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Autism spectrum disorder (ASD) – biomarkers of oxidative stress and methylation and transsulfuration cycle

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Summary

Autism spectrum disorder (ASD) affects people from all regions of the globe, regardless of nationality, living standards or social group. Currently, it is assumed that ASD pathogenesis is multifactorial because there is no one specific cause of the disorder. According to literature, ASD may result from genetic defects, metabolic disorders or exposure to environmental factors. There is a number of hypotheses that attempt to explain the intensity of emotional and behavioral symptoms or the increased sensory threshold that is characteristic of ASD. It is suggested that neurological changes may be due to oxidative stress occurring in early brain tissue development and reduced antioxidative barrier. Due to the abnormalities in the synthesis of neurotransmitters, often occurring in ASD, autism is investigated for disorders of vital biochemical processes of methylation and transsulfuration. Finding a biomarker for a disturbed oxidative-reduction equilibrium, methylation pathway pathology, or other reason could be an important diagnostic tool and the base for individual treatment for patients with varying degrees of severity. This work provides a review of the potential biological indicators for ASD taking into account the occurrence of oxidative stress and the methylation and transsulfuration cycles.

Key words: Autism Spectrum Disorders, biomarkers, oxidative stress

Introduction

Autism spectrum disorder is a term used to describe a complex group of disease entities which base on neurodevelopmental abnormalities that affect development in the areas of social interaction, cognitive functions and communication skills.

A great interest in this disease entity is caused by its complex and not fully explained etiopathogenesis. It is assumed that the background of autism has a multifactorial nature. Research to date on autistic disorders has provided a lot of relevant information on this subject, but despite the efforts of scientists from many fields that have been ongoing since the mid-20th century, it has not been possible to clearly establish the underlying causes of these abnormalities. There are hypotheses about the inherent nature of autism spectrum disorders, but the symptoms cannot be detected in prenatal tests. There are many factors in the literature that may affect the occurrence of autism. Among the reasons mentioned are genetic defects, chromosomal abnormalities, metabolic diseases, viral factors, immunological tolerance or hypoxia caused by perinatal problems [1–5].

The phenotypic characteristic of neurodevelopmental disorders is an integral part of the advances in research and clinical practice. People with ASD have a different history of 'illness' and different severity of symptoms. The hypothesis that ASD is caused by the occurrence of several different variations within several epigenetic genes enjoys increasing recognition. Interactions between environmental and genetic factors that contribute to the expression of predisposing genes are thought to have a significant impact on the appearance of autism-specific features. Specific phenotype, which arises as a result of abnormal biochemical processes under the influence of epigenetic factors, can be characterized by different biomarker profiles for different individuals [6]. The challenge and difficulty in identifying biomarkers in ASD are to correctly distinguish biomarkers representing the genetic and neurobiological changes that led to it from those whose presence is the result of an already existing disorder.

Based on the available literature, information on potential metabolic biomarkers has been collected, mainly in terms of their importance in assessing the development of autism spectrum disorders. As they are indicators of oxidative stress as well as methylation and transsulfuration cycle disorders, they seem promising when it comes to their usefulness for developing a coherent program of pharmacological therapy supplementing psychotherapeutic methods of treatment [6, 7]

Biomarkers of oxidative stress in autism

Free radicals with one unpaired electron are highly reactive systems with an oxidative nature. In the homeostasis conditions, there is a dynamic balance between the production of free radicals of oxygen and nitrogen origin and the antioxidant capacity of the cell. The physiological and pathophysiological effect of free radicals is determined by the reactivity of the oxidant itself, its subcellular location, concentration, and duration of action. Reactive oxygen species (ROS), in particular, superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl ($HO^{\bullet}$), hydroperoxide ($HO_2^{\bullet}$) radical act as relays in cellular signalling and are involved in the mechanism of destruction of pathogens, both during congenital and acquired immune responses. They are not only the first line of defence of phagocytes against pathogens, but also indirectly activate

T lymphocytes by changing the form of oxidation of intracellular glutathione, or by intensifying the production of interleukin-2 [8–10].

The excessive production of free oxygen radicals leads to the emergence of an oxidative potential advantage, i.e., oxidative stress and, consequently, the weakening of the natural mechanisms of detoxification of xenobiotics. In situations where the infection and/or inflammation lasts for several months or longer, phagocytic cells may produce excessive amounts of free radicals, which leads to undesirable damage to the neighbouring cells of the body. High concentrations of ROS initiate, among others, processes of peroxidation of polyunsaturated fatty acids in lipid membranes, leading to the oxidation of polypeptide chains, their fragmentation and conversion of amino acid residues to derivatives, which consequently reduces the biological activity of the protein. Although nucleic acids are more resistant to free radicals than polypeptides or phospholipids, they are also exposed to the oxidative effect of ROS, especially in the mitochondrial genetic material. The DNA strand is damaged because of a modification of nitrogenous bases, oxidation of deoxyribose or breaking of phosphodiester bonds by the hydroxyl radical HO• [11–15].

Reactive nitrogen species (RNS), which include free nitrogen radicals: NO•, NO_2• and peroxynitrite(III) ($ONOO^-$), analogically to ROS, may contribute to the modification of biological structures of, in particular, polypeptides and DNA [16]. Potential mutagenic and genotoxic activity is produced by excess of NO• forming harmful products of DNA deamination as well as ONOO – with oxidative activity. NO• at physiological pH may undergo autoxidation to N_2O_3. The destructive effect of N_2O_3 on DNA is the nitrosation of primary heterocyclic amines, it indirectly leads to the formation of diazonium ions, which undergo hydrolysis and finish the deamination process. As a result, deamination of adenine, cytosine and guanine leads to the occurrence of hypoxanthine, uracil and xanthine respectively. Unstable xanthine can easily depurate by creating sites devoid of nitrogenous bases in which the DNA strand breaks as a result of endonucleases activity. On the other hand, ONOO – can directly react with deoxyribose to form a sugar radical, which in the subsequent stages undergoes reactions leading to the cracking of the strand [17].

Oxidative stress, which occurs as a result of the cumulative impact of toxic environmental factors that can lead to neuronal damage in genetically predisposed individuals, may play a key role in the pathogenesis of autism spectrum disorders. The lack of balance between oxidants and antioxidants, which is a consequence of mitochondrial disorders, is increasingly being treated as a potential pathogenic factor in neurodegenerative diseases. Therefore, there is a high probability that the action of oxidative stress has serious consequences also for the development and functioning of the central nervous system of people with autism [12, 18].

Glutathione

More than 98% of total glutathione (GSH+GSSG) occurs in the reduced form (GSH) able to reduce free radicals, the rest is oxidized (glutathione disulfide – GSSG) existing as

a disulfide and S-conjugates of glutathione. The concentration of intracellular glutathione varies from 5 to 10 mM/l. The anti-oxidant effect of GSH is not only related to ROS detoxification but also with the coupling of toxic electrophilic exo – and endogenous substances and with the possibility of chelation of metal ions. In the eukaryotic cells, the GSH/GSSG ratio is an indicator of the general state of the intracellular oxidation-reduction environment. The lower level of mitochondrial glutathione is associated with neuronal sensitivity to oxidative stress. The brain is highly susceptible to oxidative stress mainly due to the limited efficiency of antioxidants, higher energy requirements and higher lipid content. It is about 2% of body weight but consumes as much as 20% of metabolic oxygen. The vast majority of energy is used by neurons. Due to the lack of potential for the production of glutathione by neurons, the brain has a limited ability to detoxify ROS, making neurons the first cell line vulnerable to oxidative stress. In children (0–2 years) and older people (41–69 years), significantly lower GSH concentration and higher GSSG were found in erythrocytes compared to people from other age groups [19]. Therefore, there are suggestions that neurological changes induced by oxidative stress occurring in the early stages of development of the brain tissue may lead to autism spectrum disorders. Numerous studies have shown a correlation between abnormal glutathione levels in various tissues, and the occurrence of autistic disorders [20]. The results of the studies on the concentration of glutathione or concentration ratios of its various forms in the biological material in persons with ASD and control groups are presented in Table 1.

Table 1. **A summary of the results of studies which measured GSH or GSH/GSSG concentrations in patients with ASD and control groups**

Biomarker	Sample	Mean value [mg/ml] ± SD	Reference
Free GSH/GSSG	Lymphoblastic cells	Control 99.14 ± 33.5 n = 10 Autistic 61.81 ± 10.6 n = 10; p < 0.001	[21]
	Mithochondria of lymphoblastic cells	Control 11.63 ± 3.9 n = 10 Autistic 5.06 ± 1.3 n = 10; p < 0.001	
GSH/GSSG	Cerebellum	Control 103.4 ± 5.9 n = 10 Autistic 48.7 ± 1.7 n = 10; p < 0.0001	[22]
	Temporal cortex	Control 113.9 ± 8.2 n = 10 Autistic 44.7 ± 3.1 n = 10; p < 0.0001	

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GSSG/GSH	Plasma	Control 0.093 ± 0.04 n = 44 Autistic 14 ± 0.05 n = 55; p < 0.0001	[23]
GSH (μM)	Whole Blood	Control 242.67 ± 32.94 n = 13 Autistic 161.16 ± 10.68 n = 15; p = 0.02	[24]
Free GSH/GSSG	Plasma	Control 7.9 ± 3.5 n = 73 Autistic 4.9 ± 2.2 n = 80; p < 0.0001	[25]
GSH/GSSG	Plasma	Control 28.2 ± 7.0 n = 73 Autistic 14.7 ± 6.2 n = 80; p < 0.0001	[26]
Free GSH/GSSG	Plasma	Control 17 ± 6,8 n = 42 Autistic 6 ± 2 n = 40; p = 0.001	[26]
GSH/GSSG	Plasma	Control 47 ± 18 n = 42 Autistic 21 ± 6 n = 40; p = 0.001	[26]
GSH/GSSG	Plasma	Control 26.07 ± 5.03 n = 2,042 Autistic 8.03 ± 2.46 n = 20; p = 0.001	[27]

SD – standard deviation; n – group size; p – level of significance

Intracellular oxidative stress causes the reduction of glutathione reductase capacity to reproduce the reduced form of glutathione. To recover intracellular redox homeostasis, GSSG is transported to plasma. Therefore, the increased GSSG value and the reduced GSH/GSSG plasma ratio is a good indicator of intracellular oxidative stress. The lower concentration of the reduced form of glutathione may also explain the possibility of recurrent infections, inflammation of the nervous tissue and digestive tract and reduced ability to remove toxic substances from the body in patients with ASD [28].

Glutathione peroxidase, catalase and superoxide dismutase

Neutralization of excess free radicals takes place thanks to an antioxidative system that involves the action of enzymatic and non-enzymatic antioxidants. The main enzymes that inhibit the formation of the most reactive hydroxyl radical are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [15, 18].

Superoxide dismutase is an enzyme catalyzing superoxide dismutation O_2• – to H_2O_2 and O_2, also inhibits lipid peroxidation by removing superoxide radicals formed during incomplete reduction of O_2. Toxic H_2O_2, a natural product of cellular metabolism, is a substrate for catalase (CAT) and glutathione peroxidase (GPx) that maintain its low concentration. GPx allows the reduction of H_2O_2 and lipid hydroperoxides in the oxidation reaction of GSH to GSSG and water. This selenium-dependent enzyme is found in the cytosol and also in the lower concentration is present in the mitochondria and extracellular fluid. In turn, catalase catalyzes the H_2O_2 decomposition reaction to water and molecular oxygen in peroxisomes. H_2O_2, which is a reaction product catalyzed by SOD, is at the same time a substrate for reactions catalyzed by CAT and GPx, between which there is a cooperation effect [29].

The results of studies on the determination of SOD, GPx and CAT antioxidant enzymes presented in Table 2 show altered activity of these enzymes in patients with autism. Investigations of oxidative stress indicators were carried out on samples of erythrocytes, plasma and serum. Erythrocytes appear to be a good material due to their simple structure and relatively large amounts of polyunsaturated fatty acids existing in cell membranes that may undergo peroxidation under the influence of ROS. H_2O_2 can diffuse easily through biological membranes and enter plasma or other cell components and thus increase the activity of antioxidants. Therefore, any changes in GPx and CAT activity in cells will also determine the activity of these enzymes in the extracellular environment [33].

Table 2. **A summary of the results of studies which measured SOD and GPx activity in ASD patients and control subjects**

Biomarker	Sample	Mean value ± SD	Reference
GPx (U/g Hb)	Erythrocytes	Control 24.81 ± 1.19 n = 25 Autistic 19.17 ± 1.16 n = 20; p < 0.005	[30]
Gpx (U/g Hb)	Erythrocytes	Control 38.01 ± 5.03 n = 41 Autistic 28.72 ± 2.64 n = 45; p < 0.05	[31]
Gpx (U/g Hb)	Erythrocytes	Control 7.45 ± 0.65 n = 9 Autistic 7.75 ± 0.93 n = 12 p > 0.05	[32]

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Gpx (U/L)	Plasma	Control 24.2 ± 6.3 n = 30 Autistic 40.9 ± 11.3 n = 27; p < 0.0001	[33]
Gpx (U/dL)	Plasma	Control 143.85 ± 61.12 n = 30 Autistic 246.88 ± 99.93 n = 30; p < 0.05	[34]
Gpx (U/mL)	Plasma	Control 0.39 ± 0.08 n = 41 Autistic 0.27 ± 0.04 n = 45; p < 0.05	[31]
SOD (U/g Hb)	Erythrocytes	Control 1,321.72 ± 46.29 n = 25 Autistic 987.8 ± 92.97 n = 20; p < 0.005	[30]
SOD (U/dL)	Erythrocytes	Control 1.24 ± 0.33 n = 30 Autistic 1.51 ± 0.45 n = 30; p < 0.05	[34]
SOD (U/g Hb)	Erythrocytes	Control 971.31 ± 239.14 n = 26 Autistic 2,123.59 ± 543.53 n = 27; p < 0.001	[35]
SOD (U/g Hb)	Erythrocytes	Control 993.17 ± 118.31 n = 41 Autistic 723.78 ± 90.03 n = 45; p < 0.05	[31]
CAT (k/g Hb)	Erythrocytes	Control 515.77±127.9 n = 26 Autistic 209.31± 61.92 n = 27; p < 0.001	[35]
CAT (U/L)	Serum	Control 0.689 ± 0.157 n = 42 Autistic 2.836 ± 0.479 n = 45; p ≤ 0.001	[36]

SD – standard deviation; n – group size; p – level of significance

The results of independently conducted experiments to determine the concentration of SOD, GPx and CAT in blood samples in children with autism syndrome showed significant differences in the concentration of these enzymes in relation to the concentrations in samples of healthy children. The obtained results are not unequivocal and do not show a clear tendency in regard to the activity of these enzymes. Impaired antioxidant capacity, manifested by reduced enzyme activity, may result from their lower production or higher consumption when trying to neutralize free radicals. Exhaustion of reserves of antioxidants may be caused by the lack of adequate and sustainable consumption of minerals such as copper and zinc, excessive exposure to xenobiotics or polymorphic changes in genes encoding enzymes that protect the cell from the appearance of oxidative stress [37]. Significantly lower concentrations of GPx and SOD in the group of people with ASD may indicate a reduced neutralization capacity of H_2O_2, superoxide anion radical and organic hydroxides. Exceeding the antioxidative capacity under GPx and CAT deficiency conditions can lead to serious damage to macromolecules. In some studies, there was an opposite relationship, i.e., statistically higher activity of antioxidant enzymes in the group of people with ASD. The hyperactivity of antioxidants is explained as a compensatory effect, preceding cellular oxidative stress [33].

Ceruloplasmin and transferrin

Ceruloplasmin inhibits peroxidation of membrane lipids by reducing the concentration of free Fe^{2+} ion catalyzing the conversion of H_2O_2 to highly reactive OH•. It acts as a ferroxidase and scavenger for superoxide anion radical O_2•, protecting polyunsaturated fatty acids found in the membranes of erythrocytes against free radicals [38].

Transferrin, partially saturated with iron ions, acts as a powerful antioxidant in human plasma until it is completely saturated. It acts as a promoter of the Fenton reaction [39]:

$H_2O_2 + Fe^{2+} \rightarrow OH\bullet + OH^- + Fe^{3+}$

In studies carried out by Chauhan et al. [40], it has been shown that levels of serum ceruloplasmin in thirteen out of nineteen, and in the case of transferrinin sixteen of nineteen studied autistic children were significantly lower than the concentrations determined in samples from healthy siblings.

Interestingly, the study in which ceruloplasmin and transferrin were determined showed the lowest levels of enzymes in children with autism who had the weakest language skills. Autistic children, who did not lose their language skills, showed serum concentrations of ceruloplasmin and transferrin similar to their healthy siblings [40].

8-isoprostane

8-isoprostane belonging to the F2-isoprostanes group is considered to be one of the most sensitive markers of polyunsaturated fatty acid oxidation and redox system dysfunc-

tion. F2-isoprostanes are formed as a result of non-enzymatic oxidation of arachidonic acid in phospholipids according to the free radical mechanism. The normal range of 8-isoprostane concentrations in human blood is between 20 and 80 pg/ml. Elevated concentrations of F2-isoprostanes were determined in biological fluids of patients suffering from allergic asthma, Alzheimer's disease, atherosclerosis and hypercholesterolemia, type I and type II diabetes, Huntington's chorea, pulmonary hypertension, cholestasis, or acute respiratory distress syndrome [41].

El-Ansary and Al-Ayadhi [42] measured concentrations of 8-isoprostane in plasma samples of 20 people with autism syndrome aged 4–12 years and 19 controls. The mean concentration of 8-isoprostane in the study group was 45.65% greater than in the control group. The hypothesis of a higher exposure of autistic children to oxidative stress was also checked by Ming et al. [43]. The authors measured the concentration of 8-isoprostane in urine samples from 33 subjects from the study group and 29 from the control group. The mean urinary 8-isoprostane concentration for the study group was significantly higher (32.92 ± 1.98 ng/mol of creatinine) than for the control group (5.71 ± 0.98 ng/mol of creatinine).

In the studies conducted by Qasem et al. [44], the presence of 8-isoprostane was detected in blood samples of autistic children at concentrations well above the upper limit of the normal range. 44 children with ASD aged 4–7 participated in the experiment. Patients were diagnosed according to the Childhood Autism Rating Scale (CARS) to determine the severity of autism from mild and moderate to severe. Based on the conducted studies, a four-fold increase in 8-isoprostane in the blood of autistic children was found in comparison to the control group. There were no significant differences in 8-isoprostane concentrations in groups differing in the severity of the autistic syndrome. In contrast, the difference in concentrations of 8-isoprostane present in the blood was statistically significant when age groups were compared. Therefore, there are suspicions that oxidative stress is important in the pathogenesis of autism especially when it occurs in the early years of life [44].

L-carnitine

L-carnitine (LC) facilitates the beta-oxidation of long-chain fatty acids and the process of removing from mitochondria medium – and short-chain fatty acids having – when in excess – a toxic effects. The results of some in vitro tests show that LC acts as a scavenger for free radicals and protects against the negative effects of oxidative stress [45–48].

Filipek et al. [49], in their work, presented results of determination of total and free L-carnitine in the serum of one hundred patients with ASD. The mean concentration of total and free L-carnitine was significantly lower for children with the autistic syndrome than the range defining the norm. The deficit of L-carnitine was accompanied by a significant increase in alanine (80% of patients had a higher concentration than the mean ± SD, $p < 0.001$) and ammonia concentration (78% of patients had a higher

concentration than the mean ± SD, $p < 0.001$) in the serum. According to the authors' suggestion, one of the theoretical reasons for the occurrence of L-carnitine deficiencies in the organisms of children with ASD might be disorders in the proper functioning of mitochondria [49].

Biomarkers of the methylation and transsulfuration cycles

Methylation is the process of adding a methyl group to a substrate molecule or substitution of an atom or group of atoms with a methyl group – CH_3. Methylation plays a key role in many different biochemical processes. It is important even in the metabolism of neurotransmitters, e.g., dopamine, noradrenaline or serotonin. It occurs during DNA and RNA repair processes, the synthesis of certain phospholipids (e.g., lecithin), hormones (e.g., melatonin) or strong antioxidants such as glutathione. In addition, as a result of methylation, numerous enzymatic processes are carried out in the liver to detoxify xenobiotics [50].

Methylation also occurs during the methionine metabolic pathway – an exogenous amino acid. Initially, methionine is converted to the S-adenosylmethionine (SAM), which in a further step transfers the methyl group to an acceptor molecule, e.g., a neurotransmitter molecule, a protein, a phospholipid or a nucleotide nitrogen base. By the action of methyltransferase the S-adenosylmethionine is being deprived of a methyl group to produce an S-adenosylhomocysteine (SAH) which is hydrolyzed to adenosine and homocysteine. Homocysteine can be converted again into methionine by reactions catalyzed by methionine synthase and methylcobalamin (vitamin B_{12}), or it can be used as a substrate for the synthesis of cysteine in the transsulfuration process. The donor of a methyl group to homocysteine methylation is the active form of folic acid – 5-methyltetrahydrofolate (5-MTHF). During transsulfuration, cystathionine is formed indirectly. The synthesis of cystathionine from homocysteine is catalyzed by vitamin B_6 and cystathionine β-synthase (CBS). Cystathionine in the presence of γ-cystathionase is hydrolyzed to cysteine (Cys), which can be used to produce glutathione. Thus, homocysteine can be remethylated to regenerate methionine in all cells by using 5-MTHF – and B_{12}-dependent methionine synthase or betaine-homocysteine methyltransferase in human liver and kidneys. The third way to remove homocysteine is the irreversible transsulfuration pathway dependent on vitamin B_6 and cystathionine β synthase, in which homocysteine is permanently removed from the methionine cycle (Figure 1) [51].

Disorders of the transmethylation and transsulfuration cycle are conditioned by genetic and environmental factors. The ability of cells to methylate depends largely on dietary factors, and most of all on the supply of methionine, folic acid and vitamins B_6 and B_{12}. Genetic polymorphisms may act synergistically with nutritional deficiencies, disrupting the natural regulatory mechanisms of metabolites, consequently increasing oxidative stress and contributing to the development of metabolic pathology. Of the common polymorphisms associated with the risk of autism, the

polymorphism of methylenetetrahydrofolate reductase (MTHFR) is one of the most frequently studied genetic correlation with autism. MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, necessary for the removal of excess homocysteine. Particularly, the 677C>T polymorphism and the 1298A>C polymorphism of the MTHFR gene is considered as a risk factor for neurodegenerative disorders that may contribute to the epigenetic mechanisms that

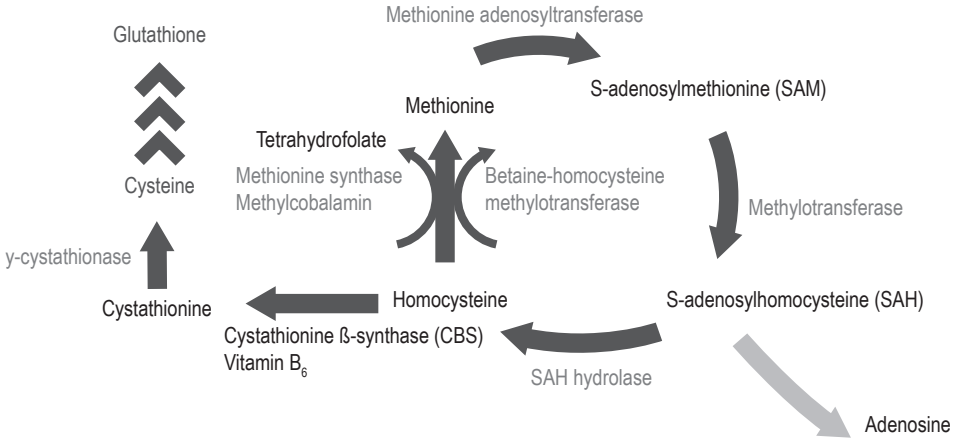

Figure 1. **Simplified scheme of methionine demethylation and homocysteine transsulfuration cycle [51]**

modify gene expression [52]. Abnormal metabolism of methionine, homocysteine and folic acid may also have a cause in the polymorphism of the gene encoding CBS. Lowering the activity of this enzyme induces homocystinuria, manifesting itself as, e.g., neurodegenerative disorders, dysfunctions of the musculoskeletal system or circulatory system. A C699T polymorphism associated with decreased CBS activity and elevated homocysteine concentrations is associated with a high CARS score in patients with autism syndrome [53].

The concentration of homocysteine in the blood depends on many factors, including diet, sex, age, and stimulants. It is usually low if the methylcobalamin – and 5-methyltetrahydrofolate-dependent methylation pathway and the transsulfuration pathway work properly. The complex metabolism of homocysteine in the body is highly dependent on the cofactors derived from vitamins. Deficiencies of vitamin B_{12}, the active form of folic acid and vitamin B6, choline and trimethylglycine are associated with hyperhomocysteinemia. Because of this, the biochemical transformation process is not working properly, and homocysteine accumulates in the body causing cell damage. Hyperhomocysteinemia is considered as an independent risk factor for

many pathological states in neurodegenerative diseases, including autism spectrum disorders [54].

Paşca et al. [55] measured, among others, total plasma homocysteine concentration of 21 children, including 12 with autism syndrome. The homocysteine concentrations of the subjects were higher (9.83 ± 2.75 μmol/L) compared to those in the control group (7.51 ± 0.93 μmol/L). C. Puig-Alcaraz et al. [56] have determined the concentration of homocysteine in the urine in a 35-person study group and a 34-person control group. They also showed a significant difference ($p < 0.05$) in homocysteine concentrations in subjects with ASD (2.24 ± 3.50 mmol/mol of creatinine) compared to healthy subjects (0.9 ± 0.58 mmol/mol of creatinine). The correlation analysis showed a significant relationship between the homocysteine concentration and the severity of disturbances in communication skills (Spearman's $r = 0.453$; $p < 0.05$) [56]. However, studies conducted by Kałużna-Czaplińska et al. [57] confirmed the beneficial effect of using B_6 and B_{12} vitamins, as well as folic acid in order to reduce the concentration of homocysteine in the urine among children with autism. Supplementation with B_6 and B_{12} vitamins was more effective in lowering homocysteine levels with the addition of folic acid. The level of homocysteine in the urine of autistic children before vitamin supplementation was 2.41 ± 1.10 mmol/mol of creatinine and after treatment 1.13 ± 0.44 mmol/mol of creatinine [57].

Homocysteine metabolism disorder in children with autism is complex and it is not easy to explain by an abnormality in the course of a single metabolic pathway. Therefore, during inefficient transmethylation, a significant increase of homocysteine and SAH concentration is observed, while the concentration of methionine and SAM are decreasing. The reversible hydrolysis reaction of SAH to adenosine and homocysteine occurs with the establishment of dynamic equilibrium. In fact, the only reason for this reaction to proceed in the hydrolytic direction is the efficient removal of the product. Thus, metabolic disturbances effectively blocking the removal of homocysteine and adenosine lead to increased SAH level. A reduced SAM/SAH value, manifesting as a methylation cycle disorder and expressing impaired cellular methylation ability, may be an important indicator of metabolic profile changes in children with autism [58, 59].

Melnyk et al. [58] showed a reduced mean SAM/SAH ratio in plasma and a reduced mean percentage of 5-methylcysteine in DNA in 68 children with autism in relation to a 40-persons being siblings of the subjects. The authors of the study also showed an elevated content of oxidized glutathione and 8-oxy-deoxyguanosine (a marker of oxidative DNA damage) in the study group compared to the group of healthy siblings. In their work, they maintain the hypothesis that the weakened antioxidant and methylation capacity of cells would affect the expression of epigenetic genes, and environmental pro-oxidants and genetic risk factors would act through changes in DNA methylation and oxidation reactions that cause damage in susceptible genes.

In the work of James et al. [59], the mean SAM/SAH ratio measured for the study group (20 subjects) was almost 50% lower compared to the control group (33 subjects). Additional measurements of the concentrations of metabolites involved in the trans-

sulfuration cycle make it easier to find the reasons for the imbalance in the methionine cycle. Low concentrations of cystathionine, cysteine, and total glutathione are consistent with a decrease in the incidence of homocysteine conversions mediated by CBS [59]. Cysteine, which is an amino acid that limits the synthesis of free glutathione, and its oxidized form – cystine (Cys-S), together cooperate as a major extracellular redox buffer. It is assumed that similar to the intracellular redox buffer (GSH/GSSG), the ratio (CyS/CyS-S) will be correspondingly lower during oxidative stress among people with autism. The analysis of plasma samples confirmed a reduced content of cysteine (202 ± 17 μmol/L for the control group, 163 ± 15 μmol/L for the study group), which was accompanied by an almost twice elevated concentration of GSSG. The value of total glutathione to oxidized ratio was therefore about 70% lower for the study group. Because of the synthesis of GSH is insufficient when the concentrations of its metabolic precursors are too low, a consistent decrease in cysteine concentration in autistic children suggests an increased sensitivity to oxidative stress [59].

Recapitulation

Autism spectrum disorder (ASD) is complex, heterogeneous, and as it is commonly believed, has a genetic background. Despite numerous reports suggesting a high level of inheritance of autism, there are still no specific genes directly responsible for this spectrum of disorders. In reference to the genetic and epigenetic factors leading to autism, in the literature many hypotheses have been proposed so far, against which it is possible to broaden diagnostic criteria and develop more accurate procedures to detect this disorder syndrome. An important goal of searching for new biomarkers of autism is to understand the processes contributing to the formation of neurobiological changes as well as to monitor their progress in order to develop an effective pharmacological therapy.

Undoubtedly, there is a good amount of research providing evidence for the association of oxidative stress with autism. Metabolic pathways disrupting the oxidative-reduction balance in cells, as well as the concentration of individual antioxidants involved in defence mechanisms against reactive oxygen and nitrogen, are the subject of many analyses. Oxidative stress caused by mitochondrial dysfunction or environmental factors is aggravated by destructive neurological processes or methylation disorders that may contribute to the development of neuropsychiatric diseases. Measurements of reduced glutathione concentration and GSH/GSSG ratio allow estimating to what extent people with autism syndrome are exposed to oxidative stress and whether their cellular antioxidant defence mechanisms are as efficient as those of healthy people. Authors of independent studies, in which the concentration of the reduced form of glutathione in blood samples of people diagnosed with autism were determined, agree that this basic antioxidant is significantly lower than in healthy people. There is, therefore, a basis to suppose that oxidative stress could be a factor contributing to the development of autism. This hypothesis is also supported by the results of studies in which other biomarkers

of the oxidative-reduction balance disorder were determined and in which statistically significant differences in concentrations between the children from the examined group (ASD) and the control group were found. The higher concentration of 8-isoprostane and lower concentrations of L-carnitine, ceruloplasmin and transferrin in the case of autistic children were related to the occurrence of linguistic disorders characteristic of this group of disorders. Other potential markers such as SOD, GPx and CAT enzymes are also the subject of studies on the relationship of oxidative stress with autism, but in this respect, the test results differ from each other giving conflicting conclusions. Thus, they cannot be considered as biomarkers specific to autism. Nevertheless, the results of these studies show a significant difference in concentrations of these antioxidants in people with autism compared to healthy children.

Recently, attention has also been paid to the occurrence of disorders of the methionine metabolic pathway in people with autism, which is of fundamental importance, among others, in the control of gene expression, detoxification of xenobiotics and production of glutathione, neurotransmitters and immune cells. Abnormal levels of homocysteine, SAM/SAH or cysteine in the plasma of autistic people are evidence of impaired ability to cooperate with interdependent methylation and transsulfuration processes and the resulting consequences, among others, for the nervous system. This dependence, unfortunately, is not only the domain of autism but also other diseases with a neurodegenerative background.

Although the results suggest a reduced 'ability' of cellular methylation and maintaining a proper antioxidant status among people affected by autism, it is worth remembering that the analysis of body fluids was carried out after the diagnosis of the illness. Thus, it cannot be unambiguously determined whether the observed methylation disruption and the ability to maintain the natural redox balance in the body contribute to the pathogenesis of autism or are simply the result of the pathophysiology of the illness.

The variety of neurological and metabolic epigenetic processes that create disorders typical for ASD syndrome causes that undertaking effective clinical therapy involves the necessity of conducting individual phenotypic therapy. So far, one universal biomarker profile has not been defined. However, the strategy of selecting appropriate and effective clinical therapy based on the comprehensive determination of biochemical ASD indicators seems to be appropriate and have a higher value than the individual determination of a biomarker associated with a single risk factor.

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