any statistically significant difference between the MF at the *HPRT* locus in styrene-exposed workers and control individuals. MF at the *HPRT* locus correlate significantly with styrene concentration at the workplace, but not with parameters of internal exposure (styrene concentration in blood, urinary mandelic acid). A significant correlation between MF at the *HPRT* locus and the parameters of cumulative exposure was recorded. These data support an assumption that MF at the *HPRT* locus may accumulate in relation to the styrene exposure over time (Publication No. III).

SSBs and SSB Endo III sites are in detail covered in Publication No. V. SSBs in DNA were significantly lower in exposed workers than in those unexposed and correlated inversely with parameters of internal exposure. This is in contrast with a previous study (Vodicka *et al.* 1999), and may be caused by differences among the examined populations. The previous study consisted of individuals exposed for 14 years on average, whereas workers in the present study were exposed to styrene for less than 4 years. On the other hand, in the present study the relatively lower SSB levels in exposed workers were associated with more efficient DNA repair capacities, whereas in previous reports DNA repair rates were not addressed. Contrasting results between this study and a previous (Somorovska *et al.* 1999) were also found in the levels of SSB Endo III sites, reflecting either abasic sites or oxopyrimidines, and were almost identical in both the exposed and control groups.

Except for DNA and haemoglobin adducts, the other biomarkers are rather non-specific, reflecting many types of various genotoxic effects due to different kind of exposure and other events, such as DNA repair in the case of SSBs. Additionally, exposure coefficient represents rather a theoretical value, assuming that styrene exposure levels are constantly the same as measured in one particular day of sampling. It is well known that levels of styrene exposure may substantially vary from day to day, depending on the character of production.

The assessment of biomarkers of genotoxic effect in the context of BD exposure is shown in Publication No. VI. Interestingly, the frequency of CAs was the highest for individuals engaged in checking and quality control (the lowest exposed) and for individuals from the mixing department (highly exposed), and lowest for individuals employed in other processes in the tire plant (medium exposed). SSBs and SSB Endo III sites were not significantly different between these groups or even between smokers and nonsmokers. More explanatory considerations about these findings will be provided in the following paragraphs.

Genetic polymorphisms of metabolising and detoxifying enzymes in individuals exposed to industrial xenobiotics

Genotype analyses of *EPHX1* (Tyr113His and His139Arg), *GSTM1* (deletion), *GSTP1* (Ile105Val) and *GSTT1* (deletion) were performed in DNA samples from styrene exposed workers and controls. (Publication No. II). The group consisting of hand-lamination workers exhibited a significantly higher frequency of low *EPHX1* activity genotype (His in *EPHX1*-Tyr113His) and significantly lower frequency of high *EPHX1* activity genotype (Arg in *EPHX1*-His139Arg). These differences in allele frequencies between styrene-exposed workers and unexposed individuals may indicate a process of selection over time, especially among styrene-exposed individuals. We may expect that individuals, who are “well equipped” to deal with styrene (and other chemicals) exposure, due to their genetic background can performe their work for many years; others would relatively rapidly quit.

The role of various biomarkers, particularly in light of possible modulatory effect of genetic polymorphisms of genes encoding for XME is discussed in

Publication No. III. DNA adducts are influenced by the heterozygosity in *CYP2E1* (7632T>A). Using multiple regression analysis, it appears that the levels of 1-SO-adenine adducts may be influenced by heterozygosity in *CYP2E1* (both 1053C>T and 7632T>A). In this study, we also demonstrate increased MF at the *HPRT* locus in styrene-exposed individuals bearing heterozygosity in *GSTP1* genotype (Ile105Val), while no such association was seen in the control group. Styrene-exposed individuals with *GSTP1* Ile/Ile genotype exhibited significantly lower MF at the *HPRT* locus as compared to those with heterozygous *GSTP1* genotype. Our study, although performed on a limited cohort, suggests that chronic styrene exposure together with genetic polymorphism in *GSTP1* may result in increased mutagenicity at the *HPRT* locus.

The effect of polymorphisms in genes coding for XME enzymes (*CYP1A1, CYP2E1, EPHX1, GSTM1, GSTP1, and GSTT1*) on CAs, SSBs, and SSB Endo III-sensitive sites was also evaluated in workers employed in tire plants, and thus were exposed to a variety of xenobiotics, mainly BD (Publication No. VI). Individuals with the low-*EPHX1*-activity genotypes exhibited the highest CAs, while those with medium and high-activity genotypes had significantly lower CA frequencies. An important additional metabolic pathway in the detoxification of reactive BD intermediates is conjugation with glutathione catalyzed by GST. We did not observe any association between the biomarkers measured in the present study and common genetic polymorphisms in *GSTM1, GSTP1*, and *GSTT1*. Interestingly, in this study we have also measured expression levels of *CYP2E1*, a gene involved in xenobiotic biotransformation, a relatively novel biomarker for biomonitoring studies. Our data suggest a trend toward higher CA frequencies in individuals with increased *CYP2E1* mRNA expression levels.

Assessment of DNA repair capacity regarding the exposure to styrene and BD

We have measured DRC using two separate DRC tests. One of them make use of the ability of specific glycosylase (oxoguanine glycosylase; OGG1) to remove 8-oxoguanine in periferal lymphocyte extracts. In second test DNA breaks induced by

γ-rays are repaired according to the individual repair capacity, and the results are expressed as amount of repaired SSBs.

Individual repair capacity to repair DNA breaks induced by γ-rays in styrene-exposed workers was significantly higher in comparison with controls (Publication No. II). BER is the DNA repair pathway most probably acting on the removal of styrene-induced DNA adducts. The stimulation of DNA repair in laminators could explain their enhanced capacity to repair γ-ray-induced DNA damage, which is known to be specifically repaired by BER.

Generally, SO induces a wide variety of different types of DNA adducts (Fig. 5 in Publication No. II). Different types of adducts at different sites of the DNA bases exhibit different biological properties, which may result in different kinetics of formation and removal, different rates of repair process and, therefore, different biological significance and mutagenic potencies

The results from two different DNA repair tests based on modified version of the comet assay are shown in details in Publication No. III and V. Capacity to repair irradiation-specific DNA damage was significantly lower in external controls than in exposed subjects from laminary plants. In the cohort exposed to the highest styrene concentrations, DRC apparently decreased. The apparent increase in DNA repair rates related to the medium styrene exposure may reflect effective activation of the DNA repair machinery. Moreover from results of these studies, we may assume that styrene exposure may result in the induction of DNA repair in humans up to a certain threshold (around 100 mg/m^3). On the other hand, an increased capacity of lymphocytes to incise 8-oxoguanine, which represents oxidative damage of lymphocytes, was recorded among highly exposed workers. Significant association between both internal and external exposure parameters and repair capacity to remove oxidative DNA damage suggests a possible role of oxidative stress in styrene-related genotoxicity.

Based on the data collected from questionnaires, we have analysed possible associations between levels of analysed biomarkers and different dietary and lifestyle factors (Publication No. IV, based on general population). Irregular exposure to a broad variety of chemicals, used in the workplace, was linked with the rates of irradiation-specific DNA repair. In addition, the consumption of coffee positively

affected irradiation-specific DNA repair rates, but decreased the capacity to repair oxidative damage.

The capacity of peripheral blood lymphocytes (PBLs) to repair γ-ray-induced SSBs and to convert 8-oxoguanine in HeLa cell DNA into SSBs were assessed also in workers employed in the tire plant (Publication No. VI). The main outcomes was, a higher DNA repair rates were detected in smokers in comparison with nonsmokers, suggesting that the various alkenes present in cigarette smoke may have induced the BER capacity to repair DNA damage. However, nonsignificant twofold higher irradiation-specific repair rates were found in workers highly exposed to xenobiotics, including BD. In our case, the DNA repair rates may be affected by the coexposure to styrene or other chemicals in the workplace, although their quantitative contribution to the total xenobiotic exposure seems rather small. Interestingly, irradiation-specific DNA repair rates moderately increased with increasing age in the study group. A significant positive association was observed between SSBs and irradiation-specific DNA repair rates. A strong positive correlation was also found between SSB endo III-sensitive-site levels and oxidative DNA damage repair rates.

In another study (Publication No. VII), we investigated possible relationships between the capacity to repair oxidative DNA damage, main confounders, parameters of exposure and parameters of genotoxic effects (SSBs, CAs, 1-Ade-adducts and *HPRT* MF). The only positive correlation was found between DRC and SSBs in females. It can be related to pronounced inter-individual variability in DNA-repair rates, differences in the levels and/or the duration of exposure, and the smaller size of the population investigated. Interestingly, unexposed smokers incised oxidative DNA damage significantly more effectively than unexposed non-smokers, and the same trend, although non-significant, was found considering the whole study group or the exposed workers only. This may be due to the generation of oxidative stress by smoking and subsequent stimulation of cellular DNA-repair activity. Moreover, possible associations between the capacity to repair oxidative DNA damage and polymorphisms in genes encoding biotransformation enzymes were analysed. Higher DNA-repair rates were observed in carriers of *GSTM1 plus* genotype, expressing functional enzyme, compared with those with a deletion in *GSTM1*.

DNA repair polymorphisms in relation to styrene and BD-induced genotoxicity (a putative modulating mode)

Genetic polymorphisms in DNA repair genes and possible links with DNA repair rates, CAs and SSBs in DNA are shown in Publication No. IV in details. In the present study, we investigated the potential links between genetic polymorphisms in genes coding DNA repair enzymes and the levels of CAs and SSBs in DNA in a central European general population. We studied polymorphisms of the DNA repair genes *XPD* Lys751Gln, *XPG* Asn1104His, and *XPC* Lys939Gln involved in NER, *XRCC1* Arg399Gln involved BER, and *XRCC3* Thr241Met involved in recombination repair and in maintaining chromosomal stability. Among all analysed SNPs, *XPD* Lys751Gln exon 23 polymorphism was a major factor influencing the level of CAs. CA frequencies were significantly lower in individuals homozygous for the variant allele C of *XPD* Lys751Gln. This effect was particularly evident in smokers, suggesting that the CC genotype results in an enhanced repair capacity towards CA-forming lesions like those induced by tobacco smoke. Additionally, moderately elevated CA levels seemed to be associated with two other polymorphisms of NER, i.e. the G allele of *XPG* Asn1104His and the C allele of *XPC* Lys939Gln. Moreover, various combinations of DNA repair genetic polymorphisms were studied to evaluate the modulating effect of `adverse' genotypes on individual susceptibility to genotoxic response.

This modulatory effects was similar on both the frequencies of CAs and SSB levels, indirectly suggesting a close reflection of DNA damage on chromosomal damage, as well as a role of NER in the repair (or formation) of SSBs. An increase in levels of SSBs was observed in individuals homozygous for *XRCC1* Arg399Gln variant allele, this association suggesting that also BER participates in modulating SSB levels. The irradiation-specific DNA repair rate is believed to represent predominantly BER. In agreement with this, the repair rate of irradiation-induced DNA damage was almost 2-fold higher in wild-type homozygote of *XRCC1* Arg399Gln. The moderate influence of XPG and XPC polymorphisms may be explained as a participation of NER.

The combination of *EPHX1* and *XPD* polymorphisms seems to modulate levels of cytogenetic endpoints among tire plant workers. (Publication No. VI).

Individuals with the combination of low *EPHX1* activity genotypes and the *AA* (wild type) and *AC* (heterozygous) *XPD* alleles exhibited higher levels of CAs than individuals with combined high *EPHX1* activity genotypes and variant allele *CC* genotype for *XPD.* This observation suggests an increased risk of genotoxic effects in individuals with particular genotype combinations,

In Publication No. VII, markers of genotoxicity were investigated in a group of styrene-exposed hand lamination workers and related to the individual genetic polymorphisms in relevant DNA repair genes and cell-cycle gene *Cyclin D1*. SSBs in DNA and CAs frequencies were the highest in individuals with wild-type *AA* genotype and the lowest in those with homozygous variant *C* allele in *XPD*, exon 23 gene. The same tendency applied for 1-SO-adenine DNA adducts, although the differences between the *XPD* genotypes were not significant. Interestingly, mutant frequencies at the *HPRT* gene were the highest in individuals with *XPD* wild-type *AA* genotype as compared to those with variant *CC* genotype. Genetic polymorphism in *Cyclin D1* gene had no significant modulating effect on any of the biomarkers of genotoxicity studied.

We studied the individual capacity to repair oxidative DNAdamage (incision step in removal of oxidized bases, mainly 8-oxoG) in workers occupationally exposed to styrene and in unexposed clerks from the same factory (Publication No. VIII). The exposed individuals showed a moderate, although non-significant increase in the capacity to incise oxidized purines compared with unexposed controls. This increase is subtle if compared with the results from a previous study, where significantly higher DNA-repair capacity in styrene-exposed individuals in comparison with the control group was observed (Publication No. V). The absence of significant differences between exposed and controls in the present study is most likely related to pronounced inter-individual variability in DNA-repair rates, differences in the levels and/or the duration of exposure, and the smaller size of the population investigated.

The relationship between the capacity to repair 8-oxoguanine, biomarkers of genotoxicity and individual susceptibility in styrene-exposed workers has ben evalutated in Publication No. VIII. Individuals with the wild-type Ser/Ser genotype for the *hOGG1* Ser326Cys polymorphism showed a higher DNA-repair capacity than those with heterozygous Ser/Cys genotype. This difference was of borderline significance. The DNA-repair capacity was significantly lower in individuals with

variant Gln/Gln genotype in *XRCC1* Arg399Gln. This trend was apparent in the whole group, as well as after stratification for exposure and sex. Significantly lower repair capacity was also found in individuals with the wild-type Lys/Lys genotype in *XPC* Lys939Gln as compared with those homozygous for the Gln/Gln variant genotype. Finally, from the general linear model analysis the DNA-repair capacity appears to be significantly modulated by polymorphisms in *GSTM1* and *XRCC1* Arg399Gln and by sex, while exposure status and smoking did not have any effect in this particular study.

Immune markers in individuals occupationally exposed to styrene

Two different immune assays, proliferative response of lymphocytes, as well as expression of adhession molecules on PBL, were evaluated in workers of a rubber factory and of a styrene plastic lamination plant (Publication No. I). Workers exposed to variety of xenobiotics, such as BD and polycyclic aromatic hydrocarbons (PAH) in a rubber factory displayed enhanced lymphocyte proliferation. On the other hand workers occupationally exposed to styrene showed a suppression of lymphocyte proliferation cultured with mitogens *in vitro.* These data suggest a different cell-mediated immune response of PBLs in workers from rubber factory versus styrene workers. Styrene increased activation of the immune system and altered leukocyte adherence in exposed workers. This interaction is a critical first step in response to immune stimuli, and alterations in leukocyte-endothelial association could be harmful to an effective response to inflammatory stimuli.

Our further interest was also to investigate the possible relationship between individual susceptibility (DNA repair polymorphisms) and individual immune response in the context of occupational exposure. It is believed that markers of genotoxicity play a role in the cascade of carcinogenic events. The same applies for immune parameters, particularly in relation to malignant cell proliferation/metastasis process. (Publication No. VII). The present report focuses on the possible modulating effect of genetic polymorphisms in cell cycle gene *Cyclin D1* and DNA repair genes on these markers. For the first time, a relationship between various genetic polymorphisms and immune markers was shown. An association between the number

of leukocytes and lymphocytes and *Cyclin D1* polymorphism seems to be logical: Cyclin D1 is involved in the cell cycle, controlling cyclin-dependent kinases and thus enabling passing the cell from G to S phase. The reason why the counts of white blood cells are affected by the *Cyclin D1* polymorphism remains unknown. The number of eosinophiles was positively associated with *XPD* variant *C* allele and negatively with *XRCC1* variant *A* allele and *XPC* variant *C* allele. No influence was exerted by any of the confounding factors examined. Immunoglobulin IgA was positively associated with *XRCC3* variant *T* allele and negatively with *XPC AC* and *CC* genotypes Both C3- and C4-complement components were decreased in individuals with *XRCC3 CT* and *TT* genotypes Both soluble adhesion molecules sL-selectin and sICAM-1 (intercellular adhesion molecule 1) were significantly associated with *XPC* gene. The relationships between various DNA repair polymorphisms and immune parameters are even more difficult to explain at the moment due to the lack of knowledge on functional aspects of the genetic polymorphisms analyzed and due to the complexity of the immune system.

CONCLUSIONS

The role of various biomarkers in assessment of genotoxic effects of industrial xenobiotics:

In our studies, we assessed the role of various biomarkers to detect genotoxicity of styrene and BD. Among biomarkers, the only specific and sensitive marker of DNA damage is represented by DNA adducts. However, methodologies for the determination of specific DNA adducts are laborious and cannot be employed on large populations, thus random variations cannot be ruled out. Additionally, styrene (as well as other xenobiotics) is capable to induce a wide spectrum of DNA adducts with different biological impacts. These adducts may be repaired via different DNA repair mechanisms, but information on adduct-specific DNA repair is very limited at the moment. If adducts are not effectively repaired, the mutation in affected site in DNA can lead to the syntesis of an altered protein. Mutations in an oncogene, tumor-suppressor gene, or a gene controlling the cell cycle can result in a clonal cell population with a proliferative or survival advantage.

Others biomarkers assessed in exposed individuals (either for measuring DNA or chromosomal damage) do not reflect the genotoxic effects of a specific exposure to styrene or BD, because their levels can be strongly influenced by others factors (for example age or exposure to another xenobiotics, like PAH, etc.).

The modulating effect of genetic polymorphisms of XME genes and of DNA repair genes in styrene and BD exposed individuals:

From our studies we can conclude that different genetic polymorphisms in XME and DNA repair genes may affect styrene and BD genotoxicity. An absorbed dose of genotoxic agent is dependent on the biotransformation and the individual susceptibility, also related to different polymorphisms in XME genes, certainly plays an important role in this process. The induced DNA damage is subsequently repaired more or less efficiently and rapidly, depending on the individual's genetic background. Particularly, factors of individual susceptibility and DRC, in combination, may substantially modulate the genotoxic outcome.

Considering these aspects, we can assume that workers with “adverse” genotypes may be at a substantially higher risk of styrene or BD genotoxicity or carcinogenicity.

The role of DNA repair capacities in styrene- and BD-exposed workers

With the employment of a specific test based on modified version of the comet assay, we have observed that DRC in styrene-exposed workers as well as workers from tire plant were higher in comparison with controls groups. The lack of accumulation of genotoxic damage over time in exposed individuals could be due to the induction of adaptive DNA-repair processes following the exposure to xenobiotics. In particular, the stimulation of DNA repair in styrene laminators could explain their enhanced capacity to repair γ-ray-induced DNA damage, which is known to be repaired specifically by BER.

The immune markers and their relationship with various genetic polymorphisms

Presented data indicate the utility of immune function assays and expression of adhesion molecules for biological monitoring for early effects in workers occupationally exposed to industrial chemicals. The associations between immune parameters and genetic polymorphisms in *Cyclin D1* and DNA repair genes seems to be important because both systems (immune and repair) represent a complex systems that play a role in the development of various diseases, including cancer.

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