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REVIEW ARTICLE

Biomonitoring and biomarkers of organophosphate pesticides exposure – state of the art

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Abstract

Human biomonitoring provides an efficient and cost-effective way to identify and quantify exposure to chemical substances, including those having deleterious effects on human organisms. Once the risk of hazardous exposure has been identified and the mechanism of toxic effects has been elucidated, an ultimate decision about how to reduce exposure can be made. A particularly high risk of exposure to hazardous chemicals is associated with the use of pesticides in agriculture, especially the use of organophosphorous pesticides (OP), which are the most widely and commonly used insecticides worldwide. There is some strong evidence that chronic exposure to these compounds may have adverse effects on health. Exposure to pesticides has been associated with an increase in the incidence of non-Hodgkin's lymphoma, multiple myeloma, soft tissue sarcoma, lung sarcoma, and cancer of the pancreas, stomach, liver, bladder and gall bladder, Parkinson disease, Alzheimer disease, and reproductive outcomes. In view of these findings, the detection of populations at risk constitutes a very important topic. The biomonitoring studies on individuals exposed to pesticides have shown an elevated level of indicators of DNA damage, such as chromosomal aberrations (CA), sister chromatid exchanges (SCE), micronuclei (MN), and recently, single cell gel electrophoresis (SCGE). The cytogenetic markers of DNA damage have become very popular and useful in providing an analytical data for risk assessment, such as internal exposure doses and early biological effects of both occupational and environmental exposure to pesticides. The article describes the usefulness and the limitations of these biomarkers in biomonitoring studies of populations exposed to pesticides, with regard to the main routes of uptake and different matrices, which can be used to monitor risk assessment in occupational settings. The article also summarizes the latest reports about biomarkers of susceptibility, and mentions other biomarkers widely used in biomonitoring studies, such as pesticide or its metabolites level.

Key words

biomonitoring, exposure, pesticides, biomarkes

INTRODUCTION

In order to recognize whether a subject is suffering from integrated exposure to a hazardous substance, or even accidental intoxication, standardized analytical procedures for diagnostic investigation of biological materials have become established and these are subsumed under the term "biomonitoring" [1].

One of the official definitions of human biological monitoring states that it is 'a systematic continuous or repetitive activity for collection of biological samples for analysis of concentrations of pollutants, metabolites or specific nonadverse biological effect parameters for immediate application, with the objective to assess exposure and health risk to exposed subjects, comparing the data observed with the reference level, and – if necessary – leading to corrective actions'.

The concept of human biological monitoring has evoked a lot of interest among individual scientists and international organizations, and nowadays biomonitoring of exposure is a useful tool for assessing environmental and occupational exposures to a given chemical. After a chemical comes into contacts with and enters the body, a toxicokinetic process is started, which includes 4 complex steps of absorption, distribution, metabolism, and excretion [2]. The measurements of specific biomarkers after the absorption step, or during each subsequent step of the process, are used to assess exposure by estimating the internal dose, which is defined as the amount of chemical absorbed into the body after an exposure has occurred. Biomonitoring of exposure to pesticides involves the measurement of an exposure biomarker, which can be pesticide(s), its metabolite(s), or reaction product(s) in biological media such as urine, blood or blood components, exhaled air, hair or nails, and tissues [3, 4].

Pesticides are widely used chemicals with unique properties designed to control pests and prevent plant disease. Many groups of pesticides can be distinguished, including insecticides, herbicides, fungicides and rodenticides. In spite of numerous benefits, the use of pesticides brings also substantial hazard to the public and environment. The active ingredients of these products are mainly organophosphates, carbamates, chlorinated hydrocarbons, and carbamide derivatives [5].

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The worldwide use of different groups of pesticides leads to global cross-contamination and unintentional exposure of humans humans [6]. Nowadays, nearly all people are inevitably exposed to pesticides due to environmental contamination or intentional use [7]. Organophosphates (OP) constitute a large class of chemical insecticides. There are more than 100 different OPs that are widely used in the agricultural industry, and to a lesser extent in home application. As a result, they are involved in more occupational poisoning cases than any other single class of insecticide. Organophosphorous pesticide residues have been detected at levels above the limit of quantification, and sometimes even exceeding maximum residue levels (MRLs) in many agricultural products; therefore, low-level dietary exposures to organophosphorus pesticides is very likely.

In addition, several cases of intentional (suicide) and accidental human exposure were described for pesticides registered for in-home use. Nevertheless, occupational exposures to organophosphorus pesticides dwarf environmental exposures; however, special populations, such as farm workers and children, may receive higher exposures [8, 9, 10].

Routes of exposure. Pesticides may enter to the body by dermal absorption, inhalation or oral absorption [11]. The design of most studies on pesticide toxicology has evolved from the concern of oral exposure via food. However, the oral route does not generally apply to farm workers exposed primarily via the skin, either by handling pesticides or re-entering spraying treated fields. Skin is the most exposed organ while spraying the pesticide on fields. Farmers are also exposed to pesticides while mixing, loading the pesticide, or while cleaning the equipment and disposing of empty containers [12]. Other activities associated with potential exposure are sowing pesticide preserved seeds, and weeding and harvesting previously sprayed crops [13]. Dermal exposure, described as a transfer of the pesticide from the surface of the foliage to the skin of the worker, depends on the amount of pesticide available for transfer, and frequency and intensity of skin contact with the treated crops [14]. There is also evidence of dermal exposure to pesticides of workers working in a greenhouse. They are exposed to pesticides at re-entry into the greenhouse after spraying pesticides on previous days, even if the restricted-entry intervals expired. The study results revealed that all pesticides used in greenhouses (hexythiazoks, azoksystrobin, imazalil) were found on cotton patches and gloves of greenhouse workers. Dermal exposure to pesticides takes place even when employees are not directly engaged in the process of spraying. It was also shown that protective gloves do not protect the workers properly, and could be the source of considerable additional exposure [11, 14-15]. There is very little quantitative information available about safe levels of chemicals for dermal exposures in the workplace or in the home. General quantitative safe levels for dermal exposures are hard to provide because of the wide variability in potential exposure parameters, such as surface area exposed and contact times [16, 17]. Salvatore *et al.* reported a low level of urinary OP insecticide metabolites observed in 73 strawberry farm workers, in spite of wearing recommended clothing and following recommended hygiene procedures [18]. Once absorbed into the skin, penetrants may cause local reaction, or may enter the circulation to produce systemic effects. In order to assess exposure and estimate potential risks, accurate quantitative data on absorption are required. Absorbent patches placed on the worker's body or clothing, or the clothing itself may be analyzed for pesticide residues [4].

EXPOSURE MARKERS

Urinary markers of pesticide exposure. Urine is the most common fluid used for biological monitoring of OP insecticide exposure, primarily because of its ease of collection and general abundance. However, urinary measurements have several limitations, such as temporal variability of the volume of collected urine, and the changeable concentrations of endogenous and exogenous chemicals from void to void. Some researchers report that more stable or representative measurements are obtained from first morning void collections rather than spot samples collected at other times of the day. When using spot urine samples, creatinine or specific mass marker should also be determined in order to normalize results for concentration and rule out overdiluted or overconcentrated samples [19]. Besides, many of the organophosphorus insecticides have common urinary metabolites that prevent identification of the parent pesticide(s) to which an individual was exposed [3, 8, 20]. Metabolic products of various OP compounds are dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylphosphate (DEP), (DETP) and diethyldithiophosphate (DEDTP). Alkyphosphates are excreted in urine as sodium or potassium salts. Unfortunately, biological limits of exposure have not yet been established, and it is complicated to interpret the results in terms of risk for human [21].

A pesticide may be either rapidly or slowly eliminated from urine. The time of sampling is of great relevance in the design of a biomonitoring study, and sufficient collection time will differ depending on the pesticide. For cyfluthrin, the half-life of elimination has been reported in the range between 5.5 or 6.4 h and 16.5 h, depending on the study. Therefore, biomonitoring studies for cyfluthrin should include urine samples taken relatively soon after exposure [4]. This procedure can be difficult to perform among farm workers or pesticide sprayers because they are constantly undergoing occupational exposure, and it poses logistical and organizational problems with collecting samples [22]. Organophosphates, such as chlorpyrifos and malathion, are very popular insecticides. They do not accumulate appreciably in humans and are rapidly metabolized and excreted in the urine, which means that the urinary metabolites of those pesticides can be measured up to several hours after an exposure has occurred. These measurements represent only a snapshot in time; thus, only exposures that occurred during the previous few hours or days can be captured. In that case, a single urinary measurement may not reflect the average exposure [23]. However, in the case of chronic exposure to those pesticides, urinary elimination may reach a steady state, which means that the chemical or metabolite present in the urine stays at a relatively constant level and reflect the average exposure. The specificity of urinary measurements is equally as high as blood measurements, but only in the case when the parent compound is excreted in urine (e.g. 2,4-dichlorophenoxyacetic acid (2,4-D), glyphosate, sulfonyl ureas).

In a study assessing exposure to atrazine (ATZ) by measuring its metabolites, it was found that the urinary metabolite profiles varied greatly among exposure scenarios,

and among persons within each exposure scenario. Although diaminochlorotriazine (DACT) appeared to be the predominant urinary metabolite detected in each exposure category (high, low and environmental exposed to this pesticide), the variation in proportion of total ATZ metabolites among persons was consistently large, suggesting that one metabolite alone could not be measured as a surrogate for ATZ exposure. To accurately classify exposure to ATZ and its environmental degradates, the multiple urinary metabolites must be measured [24]. In another study, it was shown that variability in detection for each urinary metabolite within and across individuals indicates that any single measure of urinary metabolites cannot be considered a credible indicator of exposure for an individual. Further, exposure estimation based on a urinary metabolite collected at a single time in an agricultural season are not a good indicator of population pesticide exposure. Results from these study suggest that to provide a reliable characterization of pesticide exposure it is necessary to measure numerous f pesticide urinary metabolites, as well as collect multiple samples from each participant across a single agricultural season [19].

Pesticides and their metabolites in blood. Measurement of the intact pesticide in blood is the most specific indicator of exposure to a given organophosphate pesticide; however, that kind of measurement is complex and may be hampered by the instability of the pesticide in blood [8, 23]. Because of the very short half-lives of several pesticides in the body, levels measured in blood drawn at one time point during the day may not reflect internal levels at another time during that day, much less across longer time periods. Thus, correlations observed between measured levels of given chemical and a biochemical marker or other health endpoint may be highly unstable, with no consistent relationship between the internal level of the compound and the health endpoint over time [25].

The major disadvantage related to blood measurements is the necessity of and risk associated with blood collection by invasive techniques such as venipuncture. The invasive nature of venipuncture puts some ethical limits on researchers' ability to obtain samples from children and pregnant women, who may be especially vulnerable to pesticides [26]. Obtaining blood samples in large environmental and occupational studies is also problematic. In addition, the amount of blood available to perform the analysis is often limited. Analysis is further complicated by the inherently low concentrations of OP pesticides present in the blood, compared with urinary metabolite concentrations. Therefore, ultrasensitive analytical techniques may be required [8, 23].

The advantage of using blood as a matrix for biomonitoring is that the blood measurements are specific for pesticide as the parent chemical is generally measured. As the volume of blood in a person's body is relatively constant, it is also easy to calculate the body burden (i.e. the amount of chemical relative to the amount of blood in the body) more accurately than measuring the chemical or its metabolite in urine [3, 27]. Blood can also be a valuable matrix for measuring adducts to DNA, hemoglobin or albumin. Adducts play an important role as early biomarkers of effect, providing more relevant information related to a selected health endpoint, such as cancer. Furthermore, since the lifetime of an adduct in the body is largely dependent upon the lifetime of the biomolecule itself (e.g. the life-time hemoglobin adducts are the same as single hemoglobin life-span – about 120 days) adducts enable confirmation of exposure after a longer period of time [3].

Saliva as a matrix. Although biomonitoring has been conducted primarily by utilizing biological matrices, such as blood and urine, other matrices – such as saliva – represent a simple and readily obtainable fluid. In this regard, saliva has been used to evaluate a broad range of biomarkers, drugs, and environmental contaminants, including drugs of abuse, hormones, chemotherapeutics, heavy metals, and also pesticides. However, the reliable estimate of an internal dose from a 'spot' saliva sample requires a good understanding of the pharmacokinetics of the chemical, and the relationship between the chemical concentration in the saliva and blood [10].

Several field studies have been conducted to evaluate saliva biomonitoring. The data indicate that saliva levels of pesticides can be considerably lower than blood levels, depending on the degree of protein binding that may occur. Nevertheless, the measurement of pesticides in saliva has great potential because of the convenience of sampling and analysis, and the potential accuracy of salivary concentrations as an indicator of tissue availability [3].

Sweat in biomonitoring studies. To the best of the authors' knowledge, there has just been only one study, in 1985, designed to determine if sweat could be used for monitoring pesticide levels in exposed farm workers [28]. However, many studies have monitored total blood levels of alcohol or drugs by analysis of sweat that opens the opportunity of using sweat in pesticide biomonitoring studies [29, 30].

Enzymatic changes due to exposure to pesticide. An indicator of biological response, such as enzyme activity, may be used as an internal marker of exposure. Altered cholinesterase (ChE) activity, including both erythrocyte ChE [acetylcholinesterase (AChE)] or serum ChE [butyrylcholinesterase (BuChE)], following OP exposure, is an example of such an indicator [4, 31]. The toxic effects of organophosphate insecticides are associated with the capacity of the parent chemical, or an active metabolite, to inhibit cholinesterase (ChE) enzyme activity through a process of phosphorylation [32]. Inhibition of AChE activity in the central and peripheral nervous systems is considered to be the main mechanism of OP toxicity. In addition to being found in the nervous system, AChE is present on red blood cell membranes. BuChE is synthesized in the liver and is present in serum. Both AChE and BuChE activity can be measured in blood samples as a surrogate for neuronal AChE activity. Although AChE activity in blood is thought to approximate more closely neuronal AChE activity than BuChE, both are considered to be valid marker of OP-related biological effects and environmental health, as they give early warning of OP exposure before adverse clinical health effects occur in humans and animals. Inhibition of the AChE leads to an accumulation of neurotransmitter – acetylcholine at the nerve endings, which produces the common signs of OP intoxication. Besides their inhibitory effects on AChE, there is an increasing body of evidence that OPs also induce oxidative stress through generation of reactive oxygen species, leading to lipid peroxidation and DNA damage. They may also indicate the severity of a poisoning (acute or chronic) [10, 33, 34].

In spite of the importance of the toxic effects of AChE inhibition, BuChE activity determination has been established as a screening test for low levels of exposure in routine clinical work, as it can be measured faster and easier than the RBC-AChE. However, the wide range of values of BuChE in non-exposed subjects makes it difficult to detect small degrees of inhibition. Moreover, the value of both enzyme activities may vary for reasons not associated with inhibition, for example, liver function in the case of BuChE, or erythropoiesis for RBC cholinesterase. More specifically, BuChE may be reduced for reasons other than inhibition by organophosphorus and carbamate ester. Another disadvantage is that inhibition is not measured directly, but only by reference to a 'normal' value [33, 35]. With regard to interpretation of results, a reduction to 70% of the individual AChE baseline (30% inhibition) has been suggested as an indication risk of over-exposure. Since BuChE is more sensitive, but less specific, a 50% inhibition level has been suggested as a biological limit [21]. Organophosphorus insecticide biomonitoring has primarily focused on the assessment of ChE activity in blood or the quantification of metabolites in urine [36]. However, in the latest study on the relationship between urinary pesticide metabolites and pest control operation among occupational pesticide sprayers, erythrocyte AChE failed to show a significant relationship with the length of OP operation, despite the relatively narrow inter-individual variations of this enzyme among studied subjects [22].

Another biological matrix that was used to access AChE activity was saliva. But the latest results suggest that it is not feasible to use saliva as a replacement for blood for the measurement of AChE levels. This is because of the much lower levels of AChE in saliva relative to erythrocytes, the weak correlation between the 2 measurements and the previously reported high intra-individual variation of salivary AChE [10, 37].

Nevertheless, one of the current studies has demonstrated the ability to detect chlorpyrifos metabolites in blood and saliva following exposure to this insecticide at single oral doses. Although at all the studied dose levels, the concentration of insecticide metabolite in blood exceeded the saliva concentration, the kinetics of pesticide metabolism in blood and saliva were comparable, which made saliva an alternative matrix. However, the use of saliva for chemical biomonitoring requires the utilization of very sensitive and specific analytical methods for quantitation, and the relationship between chemical concentration in blood and saliva must be established [36].

Recent studies have shown that the activity of serum γ-glutamyltransferase (GGT) may be a cumulative biomarker of exposure to various environmental chemicals. Cellular GGT is prerequisite for the metabolism of glutathione (GSH), a critical biomolecule for the conjugation of diverse chemicals. The more we are exposed to chemicals, the more GSH conjugates are formed, and more GGT is induced. Supporting this concept, serum GTT within its normal range had clear dose-response associations with a variety of chemicals, such as lead, cadmium and pesticides. However, this idea is only at the preliminary stage and needs to be verified [38].

Gene expression altered by pesticide exposure. The results of recent research investigating the influence of certain OP pesticides on the expression of the tumour suppressor gene TP53 in HepG2 cell line, have shown that the expression of TP53 mRNA was significantly higher in exposed cells than in the control cells. TP53 is a transcription factor responsible for the cellular response to DNA damage. Elevated mRNA expression of the DNA damage responsive gene in metabolically-active human hepatoma HepG2 cells suggest that OPs are genotoxic agents [39]. Abnormally high levels of p53 protein are observed in many different types of cancer. Detection of elevated levels of the protein can be found in some tumours as an early event in the neoplastic progression, and often seem to be associated with the transition to malignancy. The correlation between p53 levels and pesticide exposure, however, is not clear enough to use p53 as specific biomarker of exposure to pesticides [40]. But some studies have revealed that the distinct TP53 mutational pattern between population groups may be due to different exogenous factors. Recent research has identified that one of the risk factors that influences TP53 gene mutation is pesticide exposure in lung cancer patients residing in areas with high lung cancer incidence in the upper northern part of Thailand [41].

Markers of early biological effects. There is a paucity of data on the possible deleterious effects of chronic exposure to OP in occupational and/or environmental settings [32]. To improve the characterization of possible risks to health, dose monitoring (= exposure biomonitoring) should be complemented by studies of biological effects (= effect biomonitoring). To link the dose with any health outcomes, one would prefer to measure the biologically effective dose, the dose at the target site that induces an effect. Monitoring of biochemical effect includes studies of the formation of protein and DNA adducts, and biological effect monitoring includes additional monitoring at the subcellular level, such as changes in enzymatic activity or the formation of micronuclei (MN), chromosome aberration (CA), frequency in sister chromatid exchange (SCE), and comet formation level [1-3].

Some biological effects of pesticide exposure can be measured very early at the cellular level and may serve as indicators of exposure. Considering this fact, cytogenetic markers of DNA damage in circulating lymphocytes are widely used as a biomarker of exposure (and perhaps of effect) in those exposed to pesticides [21].

The pathway of biological measurements in biomonitoring studies is shown in Fig. 1.

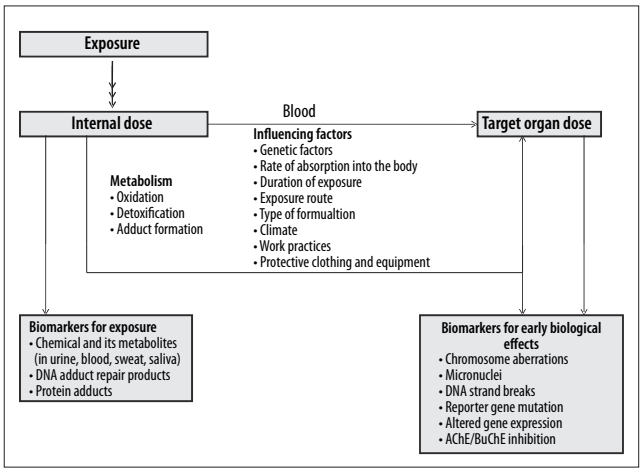

Fig. 1. Pathways for biological measurements

Chromosome aberration. The most frequently used test for genetic damage is the classical CA analysis of peripheral blood lymphocytes (PBLs). Since the 1960s, CA in PBLs has been used in occupational health surveillance programmes to assess genotoxic risks. The lymphocytes are collected from single- or multiple-timed blood samples, and prepared by a standard technique to undergo mitosis. Usually 100 or 200 metaphases per exposed and control subject are examined and the results reported in terms of the percentage of cells affected [42, 43]. A longitudinal validation study in a pooled Nordic and Italian prospective cohort (n = 5271, follow-up 13–23 years) has shown that CAs are a relevant and one of the best early biological effect biomarker for cancer risk in humans [44]. The results of another long-term study of chromosomal aberration in lymphocytes, collected from workers employed in pesticide production, have shown that the exposed group displayed a statistically increased number of aberrant cells, chromatid and chromosome breaks, acentric fragments, and dicentric chromosomes when compared with the control [45]. The presence of chromosomal aberrations is well recognized as a risk factor for cancer. Significant increase in CAs observed in association with exposure to pesticides indicate that the pesticides can lead to the development of cancer.

Several lines of evidence suggest that pesticides may be causally related to chromosomal abnormalities or genetic mutations in non-Hodgkin's lymphoma (NHL). Farmers exposed to pesticides during the high pesticide use periods, have an increased prevalence of t(14;18) translocation, which is one of the most common chromosomal abnormalities in NHL [46]. To quantify structural chromosomal aberration, such as translocations, fluorescent *in situ* hybridization (FISH) can be used. Fluorescent *in situ* hybridization with whole chromosome probes - 'chromosome painting' - provides an efficient, but expensive, approach for detecting complex, structural chromosome aberrations throughout the whole genome. Chromosome region-specific breaks probes are the cost- effective alternative to the 'chromosome painting' method; however, information provided is limited [47].

Depending on the number of DNA probes used, FISH provides information on the degree of numerical chromosome aberrations. In practice, this is usually limited to the detection of up to 4 chromosomes [26].

Sister chromatid exchange (SCE). This refers to the interchange of DNA between replication products. The technique for detecting such exchanges takes advantage of the semiconservative nature of DNA synthesis. 5'-bromodeoxyuridine (BrdU) is incorporated into the newly synthesized DNA; subsequently, colchicine or colcemid is added prior to the harvest of metaphase cells. Differential staining with Hoechst dye and Giemsa allows the newly synthesized DNA within a chromatid to be recognized, since BrdU incorporation results in much weaker staining. Any SCE appeared as a discontinuity of the stain along the chromatid [48-50]. The frequency of SCEs positively correlated with pesticide exposure. Mean number of SCEs per chromosome and proportion of high frequency cells were significantly higher among the farmers compared with the unexposed group. The same result was also obtained in every sub-category after stratification by smoking, origin, education, and age. The variables that had the most influence on the elevation of SCE were: self-preparation of the pesticides mixtures and the number of sprayings per year. The mutagenic effects of pesticides seemed to be cumulative; it was found that the SCEs frequency increased with the years of pesticide exposure [50].

Micronuclei formation. An alternative indicator of chromosome damage is the presence of micronuclei in PBLs. The frequency of micronuclei is a reliable measure of both chromosome loss and breakage, which makes it unique compared to other cytogenetic tests [51]. Micronuclei may result from small acentric chromosome fragments that are not incorporated into the daughter nuclei during cell division or non- or misrepaired whole chromosomes. The structures are enveloped by a nuclear membrane and appear as small nuclei (micronuclei) in the cytoplasm outside the main daughter nuclei [52]. The major advantage of MN assay over the traditional CA assay is that it can detect aneuploidy events. In addition, it is much less labour-intensive than the traditional CA assay. On the other hand, the MN assay has shortcomings since the cells need to survive at least one nuclear division, and some heavily damaged cells may have been lost [47]. The frequency and distribution of MN in polychromatic erythrocytes (PCEs) from bone marrow can also be used for identification of genotoxic effects of exposure to pesticide and other xenobiotics. The frequency of micronucleated polychromatic erythrocytes can be determined in samples from bone marrow and spleen, as well as from peripheral blood. However, there is a limitation in using peripheral blood erythrocytes since the spleen selectively removes micronucleated erythrocytes from the circulation in many species, e.g. humans and rats (but not mice). The results of micronucleus assay conducted in mice have shown that some of the tested pesticides produced a statistically significant increase in micronucleus frequency in mice PCEs [53, 54]. One of the advantages of the MN assay over PCEs is the ability to evaluate the clastogenic and aneugenic potential of compounds using the same end point *in vitro* and *in vivo*. Studies showed that the size of micronuclei can be used as a possible parameter to distinguish clastogens (small micronuclei, containing fragments of chromosomes) from aneugens (large micronuclei, containing whole chromosome, but with an abnormal number of chromosomes) [55]. Analysis of MN is thought to be a sensitive method for monitoring genetic damage in human populations. However, in a large study in which agricultural workers from 4 different European countries were included, the results indicated that occupational exposure to pesticides does not increase the level of cytogenetic damage when evaluated by the MN assay using peripheral blood lymphocytes and buccal epithelial cells [56].

Comet Assay. In recent years, an important tool for assessing cytogenetic/DNA damage in exposed populations has become the Comet assay, also known as single cell gel electrophoresis (SCGE) assay [57]. The comet assay is a simple and sensitive method for measuring single- and double-strand breaks in DNA. The mechanism of formation of comets (under neutral or alkaline conditions) is best understood by analogy with nucleoids, in which relaxation of DNA supercoiling in a structural loop of DNA by a single DNA break releases that loop to extend into a halo – or, in the case of the comet assay, to be pulled towards the anode under the electrophoretic field [51]. Briefly, cells with increased

DNA damage display increased migration of chromosomal DNA from the nucleus toward the anode, which resembles the shape of a comet [2]. Analysis of comets, in order to determine the level of DNA damage, includes measurement of such parameters as: comet tail length (TL = distance of DNA migration from the centre of the body of the nuclear core), tail moment (TM = tail length × % of DNA in the tail), tail intensity (TI = % of genomic DNA that migrated during the electrophoresis from the nuclear core to the tail), and % of DNA in head [51, 58, 59].

The advantages of the comet assay include the following:
1) DNA damage is measured at the single-cell level;
2) only a few cells are needed to carry out the assay (<10,000);
3) the assay can be performed on virtually any eukaryotic cell type without pre-labelling of DNA;
4) it is a very sensitive method for detecting DNA damage [60].

There are also disadvantages, such as a wide intra- and inter-individual variability of the comet assay results, as the basal level of DNA damage is influenced by a variety of factors, such as lifestyle, diet, infections, medication, air pollution, season, climate or exercise [2]. It is the method of choice for measuring the primary DNA damage in single cells, used in studies with various genotoxic agents, but it should be noted that the assay detects a mixture of lesions (single- and double-strand breaks, AP sites) [58]. The sensitivity and selectivity of the assay can be improved if lesion-specific enzymes are used to convert damaged bases to DNA breaks [51].

Glycophorin A assay (GPA). This assay is used to quantify phenotypically mutant erythrocytes. It identifies and enumerates rare variant cells lacking the expression of one allelic form of GPA – erythrocyte lineage specific surface protein - presumably due to mutations in erythroid precursor cells in the bone marrow. Two variant cell phenotypes are simultaneously measured in the GPA assay; hemizygous NØ phenotype cells lack expression of the *M*-allele but express the *N*-allele normally, while homozygous NN phenotype cells lack expression of the *M*-allele and express the *N*-allele at twice the normal level.

For the GPA assay, only individuals who are heterozygous at the MN locus can be analyzed. Increase in NN or NØ variant frequencies per million cells indicate exposure to highly toxic agents/chemicals and can be observed, for example, in chemotherapy patients. In a study which used this method to evaluate exposure to the pesticide phosphine, direct evidence of the effect of this pesticide on the frequency of GPA variants was observed. However, according to the authors'comment, the study group was small, and a larger group is required to achieve greater statistical power and see a reliable effect of exposure to this pesticide [61].

Other data show that agricultural workers exposed to diazinon in the last 6 months had a higher mean NØ frequency than those unexposed, as well as a higher NN frequency. However, there was no consistent trend of elevation in GPA variant frequencies with malathion exposure. Because the GPA assay is conducted in erythrocytes, the important exposure period is when they are maturing as stem cells 4-6 weeks before sample collection [62]. The need to use heterozygous individuals among the study populations limits the use of this assay to large populations [47]. The assay also shows significant inter-individual variation, particularly regarding the NN frequency, and both NØ and NN frequencies appear related to age and smoking [62].

CONFOUNDING FACTORS

Cytogenetic markers. Most studies of DNA damage and pesticide exposure have focused on cytogenetic endpoints, such as CA, SCE and MN, with conflicting results. Some indicate a significant increase in MN, SCE, and CA frequencies, while others do not show significant differences [7, 26, 63].

There is also a lot of uncertainty surrounding studies of pesticide exposure and genotoxic damage, including the reliability of exposure assessment, the power of the studies, suitability of control groups and the protocols used for determining genotoxicity (Table 1) [64]. Among the biomarkers for early biological effects of exposure to environmental mutagenic agents, CA and MN seem to be the most relevant. The chromosome aberration (CA) is considered to be the gold standard because the mechanisms for the induction of CAs are well understood, and the most environmental toxic substances have been shown to induce CAs [65]. Workers exposed to pesticides, and also to styrene or butadiene, and residents exposed to uranium mining and milling waste, were found to have significantly higher CAs than the respective matched controls. Increased level of observed CAs in cases compared with that in controls is therefore indicative of exposure-induced DNA repair deficiency [66]. The CAs have been found in most cancer cells and in many developmental abnormalities, which made the CA an useful biomarker for cancer risk assessment [65]. Validation studies on the relationship between MN and prediction of cancer have still to be completed. Despite this, the MN assay is one of the best candidates for wide use in public health strategies, and potentially in individual risk assessment. The sensitivity and reliability of the MN assay to detect DNA damage, as well as its ability to be applied to different kinds of cells, makes it a good method to analyze the potential cytogenetic damage of environmental pollutants, including pesticides [43, 67]. The assay, since its discovery, has been improved by the development of the cytokinesis-block micronucleus method, which allows micronuclei to be scored specifically in cells that had completed a nuclear division, to eliminate the confounding effects of variability in cell division kinetics [43]. The micronucleus test has many advantages: reliable identification of cells that have completed only one nuclear division, sensitivity and precision, speed and simplicity, the ability to screen large numbers of cells, and

Table 1. Differences in cytogenetic biomarkers results of human populations exposed to mixture of pesticide (adopted from [7])

	Analysed biomarker	Number of studies (positive/total)
Pesticide sprayers	CA	13/13
	MN	2/3
	SCE	4/7
Floriculturists	CA	5/7
	MN	3/4
	SCE	4/7
Agricultural Workers	CA	2/5
	MN	0/7
	SCE	0/2

good reproducibility [68]. The other cytogenetic markers, such as SCE and SCGE, have also been extensively used for the detection of the early biological effects of DNA-damaging agents [56].

One of the latest study of the genotoxic effect of pesticide exposure was carried out on Mexican agriculture workers. The DNA damage was detected through the SCE in PBLs and MN in exfoliated buccal cells. Significant differences were found in SCE frequencies when the exposed and non-exposed groups were compared. The results also show a correlation between exposure time and SCE frequency [69, 70]. On the other hand, no correlation was found between exposure time and MN frequency, nor between age, gender, and MN frequency [49]. The former result is consistent with the Pastor *et al.* results, which do not show any significant increase in the frequency of MN in neither peripheral blood lymphocytes nor epithelial buccal cells in relation to pesticide exposure of Polish farmers [67]. However, the results obtained in another study indicate that the mean number of cells with MN in the exposed group was significantly higher than in the control group. This indicates the fact that an increase of cells with MN in exposed workers may depend on the genotoxic potential of the pesticides [71]. Such inconsistent results in evaluating cytogenetic damage by MN or SCE assays may be due to age, genetic polymorphism, method of application, genotoxic level of the compounds used, and interaction among them [49, 67]. The comparison between results from research on cytogenetic markers undertaken in different parts of the world is also difficult because of differences in the periods, levels of exposure, type of pesticides, variety of mixtures or cocktails used in the field, and the geographic and meteorological characteristics of the agricultural areas where they are applied. Such differences refer mainly to the people who prepare the mixtures in the field, the pesticide sprayers, and the population that lives near the sprayed sites, storage rooms, greenhouses and open fields [49, 56]. There has also been reported an association between gender and cytogenetic damage. MN frequency in females was found to be 20–30% higher than in males, but no significant differences were found between genders for SCE and CA frequencies. The increase in MN frequency observed in women is attributed to aneuploidogenic events involving the X-chromosome, which is represented in MN more often than expected if equal probability is assumed between this gender chromosome and autosomes. The mechanism remains unclear [63].

Other factors that may influence the comparison between studies are smoking and drinking habits, diet or safe practice and personal protective equipment usage behaviours [63, 56]. One main critical issue in using the comet assay in human biomonitoring studies is the interpretation of data. For biological monitoring purposes, lymphocytes prepared from heparinized venous blood samples are usually used. These are surrogate cells, thus the damage detected does not reflect the damage in the target tissue [2]. The measurement of DNA strand breaks based on the SCGE or the Comet assay has been popular because of the simplicity of the assay. However, its relevance to meaningful biological effects may need to be substantiated by other more established biomarkers [65]. In fact, the comet assay is a simple and sensitive method for studying DNA damage and repair, and especially useful for human biomonitoring application. Despite the fact that each laboratory working with the comet has probably developed its own methodology of the assay, there have been many activities to harmonize the comet assay. The present state of validation in human biomonitoring, and the suggestion for a standard protocol of the alkaline comet assay has been published recently [2].

Factors that may influence the study on cytogenetic markers and hamper the comparability of results from different biomonitoring studies are summarized in Fig. 2.

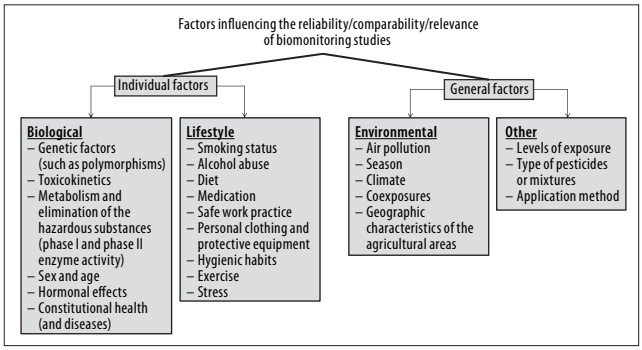

Fig. 2. Influencing factors

Polymorphisms in biotransformation enzymes and DNA-repair genes. Closely related to exposure biomarkers and biomarkers of early biological effect are biomarkers of susceptibility, which indicate increased vulnerability of individuals to diseases such as cancer, e.g. GSTM1 polymorphisms [57]. Individual responses to environmental toxicants are influenced by the metabolic capability of the individual, determined by genetic variability of the enzymes that metabolize agricultural chemicals [72]. The genetic variability of other proteins is also very likely involved in individual responses to toxicants, e.g. proteins involved in the repair of DNA damage. When detoxification and DNA repair are inefficient, metabolic products accumulate and DNA damage persists, contributing to the carcinogenic process. Most OP pesticides are believed to undergo a common metabolism in the human body by using the same monooxygenase enzyme complex responsible for the biotransformation of xenobiotic chemicals [54]. Organophosphates are primarily metabolized by hepatic *cytochrome P450 3A4* and *3A5* (Phase I enzyme) to become an active intermediate-organophosphorus-oxon. Among phase II enzymes, glutathione S-transferases (GST) are the most important group of detoxifying enzymes, followed by microsomal epoxide hydrolase (mEH) [63]. Furthermore, organophosphorus-oxon may then be hydrolyzed by *paraoxonase* (PON) to diethyl phosphate (DEP) and 4-nitrophenol, or conjugated to glutathione (GSH) [73-75]. The genotypes responsible for interindividual differences in the ability to activate or detoxify genotoxic substances are recognized as biomarkers of susceptibility to mutations, cancer, and other diseases [72].

In this respect, the polymorphic genes of cytochrome P450 (CYP), glutathione *S*-transferase M1 (GSTM1), glutathione *S*-transferase theta 1 (GSTT1), glutathione *S*-transferase P1 (GSTP1), *N*-acetyltransferase 2 (NAT2) and paraoxonase 1 (PON1) involved in the detoxification and metabolism of pesticides were selected, and their role in modulating cytogenetic effects was studied in pesticide-exposed populations. The pesticide-exposed individuals with inherited, susceptibility-associated, metabolic and DNA-repair genotypes may have increased risk of DNA damage. For example, individuals with genotype responsible for low

PON1 activity are more susceptible to parathion poisoning than individuals with higher PON1 activity [7].

Children appear to be particularly vulnerable to the effects of pesticides as they have less developed detoxification pathways, and newborn infants have low levels of the enzyme PON1 [76]. There is a study that suggest an increased risk of chronic toxicity is associated with the particular PON1 genotype [77].

In another study, DNA samples from 20 farmers and 20 controls were chasacterized for their inheritance of the polymorphic CYP2E1, GSTMI, GSTTI, and PON genes. Although the sample sizes for each of the comparison groups were small, the overall outcome was that the inheritance of 'unfavorable' alleles was frequently associated with increased cytogenetic effects (CAs: chromatid breaks, chromosome deletions, dicentrics) [73].

The latest study has revealed that *GSTP1* polymorphism (but not *PON1*, *PON2*, *GSTM1*, and *GSTT1* genotypes) in pesticide-exposed fruit growers is associated with increased DNA damage measured by the comet assay. It was also found that the polymorphism of *XRCC1* gene *(*399 *Arg-Arg)* is associated with elevated risk of DNA damage in the studied pesticide-exposed population. XRCC1 protein is exclusively required for DNA BER, strand-break repair, and maintenance of genetic stability [73-75].

Markers of acute and chronic toxicity of pesticide exposure. The primary mechanism of OP toxicity is the inhibition of acetylcholine esterase in the nervous system, leading to a variety of acute and chronic effects [39].

The immediate health effects resulting from acute high dose OP exposure have been well documented and well understood. The individuals exposed to high levels of OP can develop acute cholinergic syndrome, which is characterized by a variety of symptoms, including rhinorrhea, salivation, lachrymation, tachycardia, headache, convulsions, and death, known as a acute cholinergic syndrome. In addition, these individuals can also develop a proximal and reversible paralysis called intermediate syndrome, and organophosphate-induced delayed polyneuropathy or long-term neurologic sequelae. Although adverse effects of chronic low-level OP exposure are suspected, they have not been conclusively determined [78]. Acute toxicity of OP exposure can be evaluated by measuring the AChE inhibition level, or directly by measuring the OP pesticide concentration or its metabolites in biological matrices. On the basis of pharmacokinetic models and biomonitoring data, the OP concentrations in human tissues higher than 100 μM (10-100 μg/mL) reflect acute accidental or intentional exposure, whereas lower concentrations (0.01-1 μg/mL) represent the actual environmental exposure. These findings make measurements of OP in blood and urine very useful in differentiating between chronic and acute exposure to OP [39]. Detection of the reduction in ChE activity can serve also as biomarker of chronic exposure. However, the relationships between chronic exposure, ChE inhibition and symptoms do not, as yet, seem to be well established [32]. The chronic toxicity can be detected and evaluated by measurements of genotoxic effects of OP exposure. The cytogenetic biomarkers such as MN, SCE, CA, and SCGE occur spontaneously in proliferating cells and are regarded as a manifestation of damage to the genome. They have been extensively used for the detection of early biological effects of DNA-damaging agents also in human biomonitoring studies, and are a tool of great interest in cancer risk assessment as it is anticipated that they will allow estimation of genetic risk resulting from environmental or occupational chronic exposure to OP [68].

Health outcomes. Multiple studies indicate a wide range of pesticide-related clinical and subclinical effects, including significant positive associations between pesticide exposure and solid tumours, haematological cancers, and genotoxic effects. In addition, pesticides were found to impact on mental and emotional functioning, the nervous system – causing Parkinson's disease and other neurological diseases, and the reproductive system – causing birth defects, fertility, foetal death, and intrauterine growth retardation.

The most common clinical form of pesticide-related skin diseases is contact dermatitis, both allergic and irritant. The less common diseases include contact urticaria, erythema multiforme, ashy dermatosis, occupational acne, porphyria cutanea tarda, hair and nail disorders, and skin cancer [13, 76]. Although it is difficult to establish a connection between pesticide exposure and cancer prevalence, especially because of the high number of compounds involved, some authors evidence a greater prevalence of certain types of cancer in pesticide-exposed populations. Leukaemia, non-Hodgkin lymphomas, and incidence of multiple myeloma is higher in individuals exposed to pesticides. DNA damage and oxidative stress have been proposed as mechanisms that could mechanistically link pesticide exposures to a number of the health outcomes observed in epidemiological studies [21, 63].

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