BIOMARKERS AS BIOLOGICAL INDICATORS TO XENOBIOTIC EXPOSURE

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The presence of a xenobiotic in the environment always represents a risk for living organisms. However, to talk about impregnation there is a need to detect the toxic

in the organism, and the concept of intoxication is related to specific organ alterations and clinical symptoms. Moreover, the relationship between the toxic levels within the organism and the toxic response is rather complex and has a difficult forecast since it depends on several factors, namely toxicokinetic and genetic factors.

One of the methods to quantify the exposure to xenobiotics and its potential impact on living organisms, including the human being, is the monitoring by the use of the so-called biomarkers.

1. Definition

The National Academy of Sciences defines a biomarker or biological marker as a xenobiotically induced alteration in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample [1]. Silbergeld et al [2] defines biological markers as physiological signals that reflect exposure, early cellular response or inherent or acquired susceptibilities, which provide a new strategy for resolving some toxicological problems.

Sensu stricto, we define a biomarker as a biological response to a chemical or a group of chemical agents [3] but not the presence of the agent or its metabolites within the body (internal dose). However, there is no doubt that the measurement of a xenobiotic in a biological system or sample is a bioindicator of exposure, and thus, it could be considered like a biomarker.

Biological monitoring has advantages over environmental monitoring because it measures the internal dose of a compound. Interindividual differences in absorption, bioavailability, excretion, and DNA repair should be taken into account. Moreover, intraindividual differences, as consequence of particular physiopathological alterations occurring in a specific period of time, should also be considered. This involves an individualised biological control to evaluate the exposure to a particular xenobiotic [4]. The organism acts as an integrator of exposure and several physiological factors, which modulate the uptake of toxic. Thus, we may state that a collective can not be assimilated as a homogeneous group of individuals exposed to a xenobiotic of physico-chemical properties under reproducible and standard conditions.

The use of biological markers in the evaluation of disease risk has increased markedly in the last decade. Biomarkers are observable end points that indicate events

in the processes leading to disease. They are particularly useful in the evaluation of progressive diseases that manifest their symptoms long after exposure to initiating factors. In such cases, traditional early warning symptoms of developing disease may be lacking. At the same time, the disease, once clinically apparent, may be essentially irreversible [5].

The two main research fields in the use of biomarkers in Toxicology are Enviromental Toxicology and Industrial Toxicology, the latter being one of the most relevant and important branches of Medical Toxicology.

2. Conditions and limitations of biological monitoring

A rational biological monitoring is only possible when sufficient toxicological information has been gathered on the mechanism of action and on the toxicokinetic of xenobiotics including absorption, distribution, metabolism and excretion [6]. Biological monitoring cannot be applied for assessing exposure to substances that exhibit their toxic effects at the sites of first contact i.e. primary lung irritants or are poorly absorbed.

For other xenobiotics that are significantly absorbed and exert a systemic toxic action, a biological monitoring test may provide different information, depending on our current knowledge of the relationships among external exposure, internal exposure and the risk of adverse effects. If only the relationships between external exposure and the internal dose is known, the biological parameter can be used as an index of exposure, but it provides little information on the health risk. But if a quantitative relationship has been established between internal dose and adverse effects, and the internal-dose effects and the internal-dose response relationships are known, biological monitoring allows for a direct health risk assessment and thus for an effective prevention of the adverse effects [7].

3. Specificity of biomarkers

The ideal biomarker should have the following characteristics [8]:

a) sample collection and analysis are simple and reliable
b) the biomarker is specific for a particular type of exposure
c) the biomarker only reflects a subclinical and reversible change
d) relevant intervention or other preventive effort can be considered

e) use of the biomarker is regarded as ethically acceptable.

It is clear that if we agree with that last definition only a few biomarkers fit well. Biomarkers range from those that are highly specific such as an enzyme of the heme pathway, aminolevulinic acid dehydratase (ALAD), which is inhibited only by lead or the inhibition of acetyl choline esterase (AChE), which is specific to the organophosphorus and carbamate pesticides, to those that are non specific namely the effects on the immune system or DNA that can be caused by a wide variety of chemical agents (Table 1) [3].

An important aspect to be considered is the complementation among biomarkers that results into a higher degree of specificity. Thus, metalothionein induction may occur by exposure to a variety of metals. However, if a measurement of one specific metal is performed in a biological fluid i.e Cadmium in urine, and it is found elevated over normal values, the evaluation of the induction of metallothionein would enhance the specificity of the measurement, which in turn would increase even more if a preclinical renal alteration as renal proteinuria (i.e. $\exists_2$-microglobulin) is detected. This feature is relevant since currently there is a number of biomarkers of toxic response in different tissues, organs, and systems that are unspecific. However, the establishment of new relationships between biomarkers may contribute to increase their specificity.

4. Classification of biomarkers

Biomarkers are generally classified into three groups: exposure, effects, and susceptibility [2,3,9,10].

4.1 Biomarkers of exposure

They allow measuring the internal dose by chemical analysis of the toxic compound or metabolite in body fluids or excreta such as blood, urine and exhaled air (Table 2) [10, 11].

Internal dose may also mean the amount of a chemical stored in one or in several body compartments or in the whole body. This usually applies to cumulative toxic chemicals. For example, the concentration of polychlorinated biphenyl (PCB) in blood is a reflection of the amount accumulated in the main sites of deposition (i.e., fatty

tissues). The internal dose reflects the amount of chemical bound to the critical sites of action [12].

Bernard & Lauwerys classified the biomarkers of exposure into two subgroups, selective and non-selective, according to their selectivity test, which is based on the direct measurement of the unchanged chemicals or their metabolites in biological media. The non-selective tests are used as non-specific indicators of exposure to a group of chemicals. As an example of non-selective exposure tests, the determination of diazo-positive metabolites in urine for monitoring exposure to aromatic amines, the analysis of tioethers in urine, and the determination of the mutagenic activity of urine can be cited.

When assessing the usefulness of a particular exposure biomarker, one must consider two aspects of validity: analytical and toxicokinetic. For optimal analytical quality, standardisation is needed, but the specific requirements vary considerably between individual toxicants. Major areas of concern include: preparation of the individual, sampling procedure and sampling handling, and measurement procedure that encompasses technical factors, such as calibration and quality assurance procedures. Life events, such as reproduction and senescence, may also affect the toxicokinetic of a xenobiotic [8].

4.2 Biomarkers of susceptibility

They serve as indicators of a particular sensitivity of individuals to the effect of a xenobiotic or to the effects of a group of such compounds. Can be genetic markers that include alterations in chromosomal structure such as restriction fragment length polymorphism's (RFLPs), polymorphism of enzyme activities, etc.[13]. After the exposure of an organism to a xenobiotic it suffers a biotransformation process in two phases. In the first phase a primary metabolite, usually oxidised and more or less active, is originated by the specific action of the microsomal P450 cytochrome isoenzymic family. In the second phase, the primary metabolite is transformed into other secondary metabolite, which is usually inactive. Some individuals with a low cytochrome P450 activity will be more resistant to the generation of primary active metabolites, whereas those exhibiting a low activity of enzymes involved in the second phase will show a lower formation of phase II inactive metabolites, increasing toxicity.

Two types of susceptibility biomarkers can be distinguished: polymorphism's activating system markers and polymorphisms of detoxicating systems. Polymorphisms of activating systems are measurements of the activity of cytochrome P-450 isoenzymes. The family of cytochrome P-450 enzymes is involved in the toxicity of several xenobiotics; associated with the P-450 cytochromes there are a wide range of enzyme activities referred to as monooxygenase activities. A number of studies have suggested that the various cytochrome P-450 enzymes differ substantially in their amino acid sequences and thus they likely are encoded by distinct genes. This has been confirmed by comparisons of the complete amino acid sequences of over 71 forms of cytochrome P-450 and of the nucleotide sequences of their corresponding cDNAs and of several genes [11].

A roman numeral corresponding to its specific class designates each form of cytochrome P-450. The most important classes that constitute the different forms of cytochrome P-450 are I through IV. These cytochrome classes comprise several subclasses that are designated by a combination of a letter (A,B,C,D,..) and an identifying arabic numeral (i.e. IA1, IIC8,....). The most important are IA1 (represent the AHH activity), IIC8, IID6 and IIE1. There have been a number of studies trying to establish a relationship between specific cytochrome P450 activities and some diseases due to environmental toxic exposure, especially cancer. However, there are no definitive conclusions [14,15].

Markers of polymorphisms of detoxicating systems are measurements of the activity of conjugating enzymes such as glutathione-S-transferases, acetyltransferases, sulfotransferases, glucuronyltransferases and paraoxonase. For instance, predisposition to cancer has been correlated with genetic polymorphisms of N-acetyltransferases. N-acetyltransferase is an enzyme involved in the deactivation of aromatic amines. After acetylation there is enhanced excretion in urine.In a group of arylamine exposed workers the slow acetylators are at increased risk for bladder cancer versus rapid acetylators. Another example is glutathione-S-transferase μ, an enzyme involved in the detoxification of reactive metabolites. Half of the population has no functional allele for this enzyme and no or low enzyme activity. These persons are at increased risk to squamous cell carcinoma of the lung [11].

Finally, several organophosphates can be inactivated (hydrolysed) by

paraoxonase (PON1). Human paraoxonase exhibits an important polymorphism and in humans, three genotypes have been detected: individuals homozygous for the low activity allele, individuals homozygous for the high activity allele, and heterozygous, so paraoxonase activity can be used as a biomarker of susceptibility to organophosphorus compounds [16]. The polymorphism is also observed with the oxons of methyl parathion, chlortion and ethyl 4-nitrophenyl phenylphosphonate (EPN). However, it is not observed with the oxon of chlorpyrifos.

Several evidences suggest that high levels of serum paraoxonase are protective against poisoning by organophosphorus pesticides whose active metabolites are substrates of this enzyme. [17]. Birds, which have very low levels of serum paraoxonase are very sensitive to parathion, diazinon-oxon, and pirimiphos-oxon compared with mammals who have higher levels of this enzyme [18]. After injection of partially purified rabbit paraoxonase into rats an increased resistance to the toxic effects of paraoxon was observed. Recent studies indicate that administration of paraoxonase might have therapeutic value in case of organophosphates intoxication [19].

4.3 Response or effect biomarkers

Response or effect biomarkers are indicative of biochemical changes within an organism as a result of xenobiotic exposure. The ideal biomarkers should be early detected and be able to show adverse effects before they are irreversible. Those are the most studied biomarkers and they include modifications in some parameters of blood composition, alterations of specific enzyme activities, DNA-adducts appearance, localised mRNA and protein increases, and appearance of specific antibodies (autoantiboides) against a xenobiotic or a particular cellular fractions [10].

It is noticeable to remark that not always is easy to distinguish between an exposure and a response biomarker. Perhaps the most typical example is the formation of an DNA adduct, a exposure biomarker, which formation results from the reaction of a xenobiotic with the DNA, that in turn constitutes the cellular response. Moreover, it is evident that a particular response requires a previous exposure to the xenobiotic. Below we consider some significant examples of response biomarkers.

4.3.1.Respiratory system.

Several studies have suggested that low-molecular weight proteins (LMWP) specific for the lung might serve as peripheral biomarkers of lung toxicity [20]. A lung biomarker, measurable in serum, bronchoalveolar fluid (BAL) and sputum has recently been identified. This biomarker is a microprotein initially isolated from urine in 1974 (Urine Protein 1) of patients with renal tubular dysfunction and subsequently identified as the major secretory product of the lung Clara cells, which are non-ciliated cells localised predominantly in terminal bronchioles. This protein called Clara cell protein (CC16) is a homodimer of 15.8 kDa. Clara cells are particularly sensitive to toxic lung injury and they contain indeed most of the lung cytochrome P-450 activity, which confers them a high xenobiotic metabolising activity [21,22]. Several lines of evidence indicate that CC16 is a natural immunoregulator protecting the respiratory tract from unwanted inflammatory reactions. CC16 has been shown to inhibit the activity of cytosolic phospholipase A2, a key enzyme in inflammatory processes. Phospholipase A2 is the rate-limiting enzyme in the production of arachidonic acid, the substrate for the synthesis of prostaglandin and leukotriene mediators of inflammation [23]. By inhibiting phospholipase A2, CC16 could also prevent the degradation of lung surfactant phospholipids [24].

CC16 secreted in the respiratory tract diffuses passively by transudation into plasma from where it is rapidly eliminated by glomerular filtration before being taken up and catabolised in proximal tubule cells. Studies reviewed by Bernard suggest that CC16 in BAL fluid, sputum or serum is a sensitive and relatively specific indicator of acute or chronic bronchial epithelium injury.

A significant reduction of CC16 in serum is an indicator of Clara cells number and integrity. After adjustment for age, a linear dose-response relationship was apparent between smoking history and serum CC16, latter decreasing on average by about 15% for each 10 pack-year smoking history [25]. Serum CC16 was also found to be decreased in several occupational groups chronically exposed to silica, dust and welding fumes, and lung diseases (cancer, asthma and patients with chronic obstructive pulmonary disease) [26].

The increased concentration of CC16 in serum can also be used to detect an acute or chronic disruption of the bronchoalveolar/blood barrier integrity. Increased serum levels of CC16 have been observed in sarcoidosis and adult respiratory distress

syndrome (ARDS). This confirm that in pathological conditions the barrier between the surface of respiratory epithelium and the vascular compartment may be disrupted, upsetting the diffusional equilibrium between CC16 in serum and in the respiratory tract. The existence of an enhanced passage of proteins across the blood/brochoalveolar space barriers, for example in acute exposure in animals by inhalation or systemic routes with pneumotoxic agents (4-ipomeanol, 3-methylfuran, naphthalene, trichloroethylene, 1,2-dichloroethylene, etc.), is supported by the significant elevation of albumin, β_2-microglobulin and other plasma proteins in BAL fluids. These findings open new perspectives in the assessment of lung toxicity by suggesting that readily diffusible lung-specific proteins may serve as peripheral markers of pneumotoxicity.

4.3.2. Blood system

The most studied biomarkers of effect are those related to the alterations of heme synthesis**.** ALAD is an enzyme involved in the heme biosynthetic pathway and the assay is highly specific for lead exposure and effect. The inhibition of ALAD has been shown to be a reliable indicator of effect to lead in studies on humans and animals (specially several species of fish and birds –eagles, starlings, ducks and geese). One of the most important advantage of this biomarker in Ecotoxicology is that the animal sacrifice is not required; the effect is slowly reversed, with ALAD values returning to normal only after about 4 months [3,27-29].

Heme biosynthesis is normally closely regulated, and levels of porphyrins are ordinarily very low. Some organochlorines (OCs) cause the formation of excess amounts of hepatic highly carboxylated porphyrins. The two OCs that are most involved in inducing porphyria in mammals and birds are hexacholorobenzene (HCB) and the PCBs [3, 30].

Hemoglobin adducts are formed from exposition of several compounds (ethylene oxide, acrylamide, 3-amino-1,4-dimethyl- 5OH-pyrido-indole, 4-aminobiphenyl, 2,6-dimethylaniline,etc). Acrylamide is an important neurotoxic agent causing a peripheral neuropathy to experimental animals as well as to humans and it has been shown to be a potential carcinogen. The conversion rate of acrylamide to glycidamide (reactive metabolite epoxide responsible for the neurotoxicity) is significantly correlated with the hemoglobin adducts of acrylamide. These adducts are useful as biomarkers of

acrylamide-induced peripheral neuropathy [31]. Because of the relatively long life span of the red blood cells (four months in humans), hemoglobin adducts have advantageously been used for integrating concentrations in the blood of genotoxic substances.

4.3.3. Nervous system

Despite its obvious importance within toxicology, the area of neurotoxicity seems to be progressing more slowly than other fields with regard to biological monitoring. The complexity of the nervous system and its distinctive peculiarities, together with the problems associated with the determination of the precise targets for neurotoxic action are certainly responsible for this limited advancement. Neurochemical measurements for detecting neurotoxicity in humans are limited by the inaccessibility of target tissue. Thus, a necessary approach for identifying and characterising neurotoxicity is the search for neurochemical parameters in peripheral tissues easily and ethically obtained in humans, which could represent a marker for the same parameters in nerve tissue [32].

Perhaps, the most significant and useful example of specific biomarker of neurotoxicity is the inhibition of acetyl choline esterase (AChE) caused by organophosphorus compounds or carbamate pesticides. The enzyme activity is present in several tissues though their inhibition is generally determined from blood samples (whole blood or plasma) and brain. This biomarker has been used in human toxicology and is widely studied in ecotoxicology (birds, mammals and aquatic species). For example, inhibition of AChE in brain can be taken as proof of mortality in birds, whereas in other animals, such as fish, there is a bigger variability founding lethal inhibition in a range of 40-80% [33-35].

The decrease in AChE activity in brain may remain for several weeks after the toxic exposure, which is adequately correlated with the effect, in contrast to that occurring in blood with a lower life span. Nevertheless, measuring the blood AchE activity has the advantage of easy sampling since there is no need of animal sacrifice. [36].

Butyrylcholinesterase (BuChE), example of an unspecific biomarker of neurotoxicity, is sometimes studied in parallel with AChE but its physiological role is

unknown and its degree of inhibition is not simply related to toxic effect. Other parameters involved in neurotransmission are the target for a variety of neurotoxicant xenobiotics. The measurement of these parameters is done in red blood cells, lymphocytes and fibroblasts [32,37].

Several active bioamines are liberated from the nerve ending by exocytosis, a process which is triggered by influx of Ca^{2+} and are inactivated by reuptake and methylation mediated by catechol-O-methyltransferase (COMT). Because of its intracellular localisation, monoamine oxidase (MAO) plays a strategic role in inactivating catecholamines that are free within the nerve terminal and not protected by storage vesicles. Isoenzymes of MAO have been characterised with differential substrate specificities; MAO-A preferentially deaminates norepinephrine and serotonin, whereas MAO-B acts on a broad spectrum of phenylethylamines. MAO-B is a microsomal enzyme and the aminoacidic sequences of the enzymes from human cerebral cortex and consequently platelets were shown to be identical and platelet MAO-B activity appears to reliably reflect enzyme activity in the nervous system.

MAO-B activity is used clinically as a marker of the pharmacological effects of MAO inhibitors, such as in the treatment of Parkinson’s disease. MAO-B activity in platelets has been used as a biomarker of effects of styrene and perchloroethylene occupational exposures [38], which is known to cause dopamine depletion. Changes in MAO-B could represent an adaptive response to dopamine depletion and alternatively, styrene or its metabolite(s) might exert a direct inhibitory effect on the enzyme [37, 39].

Another example of neurotoxic biomarker, involved in delayed toxicity , is the inhibition of neuropathy target esterase (NTE). Several organophosphorus compounds (Mipafox, Methamidofos,etc), after a single dose, induce delayed neuropathy, characterised by symmetrical axonal degeneration that implicate the NTE inhibition and not the acethyl cholinesterase enzyme. Organophosphate-induced delayed neuropathy (OPIDN) is characterised by a lag period of about 1 to 3 weeks, from the moment of intoxication to the appearance of clinical symptoms. The first intoxication was described with TOCP (triorthocresylphosphate) [40-41]. In experimental assays the measurement of NTE in lymphocytes has been used as biomarker of effect and there is a good correlation between NTE activity in brain and lymphocytes after 24 h of an acute exposure to neurotoxic organophosphorus compounds [42].

In conclusion, the complexity of the nervous system does not allow a rapid and easy development of sensitive, specific and reliable biomarkers for neurotoxicity, although the biomarkers presented in this review appear promising.

4.3.4. Urinary biomarkers

Long-term exposure to certain nephrotoxic compounds (heavy metals –lead, mercury, cadmium and chromium-, halogenated hydrocarbons –chloroform-, organic solvents –toluene-, therapeutic agents –aminoglucosides, amphotericine B, acetaminophen, etc.) may cause progressive degenerative changes in the kidney. However, because of its large reserve capacity, the clinical signs of renal damage are not apparent until the injury is extensive and consequently irreversible. The prevention of renal diseases requires the use of more sensitive tests capable of detecting renal effects at a stage when they are still reversible or at least not so advanced as to trigger a progressive renal disease [43-47].

In practice, one usually recommends the determination in urine of at least two plasma-derived proteins, a high molecular weight protein (HMWP) such us albumin for the early detection of glomerular-barrier defect and a low molecular weight protein (LMWP) such as retinol-binding protein for the early screening of proximal damage (Table 3) [48,49].

Injury to the kidney can be detected by measuring the urinary activity of kidney-derived enzymes. As index of nephrotoxicity it has been proposed the lysosomal enzyme β-N-acetyl-D-glucosaminidase (NAG). Advantages of this enzyme include its stability in urine and its high activity in the kidney. The diagnostic value of NAG can be further improved by measuring the B isoenzyme (lesional form released with fragments of cell membranes) [50].

Destruction of renal tissue can also be detected by measuring in urine kidney components which, when they are quantified by immunochemical methods, are referred to as renal antigens. These have been proposed as urinary markers of nephrotoxicity and include: carbonic anhydrase, alanine aminopeptidase and adenosine deaminase-binding protein for the proximal tubule, fibronectin for the glomerulus and Tamm-Horsfall glycoprotein for the thick ascending limb of the loop of Henle [50-55].

It is important to realise that this battery of tests, does not permit the detection of

effects on all areas or segments of the kidney or nephron. No sensitive biomarker is available to detect effects on the deep medulla, the papilla or the distal tubule. There is also no biomarker to detect and follow the progression of active fibrotic processes that may insidiously and irreversibly reduce the renal function (i.e.interstitial fibrosis).

4.3.5. Immune system

Direct effects of xenobiotics can affect the immune system and can lead to decreased resistance to infections or tumours, may alter the course of autoimmunity or induce hypersensitivity reactions. Data of several immunotoxic agents (dioxin, polychlorinated biphenyls, immunotherapeutic drugs, etc.) are mainly derived from animal research (mouse and rat), although few biomarkers exist that provide specific information on the immunotoxicity in man [56,57].

The biomarkers proposed to assess immunotoxicity in man are listed in Table 4 [58] and include full blood count, antibody-mediated immunity (immunoglobulin concentrations in serum) phenotypic analysis of lymphocytes by flow cytometry, cellular immunity study, measurement of antibodies and markers of inflammatory response and finally, examination of non-specific immunity.

Although a variety of factors may modify the immune function including drugs (non-steroidal anti-inflammatory, vitamin complexes,etc.), biological parameters (sex, age, pregnancy) and other factors (diet, alcohol consumption, circadian rhythms, stress, nutritional state, sleep disturbances ,etc).

Within the field of Ecotoxicology, the resistance to infection in ducks exposed to organochloride pesticides has been studied measuring the cellular activities involved in the immune response; particularly the *in vitro* phagocytic capacity from kidney isolated macrophages in an number of species has been evaluated.

4.3.6. Biomarkers of DNA damage

At the present time many technological approaches permit the detection of covalent interactions of xenobiotics with proteins and other macromolecules. For example, several biomolecules (hemoglobin, serum albumin, etc.) have carboxyl, amino or sulfhydryl reacting groups that can interact with electrophilic compounds. Human DNA-adduct formation (covalent modification of DNA with chemical carcinogens) has

been shown to correlate with the incidence of a carcinogenic process and is a promising biomarker for elucidating the molecular epidemiology of cancer [59].

There is a sequence of events between the first interaction of a xenobiotic with DNA and consequent mutation. The first stage is the formation of adducts; the next stage, may be secondary modifications of DNA such as strand breakage or an increase in the rate of DNA repair. The third stage is reached when the structural perturbations in the DNA become fixed and the affected cells often shown altered function. One of the most widely used assays to measure chromosomal aberrations is the sister chromatid exchange (SCE). Finally, when the cells divide, damage caused by xenobiotics can lead to the DNA mutation and consequent alterations in the descent [3, 60].

Some examples of toxics capable to form human DNA adducts are given in Table 5, including polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic amines, micotoxins and alquilant chemotherapeutic agents [61-62]. The biologic monitoring to detection of human and animal DNA adducts include ^{32}P post-labelling and recently immunoassays using adduct-specific antibodies [63-64]. They can be detected in blood (lymphocytes), urine or tissue homogenates from biopsy (gastric mucosa, liver,etc.) although the study of DNA-adducts is not feasible in the routine analysis. The damage of DNA has also been studied in Ecotoxicology on several marine species (fish of fresh water, snapping turtle, etc.) that can be exposed to benzo [a] pyrene [3, 65-67].

Future investigations will focus on the implementation and design of studies to assess the association between DNA-adduct formation and cancer risk from toxic compounds. Whereas this association is strongly supported by animal studies, it remains to be ascertained whether adducts are also a necessary component of carcinogenesis in humans. Many studies are now being designed to correlate metabolic polymorphisms, urinary metabolites, chromosomal aberrations and protein and DNA-adducts and it is possible in the next future to obtain promising results from the combined use of these biomarkers in the evaluation of cancer risk.

4.3.7. Biomarkers of gene expression

The development of many tumours related with xenobiotics is associated with the aberrant expression of genes that encode proteins involved in cellular growth. This aberrant expression can involve a quantitative difference (overexpression of the protein)

and a qualitative difference (expression of a mutant form of the protein). Although these biomarkers are affected not only by toxic compounds, is very important to establish potential confounding factors and to assess of sensitivity, specificity and predictive value of these tests. The Table 6 shows the biomarkers of gene expression, which include [63,68,69]:

* growth factors
* oncoproteins: - growth factor receptors
  - other oncogene proteins
  - tumour suppresser gene proteins

These biomarkers have been studied in easily obtainable biological fluids such as serum, plasma, urine and bronchoalveolar liquid by enzyme-linked immunoadsorbent assay (ELISA), radioimmunoassay (RIA) or immunoblotting.

It has been reported that in subjects developing cancer, during the first stages of the disease, show a significant increase in those gene-expression biomarkers related with the specific cancer.

4.3.8. Biomarkers of oxidative damage

Contaminants such as polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons, heavy metals, selenium, pesticides and industrial solvents are capable of causing oxidative damage, especially by free radicals. In response to oxidative stress, there may be adaptive responses of the antioxidant systems, modification of cellular macromolecules and finally tissue damage.

Changes in the antioxidant systems and modified macromolecules can serve as biomarkers for a variety of xenobiotics. The protective systems included oxidised glutathione/reduced glutathione, glutathione reductase, catalase, superoxide dismutase and peroxidase activities, ascorbate and α-tocopherol. Macromolecules that may be affected by free-radical damage include lipids, proteins and nucleic acids [70-72].

4.3.9. Metallothioneins

Metallothioneins are small proteins with low molecular weight (approximately 61

amino acids in most mammals), cysteine-rich and capable of binding metals (Cd, Cu, Hg, Zn, Co, Bi, Ni and Ag). The cellular role of metallothioneins is complex and partially unknown; it is believed to protect cells against free heavy metal ion-induced damage. These proteins are implicated in metabolism regulation of Zn and Cu making these ions available to the cells as necessary, and may act as sulfhydryl-rich scavengers to prevent damage from stress-induced free radicals.

Metallothioneins have been proposed as biomarkers for exposure to metal ions since they are induced by the own metals . Nevertheless, recent studies indicate that they are also inducible by a variety of non-metal toxic agents (glucocorticoids) and physiological conditions (nutritional changes and pregnancy). Another limitation concern to analytical measurements. Often, methods of evaluating are slower and more expensive than analysis of metals themselves. The recent development of antibody-based methods and of messenger-RNA assays probably can make metallothionein a more valid bioindicator [58,73].

4.3.10. Others biomarkers

* *Heat stress proteins (HSP)*

Heat stress proteins are an important group of ATP-dependent proteins, which facilitate the folding of nascent proteins by preventing their aggregation and then chaperoning them to sites of membrane translocation. They were previously referred to as heat shock proteins because of their rapid appearance following heat stress, although they increased in response to a variety of xenobiotics, including metals and metalloids (especially, arsenite), heavy metals and oxidising agents [74-77]. Those biomarkers are of becoming importance in the next future.

Heat shock proteins include four types: hsp 90 (~ 90 kDa), hsp 70 (~ 70 kDa), hsp 60 (~ 60 kDa) y hsp_s of low molecular weight (~ 15-30 kDa):

* <u>hsp 90</u> (stress-90): there are two forms, the Hsp 83 is located in the eucaryotic cytosol and Grp 94 that is localised in the endoplasmic reticulum of mammals.

* <u>hsp 70</u> (stress-70): is the most widely studied and is located in the cytosol of mammals. It belongs to a multigene family with at least one form that is constitutively expressed (hsc 70) in unstressed cells, and one or more isoforms (hsp 70) that are only stress inducible. Both (hsp and hsc 70) help to refold denatured proteins that occur in

cells following heat shock or exposure to other proteotoxic stresses. Because these proteins show a very strong response to protein damage, they are ideal candidates for biomarkers of sub-lethal damage.

* hsp 60 (chaperonins 60): belongs to the family of chaperonins (cpn 60) involved in protein folding. They are localised in the mitochondria and may be useful as an organelle-specific biomarker.

* hsp_s of low molecular weight : they show substantial homology with α–crystallins of vertebrates, the may be involved in actin binding to stabilise microfilaments and have a good potential as biomarkers of effect on some targets as the cytoskeleton.

With the development of antibodies to some heat stress proteins, Western blotting is the best for evaluating heat stress proteins. Nevertheless, the heat stress protein responses have been studied only in experimental assays on laboratory species, culture systems or invertebrates.

** Eggshell thinning*

Severe eggshell thinning may be lead to breakage of eggs. This fact has been proposed as biomarker of reproductive damage. Dichloro-diphenyl-ethane (DDE), a metabolite of DDT, is the most widely studied in many species of birds (pelican, eagle,etc). The pathogenic mechanism is not yet unknown but could be related with hormone alterations that interfere in the calcium metabolism, which is essential in the formation of the eggshell.

Eggshell thickness may be estimated by two methods: one utilises direct measurement with a micrometer and the other calculates the thickness index as the weight of the shell (mg) divided by the product of shell length and breadth (mm^2) [3].

** Vitamin A, Thyroxine and Thyroxine-TBPA complex.*

Normaly, thyroxine is binding in plasma to transthyretin or TBPA (Thyroxine Binding Prealbumin) and RBP (Retinol binding protein)-vitamin A and form thyroxine-TBPA-RBP complex. Certain monohydroxymetabolites of 3,3',4,4' tetrachlorobiphenyl (OH-TCBs) formed by the monooxygenase system (Cytochrome P450IA1) compete strongly with thyroxine for its binding site upon the transthyretin. The consequence of the presence of these metabolites in blood is the quickly lost of thyroxine from

circulation and the appearance of symptoms associated with hypothyroidism. Also the binding of OH-TCBs to transtyretin can cause a conformational change which leads to a reduction in the attachment of the RBP to TBPA. RBP and attached retinol (vitamin A) is then lost from the blood by glomerular filtration (this complex has a low molecular weight). The consequences of this feature are the appearance of hypovitaminosis A (dermal and epithelial lesions [78]. These finding have been shown in laboratory studies with mice, rats and monkeys [79]. The increase of thyroxine and vitamin A in urine represent a biomarkers of TCBs exposition.

* *Biomarkers for anticoagulant rodenticides*

Warfarin and second generation anticoagulant rodenticides (flocoumafen) act as inhibitors of the vitamin K cycle, which operates in the liver of vertebrates. These compounds act by competing with vitamin K or derivatives and the consequence of this antagonism is a failure of the vitamin K cycle to carboxylate the precursors of clotting proteins. This leads to an extension of blood clotting time, haemorrhaging and death. The effects of anticoagulant rodenticides may be detected at two different levels: monitoring changes in the vitamin K cycle or monitoring increases in precursors of clotting proteins in blood. The development of ELISA assays in the last years have allowed the detection of forerunners of clotting proteins in mammals and birds [3].

* *Plant biomarkers*

Plants have widely been used as biomonitors to analyse the environmental impact of pollutants (especially gaseous air pollutants). Specific biomarkers have been identified in sensitive plants. In a few cases, it is known that excess of a specific compound will give rise to the production of a metabolite, which is different between tolerant and sensitive plants.

For example, in the presence of an excess of selenium, Se-sensitive plants fail to differentiate between S and Se and incorporate Se in sulphur amino acids (essentially, selenomethionine and selenocysteine) leading to the synthesis of enzymes of lower activity (selenoproteins), which can lead to plant death. On the contrary, Se-tolerant plants biosynthesise and accumulate non-protein seleno-amino acids (such as selenocystathioneine and Se-methylselenocysteine) which do not cause metabolic

problems for the plant. Thus, the occurrence of selenoproteins in plants are excellent biomarkers, although their use has not been widely reported.

Another example is the synthesis of fluorocitrate after an exposure to an excess of fluorine. The plants synthesise fluoroacetyl-CoA and then convert it, via the tricarboxylic acid cycle to fluorocitrate. This compound is not recognised by aconitase and as a result the fluorocitrate is accumulated being a very reliable biomarker for fluorine poisoning.

Phytochelatins are proteins plenty of sulfhydryl groups and synthesised during exposure to a heavy metal and anions as SeO_4^{2-}, SeO_3^{2-} y AsO_4^{3-} . Dose and time dependent relationships have been established under laboratory conditions for cadmium, copper and zinc. Nevertheless, for biological monitoring more research is needed.

Plant biomarkers respond to a wide variety of environmental compounds and may be useful to indicate a hazard to plant life. For example, the activity of the enzyme peroxidase has been used to biomarker exposure of plants to air pollution, especially SO_2. Changes of enzyme systems during the development of the plant (seasonal and climatic processes) are not yet well enough known and plant biomarkers are not as well advanced as animal end point though a good future in the ecotoxicology field has been predicted [3].

In conclusion, the markers of biological toxicity represent an important tool in Toxicology by three main reasons:

1) they permit to estimate the biological effect on target tissue;

2) they are markers of subclinic alterations and sensible indicators of pathology and thus, may be useful in diagnostic and preventive strategies;

3) the biomarkers consider inter- and intra-individual variability in the response to xenobiotics.

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Table 1. Biomarkers listed in order of decreasing specificity to xenobiotics [3]

Biomarker	Xenobiotic
Inhibition of ALAD	Lead
Inhibition of AchE	Organophosphorus Compounds (OPIs) and Carbamates
Induction of metallothionein	Metals (cadmium)
Eggshell thinning	DDT, DDE, Dicofol
Porphyrin	Organochlorines Compounds (OCIs)
Heat shock proteins	Metals and OCIs.
Immune response	Metals, OCIs and polycyclic aromatic hydrocarbons (PAHs)
DNA and hemeglobin adducts	HAPs, nitrosamine, aromatic amines,chemoterapeutic agents

Table 2. Examples of selective exposure biomarkers

I. INORGANIC COMPOUNDS

Cadmium	Cd	urine	< 2 µg/g creat.
	Cd	blood	< 0.5 µg/100 ml
Mercury	Hg	urine	< 5 µg/g creat.
	Hg	blood	< 1 µg/100 ml
Lead	Pb	blood	< 30 µg/100 ml
	Pb	urine	< 50 µg/g creat.
	Pb (after 1g EDTA)	urine	< 600 µg/24 h
Cinc	Zn	urine	< 0.7 mg/g creat.
	Zn	serum	< 15 µg/L

II. ORGANIC COMPOUNDS

n-Hexane	2-hexanol	urine	0.2 mg/g creat.
	2,5-hexanodione	urine	2 mg/g creat.
Benzene	phenol	urine	< 20 mg/g creat.
	benzene	blood	< 5 µg/100 ml
	benzene	exhaled air	< 0,022 ppm
Styrene	mandelic acid	urine	1g /g creat.
	phenylglioxilic acid	urine	350 mg/g creat.
	styrene	blood	0.055 mg/100 ml
	styrene	exhaled air	18 ppm
Aniline	aniline	urine	0.75 mg/g creat.
	p- aminophenol	urine	10 mg/g creat.
	metahemeglobin	blood	< 2 %
Ethylenglicol	oxalic acid	urine	< 50 mg/g creat.
M-n-butylcetone	2,5 hexanodione	urine	4 mg/g creat.
Acetone	acetone	urine	< 2 mg/g creat.
	acetone	blood	< 0.2 mg/100 ml

Table 3. Biomarkers of renal effects [44]

Serum	
* *Markers of glomerular filtration*	Cretainine, β_2-microglobulin
* *Markers of the glomerular basal membrane (GBM) integrity*	Laminin and anti-GBM antibodies
Urine	
* Plasma-derived proteins	
High molecular weight	Albumin, transferrin
Low molecular weight	β_2-microglobulin, retinol binding protein, α_1-microglobulin, Clara Cell protein, α-amilase
Kidmey-derived components	
Enzymes	Gluathione-*S*-transferase, β-N-Acetylglucosaminidase
Antigens	
* Glomerulus	Fibronectin, laminin
* Proximal tubule	Brush border antigens (alkaline phosphatase)
* Loop of Henle	Tamm-Horsfall glycoprotein
Others	Glycosaminoglycans, prostanoids

Table 4. Biological markers of immunotoxicity in humans [58]

A) Full blood count (include the lymphocytes count).

B) Study of antibody-mediated immunity:

- immunoglobulin concentrations in serum (IgM, IgG, IgA, IgE)

C) Phenotypic analysis of lymphocytes by flow cytometry:

- surface markers (CD3, CD4, CD8, CD20, CD23, etc)

D) Study of cellular immunity:

- delayed-type hypersensitivity on skin
- natural immunity to blood group antigens (anti-A, anti-B)

E) Autoantibodies and markers of inflammatory response:

- C-reactive protein
- Autoantibodies to nuclei, DNA and mitochondria

F) Measure of non-specific immunity:

- Interleukines analysis (ELISA or RT/PCR)
- Natural Killer cell activity (CD56 or CD60)
- Phagocytosis (chemiluminescence)
- Measurement of complement components

Table 5. Xenobiotics capables of human DNA-adducts formation

***N-* nitrosamines**	4-(N-nitrosomethylamino-1-(3-pyridyl)-1-butanone (NNK) N-nitrosodimethylamine (NDMA) Diethylnitrosamine (DEN)

Polycyclic Aromatic Hidrocarbons	Benzo-a-pyrene (BP) 7,12 Dimethylbenzo[a] antracene (DMBA)
Aromatic Amines	2- acetylaminophluorane (2-AAF) 4- aminobiphenyl (4-ABP) 4- iminobiphenyl (4-IBP)
Heterocyclyc Amines	2-amino-3,8-dimethylimidazo-quinoxaline
Micotoxins	Aflatoxin B_1 Ochratoxin A
Chemotherapeutic Agents	Cisplatin Mitomycin C Procarbazine Dacarbazine 8-methoxypsoralen
Others	Ulltraviolet light Oxidative damage Malondialdehyde (endogenous)

Table 6. Biomarkers of gene expression [69]

I) Growth factors

*** *Platelet-derived (PDGF)*:** breast cancer, various carcinomas, sarcomas, lynphomas, lung fibrosis, pneumoconiosis, atherosclerosis.

*** *Transforming α (TGFα)*:** breast cancer, various carcinomas and pneumoconiosis.

*** *Transforming β (TGFβ)*:** liver cancer, bladder cancer, breast cancer, leukemia, liver

and lung fibrosis and pneumoconiosis.

* ***Fibroblast (bFGF*)**: kidney cancer, bladder cancer and others carcinomas.

* ***Epidermal (EGF)***: stomach and ovarian cancer.

* ***Insulin-like (IGF):*** bladder cancer, ovarian cancer, hepatitis and cirrhosis.

* ***Hepatocytes (HGF)***: liver cancer, hepatitis and cirrhosis.

II) ONCOPROTEINS

II.a) Growth factor receptors:

* ***Transmembrane growth factor receptors*** *(encoded by the erbB-2 oncogene)* : bladder cancer, ovarian cancer, liver cancer, lung cancer and others.

* ***Epidermal growth factor receptor -EGFR-*** *(encoded by the c-erB-1 oncogene)*: lung cancer and other carcinomas.

II.b) Oncogene proteins:

* ***membrane-associated G proteins or p21 [21kDa]*** *(encoded by the ras oncogene)*: lung cancer, colon cancer, liver angiosarcoma and others.

* ***nuclear DNA-binding protein [64 kDa] or p54 [54 kDa]*** *(encoded by the myc oncogene)*: lung cancer, colon cancer, bladder cancer.

II.c) Tumor suppressor gene proteins*:*
* ***nuclear phosphoprotein p53 [53 kDa]*** *(encoded by the suppressor tumour gene p53):* liver cancer, breast cancer, lung cancer colon cancer and lymphoma.