

Mechanistic Biomarkers in Toxicology (I)

Toksikolojide Mekanistik Biyogöstergeler (I)

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ÖZ

Biyogöstergeler önemli, güvenilir, uygulanabilir, tekrarlanabilir ve genellikle pahalı olmayan parametrelerdir. Biyogöstergelerin insan sağlığı açısından önemli rolü olmakla birlikte, özellikle mekanistik biyogöstergelerin toksik etkilerin belirlenmesinde ve hastalıklardan korunmada önemi büyüktür. Biyogöstergeler hastalıkların tanısını, gidişatını, tekrarlanma ve yayılma olasılığını, tedavinin etkinliğini gösterebilir. İlaveten birçok kimyasal, biyolojik ve fiziksel ajanlara maruz kalma düzeyini gösterir.

Bugün, biyogöstergelerin geliştirilmesi ve uygulanması için oluşumlarının altında yatan mekanizma bilgilerinin bilinmesi gerektirir. Bu nedenle bu derlemede olası mekanistik biyogöstergeler üzerinde odaklanılmıştır.

Anahtar Kelimeler: Mekanistik, Biyogöstergeler, Toksikoloji

ABSTRACT

The important parameters which are reliable, applicable, reproducible, and generally inexpensive are biomarkers. However, all biomarkers have a significant role in human health, especially mechanistic biomarkers, which are the most important for the prevention of toxic effects and disease. They demonstrate the possibility of diagnosis, prognosis, recurrence, and spreading of disease; furthermore, they show exposure level to many chemical, biological and physical agents.

Today, developing and applying biomarkers requires knowledge of mechanisms underlying their production. Therefore, in the present review, it has been focused on possible mechanistic biomarkers.

Keywords: Mechanistic, Biomarker, Toxicology

Introduction

The National Institutes of Health (NIH) has defined the biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes or pharmacological responses to a therapeutic agent."^{1,2} Parameters like glomerular filtration rate or recording blood pressure at different time intervals are examples of biomarkers.

Generations of epidemiologists, physicians, and scientists have used various biomarkers to

study human disease. Biomarkers have been used in the management and diagnosis of cardiovascular disease, infections, genetic disorders, cancer, and many other diseases.³ The time course of injury and underlying molecular mechanisms are reflected by measuring biomarkers. Accurate diagnosis, prognosis, and treatment regime can be applied to the patient by analysing different biomarkers.² Periodic surveillance of biomarkers also serves as a tool to conclude if the treatment protocol or daily dietary habits are improving the patient's condition.² Periodic follow-up of biomarkers will provide the health care personnel with important information about efficacy or toxicity of the treatment regime and act as a border for clinical trials, with the final goal of treating the patients with safe and effective medical therapies.²

Biomarkers are generally classified as biomarkers of (i) exposure, (ii) effect, and (iii) susceptibility.^{2,4} The exposure biomarker is considered an early marker, which is a result of an interaction between a chemical agent and a target molecule.⁴ Therefore, these biomarkers are essential and valuable in collaboration with biomarkers of early disease detection in order to develop personalized medical treatment strategies.² The biomarker of effect is considered as a late marker. It is used for measuring the burden of injury or damage caused by different agents on the target organ. These biomarkers objectively and accurately measure the overall health status of the patient, usually after being exposed to an agent or disease.⁴ Susceptibility biomarker is used as a guide to inherent or gained ability of the body to respond to difficulties. It is due to being exposed to a disease or chemicals.^{4,5}

Biomarker development requires detailed and considerable knowledge. A growing number of researches about the pathogenesis of the disease, molecular changes, and alterations in biochemical pathways underlying toxic effects should be done. This mechanistic information causes the formation of mechanistic biomarkers. Mechanistic biomarkers cover exposure, effect, susceptibility biomarkers, and leads to the generation of new ones.^{6,7} Therefore, they have the highest potential for assisting with clinical decision making. The best example of a mechanistic marker is a genetic trait index, which is commonly used for the diagnosis of certain diseases. Mechanistic biomarkers provide information regarding the prognosis of the patient and the probability of response to various treatment options; however, it is not used for following-up of progression or response to medical therapy that is applied.^{6,8} It is important to note that many biochemical processes such as oxidative stress, alterations in biotransformation, alteration in protective and repairing systems, and organelle damage are the mechanistic information that leads to detectable biomarkers.

Oxidative Stress Biomarkers

In 1985 Helmut Sies defined "oxidative stress as a disturbance in the prooxidant-antioxidant balance in favour of the former."⁹ Oxidants are mostly produced by cellular metabolism. The antioxidant system of the body quickly eliminates small amounts of the oxidants, but, in some cases, their damage to macromolecules (proteins, lipids, DNA, and carbohydrates) are too profound (Figure 1). Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radicals, hydroxyl radicals are common by-products of metabolic activities.^{2,10} ROS are synthesized during mitochondrial respiration, inflammation, immune system activity, and many other activities.^{2,11-13} In addition, the increased oxidative stress level increases the production of ROS through Fenton reaction (reduction of iron by superoxide).^{2,11,14} Excess amounts of ROS interfere with the physiological activity of mitochondria and result in adenosine triphosphate (ATP) depletion. An increase in oxidative stress levels has been associated with numerous diseases or toxicities. There is a substantial amount of evidence revealing the association between oxidative stress and different diseases such as cancer, diabetes, infections, cardiovascular and neurodegenerative diseases, and the aging process (Figure 2).^{2,12,15}

Markers of oxidative stress are used to evaluate the nature and effect of reactive oxygen species. Measuring ROS may be a useful marker, but it is not stable; detecting it requires invasive methods, and the results may lack specificity. Thus, scientists measure the by-products of the reaction of ROS with other biomolecules that are more stable. Some of the surrogate markers are nitrite and nitrate levels, products of lipid peroxidation, and levels of oxidized proteins.¹¹ Figure 1 shows the effect of ROS on macromolecules also some of the end products.

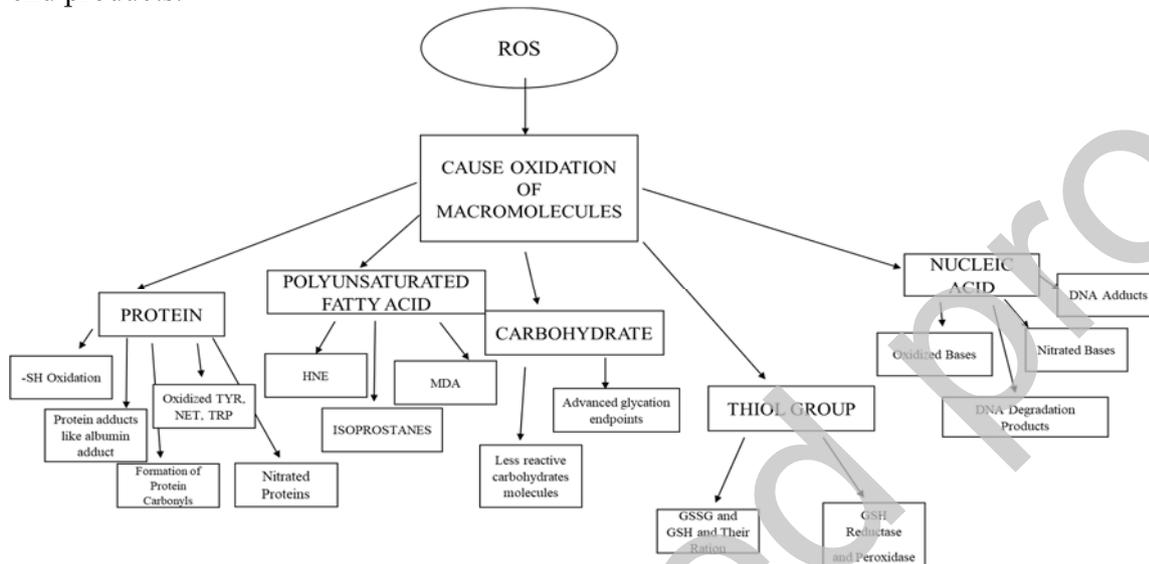


Figure 1: Reactive oxygen species (ROS) cause oxidation of macromolecules. As a result of this oxidation, the end products of the oxidation process has been mentioned. These end products are being used as a biomarker to detect the presence of oxidative damages(drawn by authors)

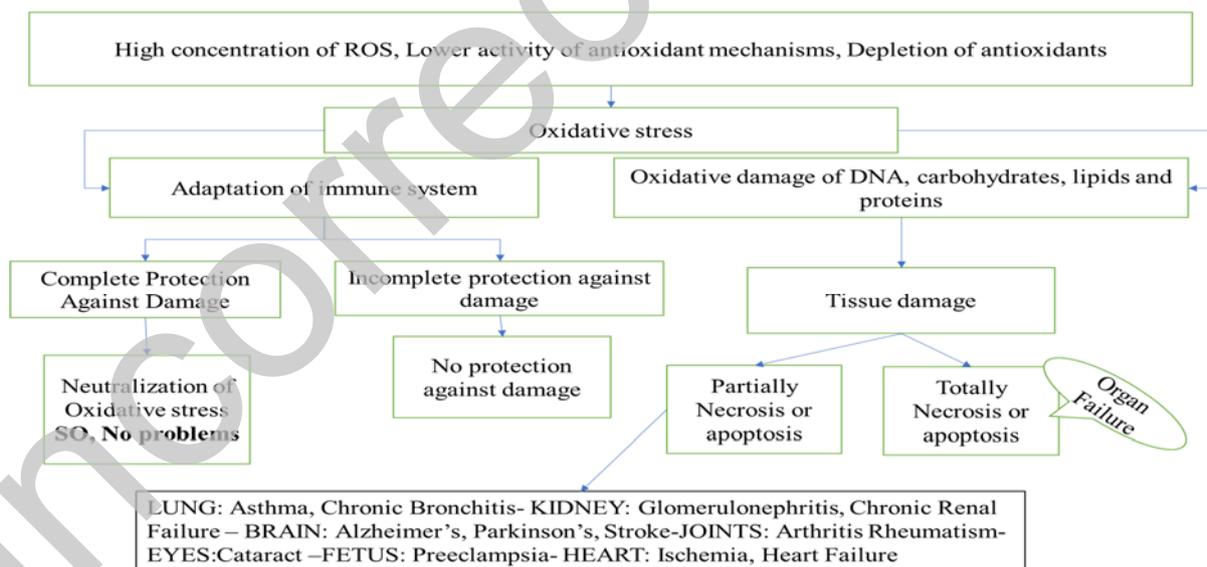


Figure 2: Oxidative stress in general pathogenesis of the disease(drawn by authors)

Lipid peroxidation is a cascade of reactions due to ROS attack on lipids in the cell membrane and has been implicated in various diseases such as hypertension, Alzheimer's disease, cancers, and many other disorders.¹⁶⁻¹⁹ The burden of lipid peroxidation can be measured by analysing thiobarbituric acid, N-epsilon-hexanoyl-lysine, malondialdehyde (MDA), 4-hydroxy-nominal (HNE) and F2-isoprostane 15(S)-8-iso-prostaglandin F2α (15(S)-8-iso-PGF2α) which are the by-products of lipid peroxidation.¹⁷⁻²⁰

Antioxidants

The human body is equipped with different antioxidant systems that serve as a counterbalance to the effect of oxidants. The antioxidant defence involves several strategies; in explanation, the enzymatic and non-enzymatic mechanisms. The enzymatic mechanisms such as superoxide dismutase and non-enzymatic defence systems protect the cells against free radicals and ROS. Antioxidants like alpha-tocopherol scavenge the oxidants (which damage cell membranes and cause lipid peroxidation) or ascorbate trap ROS.¹⁵

Glutathione (GSH) is a three-peptide molecule that contains cysteine, glycine, and glutamate, and it is the most critical molecule of the antioxidant system. GSH plays a significant role in detoxification of aggressive electrophilic molecules such as radicals, epoxides, and halides by conjugation reactions. GSH is the major thiol in the body and a perfect reductant molecule, which prevents oxidative damage.²¹ The ratio of reduced to oxidized GSH indicate the redox balance of the cell. This redox balance is an indicator of the overall health of the cell.²²

Dysregulations in GSH synthesis and its concentration are considered an important biomarker in the diagnosis of diseases such as HIV, cancer, inflammation, tuberculosis, Alzheimer's Disease, and many others.²³⁻²⁵ Evaluation of the GSH pathway will reflect the status of the antioxidant system, which may elucidate underlying various pathology aetiologies. Among the enzymes that have a role in the antioxidant system, glutathione peroxidases (GSH-Pxs) consist of 4 enzymes^{26,27} (Table 1), all of which contain selenium. They are hydrogen and lipid peroxide scavengers. The hydrogen peroxide is produced during the metabolic process in the cell; also, its amount increases in oxidative stress.¹⁵

Table 1: Different GPX enzymes

The Enzyme	Location
Glutathione Peroxidases I, neutralizes hydrogen peroxide and protects hemoglobin from oxidative damage. ^{28,29}	Cell cytosol
Glutathione Peroxidases II; this isoenzyme level increases in different cancers such as prostate, hepatocellular, and breast cancers. ^{29,30}	Cell cytosol, especially in the gastrointestinal tract
Glutathione Peroxidases III; It is a glycoprotein ^{29,30}	Plasma
Glutathione Peroxidases IV; It is activated in case of free radical damage, serum cholesterol, and lipoproteins ²⁹	Mitochondria

Another essential enzyme in the antioxidant system is superoxide dismutase (SODs), which eliminates superoxide radicals.³¹ There are 3 different metalloenzyme forms of SODs; the cytoplasmic Cu/Zn-SOD, the mitochondrial Mn-SOD, and the extracellular EC-SOD, all of which require cofactors (Cu or Mn) for their activity.^{32,33} As a result of the neutralization of superoxide, hydrogen peroxide is formed. Catalase and glutathione peroxidase enzymes then

catalyze this H₂O₂. Since all these enzymes depend on each other, fluctuations in their levels will affect the overall antioxidant system.^{15,34}

Biomarkers Related to Biotransformation

Biotransformation is the process of enzymatic transformation of xenobiotics to the metabolites, which can be excreted. However, in some cases, the metabolites may be toxic and reactive electrophiles. These toxic metabolites lead to cell damage or cell death. Measurement of active metabolites (like measuring morphine as the active metabolite of codeine biotransformation), determining the effect of reactive metabolite on macromolecules and analysing the end product (like measuring mercapturic acid or hippuric acid in urine samples), measurement of enzyme activity, which is responsible for xenobiotics metabolism, are some of the biomarkers related to biotransformation.²

The effect of xenobiotics or toxins is dependent on their metabolism, which is controlled by the action of enzymes. Any modification in the activity of these enzymes results in a change of xenobiotics fate. Metabolizing can be altered by enzyme induction or inhibition. Enzyme induction or inhibition has been studied as a biomarker for measuring responses to environmental pollutants, exposure to various drugs, or even drug interactions.³⁵ For example, chronic alcohol usage results in the induction of the 2E1 enzyme. The induction of this enzyme will alter the fate of some drugs that are metabolized by it.³⁶ Organophosphate pesticides reversibly or irreversibly bind to cholinesterase enzyme and inhibit it. This inhibition prevents neurotransmitter (acetylcholine) degradation.³⁷ Quinidine is a potent CYP2D6 inhibitor.³⁸ In polypharmacy, inhibition or induction of enzymes is very important. The first or second drug interferes with the other drug's biotransformation, and the outcome is either toxicity or no therapeutic activity.³⁹

Differences in genetic traits that cause a difference in expression and the activity of enzymes are the primary source of susceptibility to various diseases. The mutations and alterations in genes can be detected in 1% of populations, and it is called genetic polymorphism.

Polymorphisms in phase I and phase II biotransformation enzymes or DNA repair enzymes can be a biomarker. Polymorphism of glutathione S-transferase (GST), N-acetyl transferase (NAT) and CYP1A2, 2A6, 2D6, 2E1 has been studied² in various conditions, as an example polymorphism in CYP2C9 causes the patient to need-less doses of warfarin so that patient will be susceptible to increased risk of bleeding and in case of CYP2C19, increased risk of anticonvulsant side effects.⁴⁰ People who are CYP2D6 polymorphic need higher doses of fluoxetine to show the same plasma levels as those have normal CYP2D6.⁴¹

Furthermore, protein expression and function can be altered as a result of molecular response to signals, post-translational modifications, and many other factors.⁴⁰ Additionally, measuring the ratio of the parent compound to the metabolite (metabolite ratio) is considered an applicable and practicable biomarker. Measuring metabolic ratio is a valuable indicator of the metabolism rate. If the ratio is high, the patient is a poor metabolizer and vice versa. Codeine is converted to morphine by CYP2D6 in order to show its analgesic effect. However, in poor metabolizers, codeine is not a good analgesic.⁴²

DNA

DNA damage is a sign of several disorders such as colon cancer, chronic renal damage, and aging-related problems.^{43,44}

DNA damage can be caused by endogenous agents such as various metabolic by-products, and environmental factors such as ultraviolet and ionizing radiation.^{45,46} Reactive oxygen species are source DNA damage too. The degree of DNA damage can be used as a biomarker to assess oxidative stress in various conditions such as pancreatic and mammary cancers and the damage from ionizing radiation, which is used for radiotherapy of cancer patients.⁴⁶

Certain compounds undergo bioactivation reactions that result in the production of potentially carcinogenic metabolites. These metabolites are carcinogenic because they react with the

DNA and make DNA adducts. For example, metabolism of benzo (alpha) pyrene results in the formation of cation radical, this radical form DNA adducts, in exposure to tobacco smoke or coal.⁴⁷ Also, reactive oxygen and nitrogen species can directly interact with DNA.⁴⁸ This interaction causes oxidation of DNA and produces DNA adducts. The most important of these oxidative DNA adducts are 8-hydroxydeoxyguanosine (8-OHdG), thymine glycol, hydroxymethyl uracil, 8-hydroxydeoxyadenine, and formylamidopyrimidine. Measurement of oxidized bases from the urine sample is good indicators of the oxidative damage of the nuclear DNA that takes part in the carcinogenesis process and are important prognostic factors for some cancers.⁴⁸

Damage to mitochondrial DNA is mainly due to oxidative stress damage. The DNA repair systems are incomplete in the mitochondria, which causes increased susceptibility and mitochondrial dysfunction. Therefore, this damage directly interferes with oxidative phosphorylation and results in the induction of apoptosis and cell death. Increased levels of 8-OHdG in biological samples can be a surrogate marker for mitochondrial DNA damage.⁴⁸ DNA repair systems have an important role in repairing DNA damage at first sight. If the DNA repair systems are defective or overwhelmed, the risk of cancer and various diseases related to aging increases.⁴⁸

Cofactors: NAD⁺ and NADP⁺

Cofactors mediate a wide range of biological reactions. Nicotinamide adenine dinucleotide [NAD/ reduced form is NAD(H)], nicotinamide adenine dinucleotide phosphate [NADP/ and its reduced form is NADP(H)] and adenosine triphosphate (ATP) are the ones that are important mechanistic biomarkers. NAD⁺ was first discovered in 1906.⁴⁹⁻⁵² NAD⁺ and NADH, play an important role in many metabolic processes such as glycolysis, mitochondrial oxidative phosphorylation, oxidation of fatty acids, citric acid cycle, and many other oxidation-reduction (redox) reactions.⁵¹⁻⁵³ Its effects show circadian change.^{50,51}

Fluctuations in the NAD⁺ level have a significant effect on cell function and metabolism. As shown in Figure 3, NAD⁺ as co-substrate for 3 important enzymatic activity (sirtuin, poly (ADP- ribose) polymerases (PARPs) and redox enzymes) has gained attention recently. CD 38/CD157 are ectoenzymes that consume mitochondrial NAD⁺ and degrade it to cyclic ADP ribose and nicotinamide (NAM). CD38 activity increases with age, resulting in increased NAD⁺ consumption and depletion of NAD⁺ reserves. Overexpression of CD38 in chronic inflammation and chronic lymphocytic leukaemia and mitochondrial NAD⁺ is depleted in these diseases. PARPs are playing a role in epigenetics, repair of DNA, and chronic inflammation. An increase in expression of PARPs results in NAD⁺ consumption and reduction of the NAD⁺ pool. Sirtuin is important as a factor increasing the life span of cells. Sirtuin pool decreases by aging along with the NAD⁺ pool. Thus, increasing the NAD⁺ pool enhances the lifecycle of the cells.⁵⁴ Sirtuin acts as a tumour suppressor by regulating transcription, programming metabolic pathways of the cells, and increasing cell resistance against oxidative stress.^{51,52} Through these enzymes, NAD⁺ affects energy balance, stress response, and cellular homeostasis.^{50,54,55} Fluctuations in NAD⁺ levels result in fluctuations in protein levels, which are dependent on NAD⁺, and as a result, these proteins are significant in carcinogenesis.⁵¹

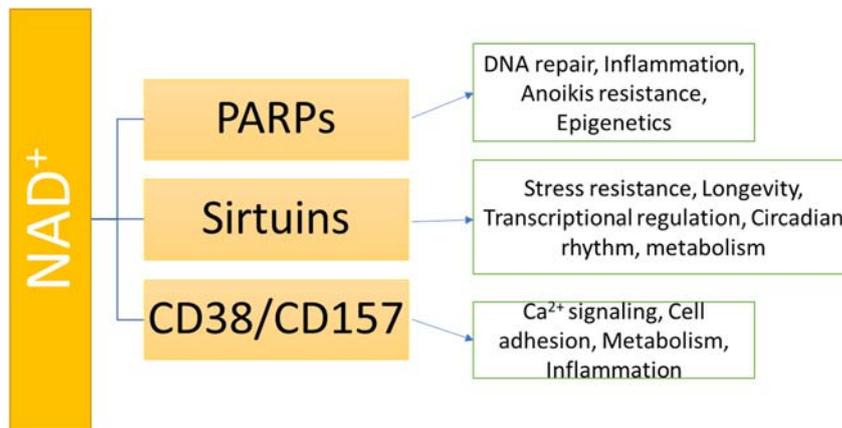


Figure 3: NAD⁺ is a co-enzyme for the function of PARPs, sirtuins, and cyclic ADP-ribose synthases (CD38/CD157). Fluctuations in the NAD⁺ level affect the biological processes which are dependent on these enzymes.⁵¹

Maybe increasing NAD⁺ levels reduces the risk of cancer, but this increase leads to increased activity of PARPs enzymes. The PARPs help to protect and repair DNA, even in cancer cells. PARPs cause overexpression of inflammatory genes, which are responsible for the increased incidence of hormone-dependent tumours.⁵¹ Sirtuin is more sensitive to fluctuation in NAD⁺ levels. Different sirtuin isoforms act as a tumour suppressor by altering transcription and rescheduling cell metabolic activity.⁵¹

Aging is an essential factor in decreasing NAD⁺ synthesis. Aging means being susceptible to chronic inflammation, circadian rhythm changes, and fluctuations in microRNA gene expression. All those factors mentioned above decrease nicotinamide phosphoribosyltransferase (Nampt) activity, which is an important enzyme in NAD⁺ synthesis. Nampt is a rate-limiting enzyme in the NAD⁺ salvage biosynthesis pathway from nicotinamide. Decreased Nampt activity results in reduced synthesis of NAD⁺, increased NAD⁺ degradation, and higher risk for age-related diseases.⁵⁰

Nicotinamide adenine dinucleotide phosphate (NADP⁺), is being formed by the addition of phosphate to NAD⁺. NADP⁺ and NADPH are critical cofactors, fighting against oxidative stress and play a role in the synthesis of nucleic acid, fatty acids, and cholesterol.⁵³⁻⁵⁵

Thus, these redox couples act as a substrate for the majority of enzymes. They play an active part in cellular redox homeostasis. The deficiency of any of them disrupts this homeostasis, which results in oxidative stress, the onset of disease, and energy impairment.

Polyamines: Ornithine Decarboxylase

Polyamines are small, cationic amines derived from amino acids. They are required for healthy cell growth; however, they involve in cancer cell proliferation as well.^{1,56-59}

Putrescine, spermidine, and spermine are three main polyamines in eukaryotes and prokaryotes.^{56,58} Dietary polyamines or endogenous polyamines produced by the gut microbiota and those that are synthesized in the cytoplasm are the chief sources for all cells and tissues.^{57,60} Since they are significant for cell function, their levels are strictly regulated by maintaining a balance between synthesis, degradation, and uptake. Ornithine decarboxylase (ODC) plays a critical role in the biosynthesis of polyamines. Increased levels of ODC enzyme in blood has been reported in regenerating tissues and also in cancer.^{56,57}

Along with chemical cancer promoters, which result in ODC increase, some environmental and genetic factors like ultraviolet light can result in increased ODC gene expression. ODC levels have been reported to increase in skin, lung, and prostate cancers.^{56,57,60-62}

S-adenosylmethionine is another enzyme in polyamine synthesis which forms acetylated polyamines. Both the parent polyamines and the acetylated derivatives (e.g., N₁-acetyl spermidine, N₈-acetylspermidine, N₁-acetylspermine, and N₁, N₁₂-diacetylspermine) can be detected in urine and have been associated with cancer.⁶⁰

Tumour cells with high polyamine production, show an increased synthesis of proteinases and cathepsins, which destroy the surrounding tissue. These also induce hypoxia, which results in higher uptake of polyamines by cells and results in increased proliferation rate.^{56,58}

Pteridine pathway: Folate and Neopterin

Pteridines are bicyclic nitrogenous ring system pyrazino-(2,3-d)-pyrimidine derivatives, which bears small substituents such as neopterin and biopterin and are called unconjugated pteridines. The derivatives with a larger residue like folic acid and riboflavin are called conjugated pteridines.⁶³

Some of the crucial cellular mechanisms depend on folate as the source of one-carbon in DNA synthesis and methylation of protein. Thus, folate plays a significant role in DNA synthesis.⁶⁴⁻⁶⁸ Dihydrofolate reductase and thymidylate synthase enzymes have been used as targets chemotherapy; thus, this makes conjugated pteridines a good candidate as biomarkers.⁶⁹ Folate deficiency leads to different disorders and diseases.⁷⁰

Among folate derivatives, 5-methyltetrahydrofolate (5-methyl THF) is found in circulation, which acts as a co-substrate in the conversion of homocysteine to methionine, as shown in Figure 4. DNA mutations and strand breakage can also be the result of an increase in the replacement of uracil instead of thymidine. These are because 5,10-methyl THF decreased.^{66,71} Moreover, decreased levels of 5-methyl THF will lead to reduced levels of s-adenosylmethionine, which will cause an activation of oncogenesis and increased DNA damage.⁷²⁻⁷⁴ For this reason, folate level can be a useful biomarker in predicting or diagnosing cancer.^{64,66}

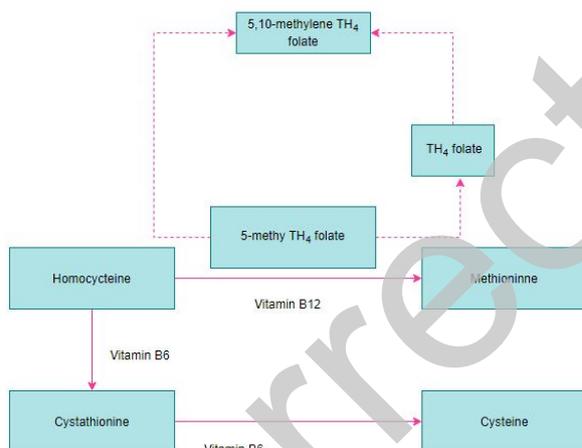


Figure 4: Conversion of homocysteine to methionine.⁷⁵

The relationship between folate and cancer is directly related to its dosage. Little doses of the folate increase the risk of cancer; on the other hand, higher doses of folate will reversely inhibit dihydrofolate reductase enzyme. Another risk factor for carcinogenesis is the circulating unreduced form of folate. Antifolate medications have been used widely in cancer therapy to inhibit the single-carbon metabolism, which is necessary for cell proliferation in cancerous tissue.^{66,70} Other agents like chronic alcohol usage,⁷⁶ antacids,⁷⁷ general anesthetics⁷⁸ cause depletion or alteration in folate level.

Additionally, diseases such as Crohn's Disease, celiac disease, and some kind of cancers result in folate depletion. Evaluating the folate levels are important in patients who have been on diuretic therapy for long terms like furosemide and amiloride. These medications cause an increase in folate elimination.⁷⁰

Measuring the folate levels, even in the process of testing new therapeutic agents considered a vital biomarker because of its essential role in DNA biosynthesis and red blood cell synthesis. The depletion of folate levels increases the rate of cardiovascular and neuronal disorders.⁷²

Unconjugated pteridines and their derivatives have a role as intermediates in the metabolism, and their biological concentrations have been shown changed in various disease processes. It has been shown that unconjugated pteridines could be measured in serum, cerebrospinal fluid, and urine.⁷⁹ Neopterin, as an unconjugated pteridine, has been one of the early biomarkers for cancer, systemic diseases, infectious and/or inflammatory diseases such as HIV, rheumatoid arthritis, Behçet disease, and acute myocardial infarction. Neopterin has gained its popularity among scientists because it is highly fluorescent, and it can be synthesized easily by gamma interferon activated macrophages and monocytes.^{11,80,81} 7,8-dihydroneopterin, a form of neopterin produced by macrophages, acts as a radical scavenger and inhibits free radicals that are formed during lipid and protein oxidation. 7,8-dihydroneopterin is hydroxyl, superoxide and peroxy scavenger. Surveillance of the neopterin in body samples is a good indicator of the levels of free radicals in tissues and cells.⁸² It has also been reported that the Austria government have been using neopterin screening test in donated blood to be sure about their safety.⁸³ It predicts the patients' inflammatory status.⁸⁴⁻⁸⁶

Conclusion

Generally, biomarkers are being used to measure the response of the biological systems. In the field of toxicology, biomarkers are practical tools to understand mechanisms of toxicity. They are also useful in risk management and assessment. In the toxicological aspect, it is a known fact that biomarkers play an important role in the prevention and reduction of harmful effects of different chemicals and agents. Mechanistic biomarkers have been used as a tool in diagnosis, treatment, and monitoring course of treatment of different disease like cancers, Alzheimer's Disease, immunological disorders, and many other pathologies. In conclusion, reliable and applicable proper biomarkers that are in accord with ethical rules are beneficial for human health; nevertheless, there is still a need for further research to define ideal biomarkers for different fields of life sciences.

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