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REVIEW

Mechanistic Biomarkers in Toxicology

Toksikolojide Mekanistik Biyobelirteçler

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ABSTRACT

Biomarkers are important parameters that are reliable, applicable, reproducible, and generally inexpensive. All biomarkers have a significant role in human health, especially mechanistic biomarkers, which are the most important for the prevention of toxic effects and diseases. They demonstrate the possibility of diagnosis, prognosis, recurrence, and spread of disease. Furthermore, they show the exposure levels to numerous chemical, biological, and physical agents. To date, the development and application of biomarkers require the knowledge of mechanisms underlying their production. Therefore, the present study focused on the possible mechanistic biomarkers.

Key words: Mechanistic, biomarker, toxicology

ÖZ

Biyobelirteçler önemli, güvenilir, uygulanabilir, tekrarlanabilir ve genelikle maliyeti uygun parametrelerdir. Biyobelirteçlerin insan sağlığı açısından önemli rolü olmakla birlikte özellikle mekanistik biyobelirteçlerin toksik etki ve hastalıklardan korunmada önemi büyüktür. Biyobelirteçler hastalıkların tanısını, gidişatını, tekrarlama ve yayılma olasılığını, tedavinin etkinliğini gösterebilir. İlave olarak birçok kimyasal, biyolojik ve fiziksel ajanlara maruz kalma düzeyini gösterir. Bugün biyobelirteç geliştirilmesi ve uygulanması, bunların oluşumunun altında yatan mekanizma bilgilerini gerektirir. Bu nedenle bu yazıda olası mekanistik biyobelirteçler üzerinde yoğunlaşılmıştır.

Anahtar kelimeler: Mekanistik, biyobelirteç, toksikoloji

INTRODUCTION

The National Institutes of Health defines a 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, such as glomerular filtration rate or recorded blood pressure at different time intervals, are examples of biomarkers. Generations of epidemiologists, physicians, and scientists have used various biomarkers to study human diseases. Biomarkers have been used in the management and diagnosis of cardiovascular diseases, infections, genetic disorders, cancer, and others.[3] The time course of injury and underlying molecular mechanisms are reflected by the measuremment of biomarkers. Accurate diagnosis, prognosis, and treatment regime can be applied to a patient by analyzing different biomarkers.[2] Periodic surveillance of biomarkers also serves as a tool to determine whether a treatment protocol or daily dietary habits are improving the patient's condition.[2] The periodic follow-up of biomarkers will provide the health care personnel with important information about the 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] Exposure biomarkers are considered early markers, which result from the 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 to develop personalized medical treatment strategies.[2] The biomarkers of effect are considered late markers. They are used to measure the burden of injury or damage caused by different agents on the target organ. These biomarkers are also employed to objectively and accurately measure the overall health status of patients, usually after being exposed to an agent or disease.[4] Susceptibility biomarkers are used as a guide to inherent or gained ability of the body to respond

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to difficulties. These markers are the result of exposure to diseases or chemicals.[4,5]

Biomarker development requires detailed and considerable knowledge. Studies about the pathogenesis of diseases, molecular changes, and alterations in biochemical pathways underlying toxic effects should be conducted. Such mechanistic information causes the formation of mechanistic biomarkers. Mechanistic biomarkers cover exposure, effect, and susceptibility biomarkers and lead 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 patient prognosis and the probability of response to various treatment options; however, it is not used for follow-up of progression nor response to an applied medical therapy.[6,8] Numerous biochemical processes, such as oxidative stress, alterations in biotransformation, alteration in protective and repair systems, and organelle damage, are the mechanistic information that lead to detectable biomarkers.

Oxidative stress biomarkers

In 1985, Helmust Sies defined "oxidative stress as a disturbance in the prooxidant-antioxidant balance in favor of the former".[9] Oxidants are mostly produced by cellular metabolism. The antioxidant system of the body quickly eliminates small amounts of oxidants, but in certain cases, they cause remarkably profound damage to macromolecules (proteins, lipids, DNA, and carbohydrates) (Figure 1). Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radicals, and hydroxyl radicals, are common by-products of metabolic activities.[2,10] ROS are synthesized during mitochondrial respiration, inflammation, immune system activity, and other processes.[2,11-13] The increase in 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 also been associated with numerous diseases or toxicities. A substantial amount of evidence reveals 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 ROS. The measurement of ROS may be a useful marker, but such method is unstable; their detection 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. Surrogate markers include nitrite and nitrate levels, products of lipid peroxidation, and levels of oxidized proteins.[11] Figure 1 shows the effect of ROS on macromolecules and several end products.

Lipid peroxidation is a cascade of reactions due to ROS attack on lipids in the cell membrane, and it has been implicated in various diseases, such as hypertension, Alzheimer's disease, cancers, and other disorders.[16-19] The burden of lipid peroxidation can be measured by analyzing thiobarbituric acid, N-epsilon-hexanoyl-lysine, malondialdehyde, 4-hydroxy-nominal, and F2-isoprostane 15(S)-8-iso-prostaglandin F2α, which are 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 defense involves several strategies, namely, enzymatic and non-enzymatic mechanisms. Enzymatic mechanisms, such as superoxide dismutase (SOD), and non-enzymatic defense systems, protect cells against free radicals and ROS. Antioxidants, including alpha-tocopherol, scavenge 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 the 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 that prevents oxidative damage.[21] The ratio of reduced-to-oxidized GSH indicates the redox balance of the cell. This redox balance is an indicator of the overall health of cells.[22] Dysregulations in GSH synthesis and its concentration are considered important biomarkers in the diagnosis of diseases, such as human immunodeficiency virus, cancer, inflammation, tuberculosis, Alzheimer's disease, and numerous others.[23-25] The evaluation of the GSH pathway will reflect the status of the antioxidant system, which may elucidate various underlying pathology etiologies. Among the enzymes that participate in the antioxidant system, GSH peroxidases (GSH-Pxs) consist of four enzymes (Table 1),[26,27] all of which contain selenium. These GSH-Pxs are hydrogen and lipid peroxide scavengers. Hydrogen peroxide is produced during cell metabolic processes, and its amount increases under 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

GPX: Glutathione peroxidase

Another essential enzyme in the antioxidant system is SOD, which eliminates superoxide radicals.[31] SOD has three

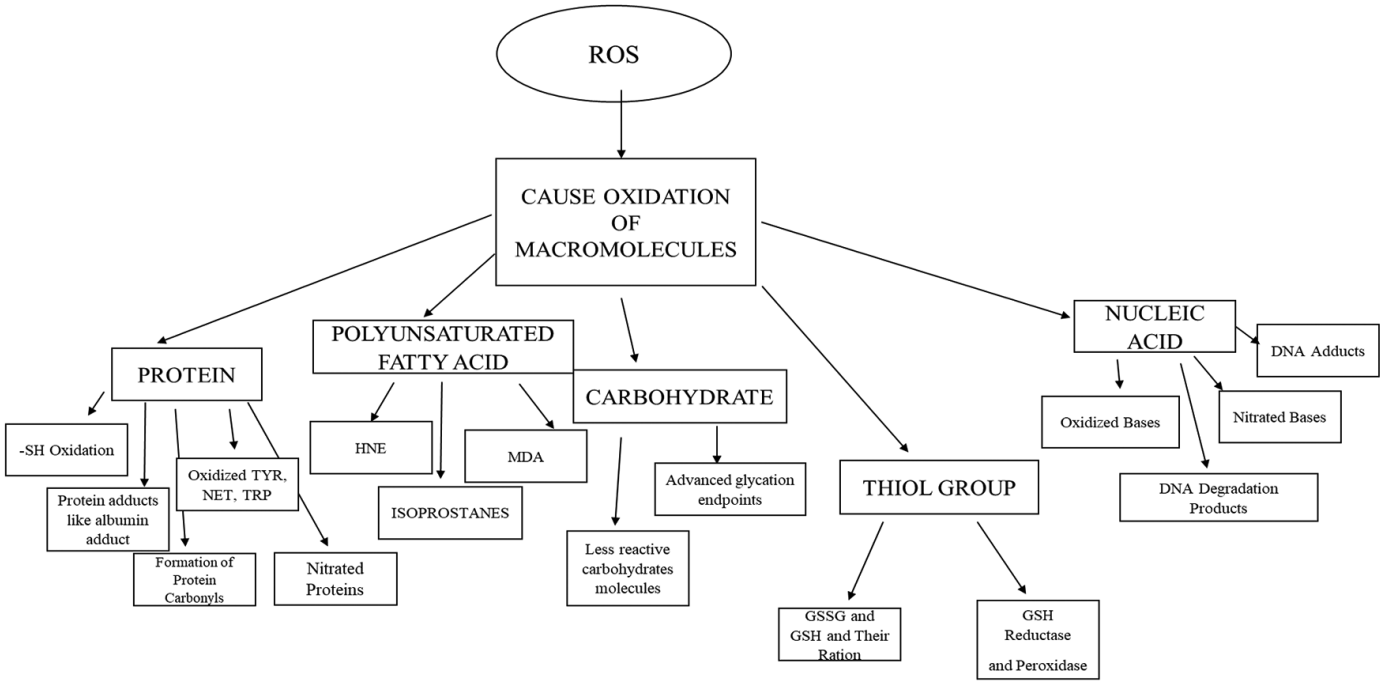

Figure 1. ROS cause the oxidation of macromolecules. As a result of this oxidation, the end products of the oxidation process have been mentioned. These end products are used as biomarkers to detect the presence of oxidative damages (drawn by authors)

ROS: Reactive oxygen species

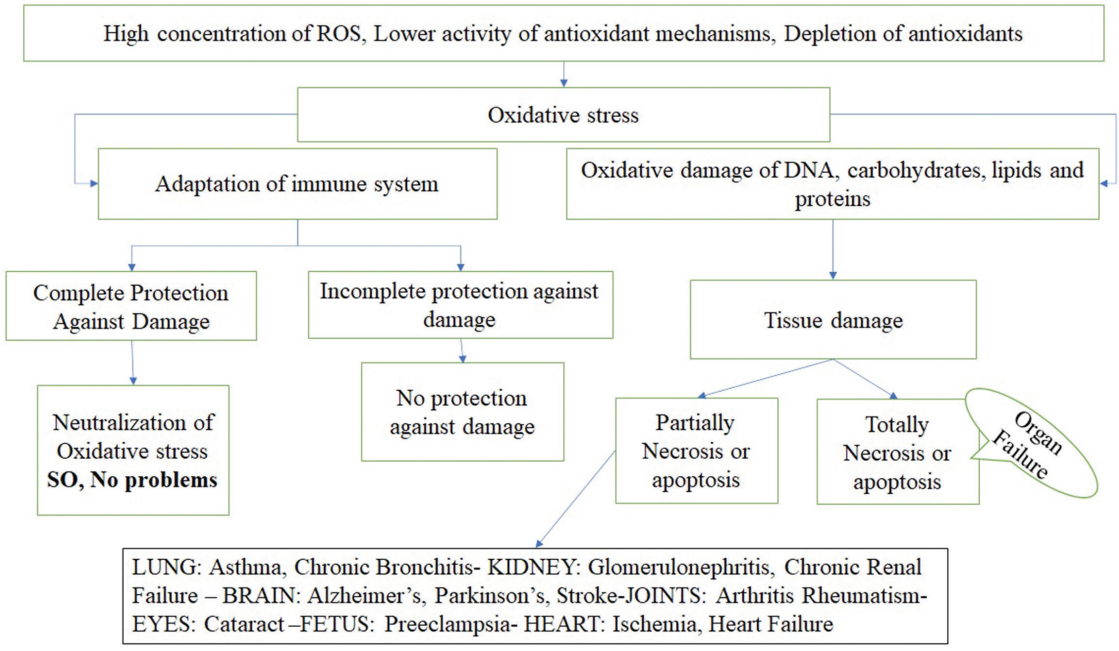

Figure 2. Oxidative stress in the general pathogenesis of diseases (drawn by authors)

metalloenzyme forms, namely, cytoplasmic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular EC-SOD, all of which require cofactors (Cu or Mn) for their activity.[32,33] Hydrogen peroxide formation results from the neutralization of superoxide. Catalase and GSH-Px enzymes then catalyze this H_2O_2. Given that 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 excretable metabolites. However, in certain cases, the metabolites may be toxic and reactive electrophiles. These toxic metabolites lead to cell damage or cell death. The measurement of active metabolites (such as morphine as the active metabolite of codeine biotransformation), determining

the effect of reactive metabolites on macromolecules and the analysis of end products (such as of mercapturic acid or hippuric acid in urine samples), and measurement of enzyme activity, which is responsible for xenobiotics metabolism, are several 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 in the fate of xenobiotics. Metabolization can be altered by enzyme induction or inhibition. Enzyme induction or inhibition has been studied as a biomarker for the measurement of responses to environmental pollutants, exposure to various drugs, or drug interactions.[35] Chronic alcohol usage results in the induction of the 2E1 enzyme. The induction of this enzyme will alter the fate of specific drugs that are metabolized by it.[36] Organophosphate pesticides reversibly or irreversibly bind to and inhibit cholinesterase. This inhibition prevents neurotransmitter (acetylcholine) degradation.[37] Quinidine is a potent CYP2D6 inhibitor.[38] In polypharmacy, the 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 the toxicity or absence of a therapeutic activity.[39]

Differences in genetic traits that cause differences in the expression and activity of enzymes are the primary cause of susceptibility to various diseases. The mutations and alterations in genes can be detected in 1% of populations, a phenomenon called genetic polymorphism. Polymorphisms in phase I and II biotransformation enzymes or DNA repair enzymes can be biomarkers. The polymorphisms of GSH S-transferase, N-acetyl transferase, and CYP1A2, 2A6, 2D6, and 2E1 have been studied[2] in various conditions; as an example, the polymorphism of CYP2C9 causes the patient to need less doses of warfarin, which will increase the susceptibiltiy of the patient to increased risk of bleeding and in the case of CYP2C19, the increased risk of anticonvulsant side effects.[40] People who are CYP2D6 polymorphic need high doses of fluoxetine to show the same plasma levels as those with normal CYP2D6.[41]

Furthermore, protein expression and function can be altered as a result of molecular response to signals, post-translational modifications, and other factors.[40] Additionally, the measurement of the parent-compound-to-metabolite (metabolite ratio) ratio is considered an applicable and practicable biomarker. The measurment of 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 to show its analgesic effect. However, in poor metabolizers, codeine is a poor 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] ROS also cause DNA damage. The degree of DNA damage can be used as a biomarker to assess the oxidative stress in various conditions, such as pancreatic and mammary cancers and the damage from ionizing radiation that 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 form DNA adducts. The metabolism of benzo (alpha) pyrene results in the formation of a cation radical, which forms DNA adducts, during exposure to tobacco smoke or coal.[47] In addition, reactive oxygen and nitrogen species can directly interact with DNA.[48] This interaction causes the 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. The measurement of oxidized bases from urine samples is a good indicator of the oxidative damage of nuclear DNA, which occurs during carcinogenesis, and an important prognostic factor for certain cancers.[48]

Damage to mitochondrial DNA is mainly caused by oxidative stress damage. The DNA repair systems are incomplete in the mitochondria, which increases the susceptibility and mitochondrial dysfunction. Therefore, this damage directly interferes with oxidative phosphorylation and results in the induction of apoptosis and cell death. The 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: Nicotinamide (NAM) adenine dinucleotide (NAD)⁺ and NAM adenine dinucleotide phosphate (NADP)⁺

Cofactors mediate a wide range of biological reactions. NAD [reduced form: NAD(H)], NADP [reduced form: NADP(H)], and ATP are important mechanistic biomarkers. NAD^+ was first discovered in 1906.[49-52] NAD^+ and NADH play an important role in various metabolic processes, such as glycolysis, mitochondrial oxidative phosphorylation, oxidation of fatty acids, citric acid cycle, and other oxidation-reduction (redox) reactions.[51-53] Its effects determine the 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 a co-substrate for three important enzymatic activities [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 NAM. The CD38 activity increases with age, resulting in the increased NAD^+ consumption and depletion of NAD^+ reserves. CD38 is overexpressed in chronic inflammation and chronic lymphocytic leukemia, whereas mitochondrial NAD^+ is depleted in these diseases. PARPs play a role in epigenetics, DNA repair, and chronic inflammation. An increase in the expression of PARPs results in NAD^+ consumption and reduction of the NAD^+ pool. Sirtuin is an important factor that increases the life span

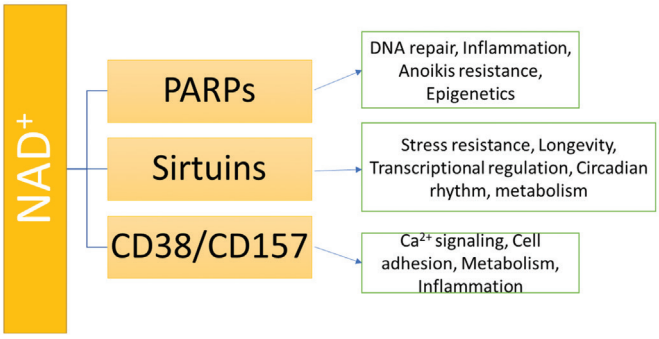

Figure 3. NAD^+ is a co-enzyme for the function of PARPs, sirtuins, and cyclic ADP-ribose synthases (CD38/CD157). Fluctuations in the NAD+ level affect the biological processes that are dependent on these enzymes[51]

NAD: Nicotinamide adenine dinucleotide, PARPs: Poly (ADP-ribose) polymerases, ADP: Adenine dinucleotide phosphate

of cells. Sirtuin pool decreases by aging along with the NAD^+ pool. Thus, increasing the NAD^+ pool enhances the life cycle of cells.[54] Sirtuin acts as a tumor suppressor by regulating transcription, programming the metabolic pathways of cells, and increasing cell resistance against oxidative stress.[51,52] Through these enzymes, NAD^+ affects the 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 thus, these proteins are significant in carcinogenesis.[51]

The increase in NAD^+ levels possibly reduces the risk of cancer, but this increase leads to the increased activity of PARP enzymes. PARPs promote the protection and repair of DNA, especially in cancer cells. PARPs cause the overexpression of inflammatory genes, which are responsible for the increased incidence of hormone-dependent tumors.[51] Sirtuin is sensitive to the fluctuation in NAD^+ levels. Different sirtuin isoforms act as tumor suppressors by altering transcription and rescheduling the cell metabolic activity.[51]

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

$NADP^+$ is formed by the addition of phosphate to NAD^+. $NADP^+$ and NADPH are critical cofactors, fighting against oxidative stress and playing a role in the synthesis of nucleic acids, 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 in any of them disrupts this homeostasis, which results in oxidative stress, disease onset, and energy impairment.

Polyamines: Ornithine decarboxylase (ODC)

Polyamines are small, cationic amines derived from amino acids. They are required for healthy cell growth; however, they are also involved in cancer cell proliferation.[1,56-59] Putrescine, spermidine, and spermine are the main polyamines in eukaryotes and prokaryotes.[56,58] Dietary 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] Given their significance in cell function, their levels are strictly regulated by maintaining a balance between synthesis, degradation, and uptake. ODC plays a critical role in the biosynthesis of polyamines. Increased levels of ODC enzyme in blood has been reported in regenerating tissues and in cancer.[56,57]

Along with chemical cancer promoters, which result in ODC increase, several environmental and genetic factors, such 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, which forms acetylated polyamines, is another enzyme in polyamine synthesis. Both the parent polyamines and acetylated derivatives (e.g., N_1-acetyl spermidine, N_8-acetylspermidine, N_1-acetylspermine, and N_1, N_{12}-diacetylspermine) can be detected in urine and have been associated with cancer.[60]

Tumor cells with a high polyamine production show an increased synthesis of proteinases and cathepsins, which destroy the surrounding tissue. These cells also induce hypoxia, which results in the increased uptake of polyamines by cells and results in an increased proliferation rate.[56,58]

Pteridine pathway: Folate and neopterin

Pteridines are bicyclic nitrogenous ring system pyrazino-(2,3-d)-pyrimidine derivatives that bear small substituents, such as neopterin and biopterin, and are called unconjugated pteridines. The derivatives with a large residues, such as folic acid and riboflavin, are called conjugated pteridines.[63]

Several crucial cellular mechanisms depend on folate as the source of 1-carbon in DNA synthesis and methylation of protein. Thus, folate plays a significant role in DNA synthesis.[64-68] Dihydrofolate reductase and thymidylate synthase have been used as targets in chemotherapy, thus rendering conjugated pteridines as good candidate biomarkers.[69] Folate deficiency leads to different disorders and diseases.[70]

Among folate derivatives, 5-methyltetrahydrofolate (5-methyl THF) is found in circulation, and it acts as a co-substrate in the conversion of homocysteine to methionine (Figure 4)[71]. DNA mutations and strand breakage can also be the result of an increase in the replacement of uracil instead of thymidine. These events occur due to the decrease in 5,10-methyl THF.[66,72] Moreover, the decreased levels of 5-methyl THF will lead to the reduced levels of s-adenosylmethionine, which will cause the activation of oncogenesis and increase DNA damage.[73-75] For this reason, folate level can be a useful biomarker in predicting or diagnosing cancer.[64,66]

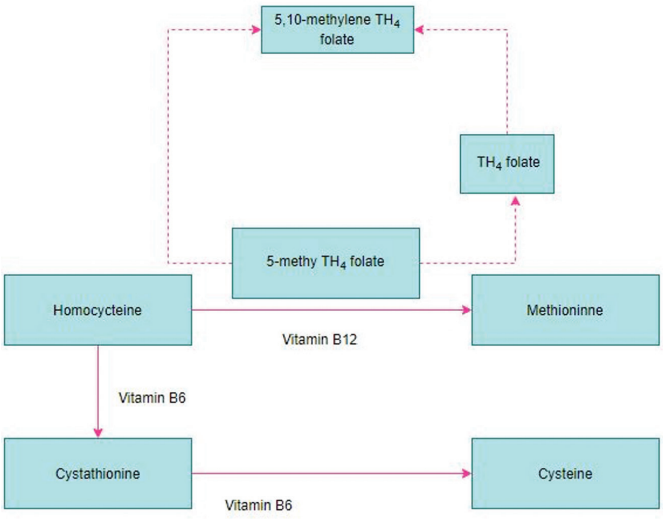

Figure 4. Conversion of homocysteine to methionine[71]

The relationship between folate and cancer is directly related to its dosage. Low doses of folate increase the risk of cancer. On the other hand, high doses of folate will reversely inhibit dihydrofolate reductase. Another risk factor for carcinogenesis is the circulation of the unreduced form of folate. Antifolate medications have been used widely in cancer therapy to inhibit single-carbon metabolism, which is necessary for cell proliferation in cancerous tissue.[66,70] Other agents, such as chronic alcohol usage,[76] antacids,[77] and general anesthetics,[78] cause depletion or alteration in folate levels.

Additionally, diseases, such as Crohn's disease, celiac disease, and several kind of cancers, result in folate depletion. The evaluation of folate levels is important in patients who have been on long-term diuretic therapies, including those using furosemide and amiloride. These medications increase the elimination of folate.[70]

The measurement of folate levels in the process of testing new therapeutic agents is considered a vital biomarker because of the essential role of folate in DNA biosynthesis and red blood cell synthesis. The depletion of folate levels increases the rate of cardiovascular and neuronal disorders.[73]

Unconjugated pteridines and their derivatives act as intermediates in metabolism, and their biological concentrations have shown changes in various disease processes. Unconjugated pteridines can be measured in the serum, cerebrospinal fluid, and urine.[79] Neopterin, as an unconjugated pteridine, is 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 became popular 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 a hydroxyl, superoxide, and peroxyl scavenger. Surveillance of neopterin in body samples is a good indicator of the levels of free radicals in tissues and cells.[82] The Austrian government has been using neopterin screening to test donated blood to ensure their safety.[83] Neopterin screening can also be used to predict a patient's inflammatory status.[84-86]

CONCLUSION

In general, biomarkers are used to measure the response of biological systems. In the field of toxicology, biomarkers are practical tools to understand the mechanisms of toxicity. They are also useful in risk management and assessment. From the toxicological aspect, 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 of the treatment course of different diseases, such as cancers, Alzheimer's disease, immunological disorders, and other pathologies.

In conclusion, reliable and applicable proper biomarkers that accord with ethical rules are beneficial for human health. Nevertheless, further research is still needed to define ideal biomarkers for different fields of life sciences.

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