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REVIEW ARTICLE

LINK BETWEEN METHYL NUTRIENTS AND THE DNA METHYLATION PROCESS IN THE COURSE OF SELECTED DISEASES IN ADULTS

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ABSTRACT

DNA methylation is a reversible epigenetic modification that plays a crucial role in transcriptional gene silencing. Both excessive (hypermethylation) and reduced DNA methylation (hypomethylation) can contribute to the disturbance of the proper course of many important processes in the human body. The aim of the study was to discuss the relationship between methyl nutrients and the DNA methylation process in the course of selected diseases in adults. Methyl nutrients include folates (vitamin B9), riboflavin (vitamin B2), cobalamin (vitamin B12), pyridoxine (vitamin B6) and choline (vitamin B4), as well as methionine and betaine. These substances play the role of both substrates and cofactors in transformations related to one-carbon metabolism. The deficiency of methyl nutrients in the body can lead to disturbances in SAM synthesis, which is the primary donor of methyl groups in the DNA methylation process. However, the mechanism explaining the discussed relationship has not been fully explained so far. Both the concentration in the body and the intake of folate and vitamin B12 in the diet can, to some extent, have an effect on the level of DNA methylation in healthy people. In comparison, data on the effect of excessive intake of vitamin B12 in the diet on the risk of cancer development are inconsistent. An adequate betaine and choline intake in the diet might not only affect the overall improvement of the DNA methylation profile, but, to some extent, also reduce the risk of cancer, the effect of which can depend on the content of folic acid in the body. Research results on the effect of supplementation of methyl nutrients on the DNA methylation process are inconclusive. It is therefore necessary to conduct further research in this area to draw clear conclusions.

Key words: *DNA methylation, diet, methyl nutrients, one-carbon metabolism, epigenetics*

STRESZCZENIE

Metylacja DNA jest odwracalną modyfikacją epigenetyczną, która odgrywa kluczową rolę w transkrypcyjnym wyciszaniu genów. Zarówno nadmierna (hipermetylacja), jak i zmniejszona metylacja DNA (hipometylacja) mogą przyczyniać się do zaburzenia prawidłowego przebiegu wielu istotnych procesów zachodzących w organizmie człowieka. Celem pracy było omówienie związku między metylowymi składnikami diety a procesem metylacji DNA w przebiegu wybranych chorób u osób dorosłych. Do metylowych składników pokarmowych zaliczane są: foliany (witamina B9), ryboflawina (witamina B2), kobalamina (witamina B12), pirydoksyna (witamina B6) i cholina (witamina B4) oraz metionina i betaina. Substancje te pełnią zarówno rolę substratów, jak i kofaktorów w przemianach związanych z metabolizmem jednego atomu węgla. Niedobór metylowych składników pokarmowych w organizmie może prowadzić do zaburzeń w syntezie SAM, będącej podstawowym donorem grup metylowych w procesie metylacji DNA. Mechanizm wyjaśniający omawianą zależność nie został jednak dotychczas w pełni wyjaśniony. Zarówno stężenie w organizmie, jak i podaż w diecie folianów oraz witaminy B12 mogą w pewnym stopniu oddziaływać na poziom metylacji DNA u osób zdrowych, przy czym dane dotyczące wpływu nadmiernej zawartości witaminy B12 w diecie na ryzyko rozwoju nowotworów są niespójne. Odpowiednia podaż betainy i choliny w diecie może wpływać korzystanie nie tylko na ogólną poprawę profilu metylacji DNA, ale w pewnym stopniu również na zmniejszenie ryzyka wystąpienia nowotworów, przy czym efekt ten może być zależny od zawartości kwasu foliowego w organizmie. Wyniki badań odnoszące się do wpływu suplementacji metylowymi składnikami pokarmowymi na przebieg procesu metylacji DNA są niejednoznaczne. Konieczne jest prowadzenie dalszych badań w tej dziedzinie w celu sformułowania jednoznacznych wniosków.

Słowa kluczowe: *metylacja DNA, dieta, metylowe składniki pokarmowe, metabolizm jednego atomu węgla, epigenetyka*

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ABBREVIATIONS

ADCY3 – *adenylate cyclase type 3*
BHMT – *betaine homocysteine methyltransferase*
BMI – *body mass index*
BRCA1 – *breast cancer gene 1*
C – *cytosine*
CBS – β-cystathionine synthase
CG – *cytosine-guanine*
CIN – *cervical intraepithelial neoplasia*
CpG – *cytosine-phosphate group-guanine*
DMG – *dimethylglycine*
DNA – *deoxyribonucleic acid*
DNMTs – *DNA methyltransferases*
EC-SOD – *extracellular superoxide dismutase*
ESR1 – *estrogen receptor 1*
GST – *glutathione S-transferase*
HDACs – *histone deacetylases*
HATs – *histone acetyltransferases*
HPV – *human papilloma virus*
LINE-1 – *long interspersed nucleotide element-1*
MBDs – *methyl-CpG binding domains*
MBPs – *methyl-CpG binding proteins*
MLH1 – *human MutL homolog 1*
MTHFR – *methylenetetrahydrofolate reductase*
PBMCs – *peripheral blood mononuclear cells*
RAPGEF4 – *rap guanine nucleotide exchange factor 4*
RARB – *retinoic acid receptor beta*
SAH – *S-adenosyl-L-homocysteine*

INTRODUCTION

Every human body contains a unique genome, which consists of two main components. The first is a double-stranded deoxyribonucleic acid (DNA) packed in condensed chromatin form that contains specific nucleotide sequences (genetic information record), and the second is methylation. Methylation is a complex molecular mechanism that regulates the course of numerous biochemical processes responsible for the correct reading of the genetic information stored in DNA, maintains genome stability, and controls the course of gene transitions [26, 42]. Methylation is one of the epigenetic processes that is not related to modifications of nucleotide sequences in DNA [17, 19, 33, 42]. While epigenetic changes occur at every stage of life and can be inherited, they do undergo modification under the influence of external factors, such as: lifestyle (including, in particular, diet, weight disorders and physical activity), as well as drugs and toxic substances [19, 22, 42].

In recent years, there has been a significant progress in studies on nutriepigenetics, including the analysis of nutrients influence on epigenetic processes, especially on their effect on modifying susceptibility to the development of many chronic diseases. It has been indicated that some nutrients may influence the regulation of gene expression. More and more attention is also paid to the analysis of the influence of selected nutrients on the course of the DNA methylation process [3, 13, 22, 26]. DNA methylation plays a key role not only in controlling processes in determining the gene expression level in cells, but also regulates the course of important biological processes taking place in the human body, such as genomic imprinting and X-chromosome inactivation [14, 26, 32, 33, 42]. DNA methylation may also condition the silencing of genes related to the aging process [20, 44]. Both excessive (hypermethylation) and reduced DNA methylation (hypomethylation) may contribute to the abnormalities of the proper course of many key body processes [3, 10, 13, 44]. Abnormalities in the DNA methylation profile are very often associated with the development of genetic diseases (including *Prader-Willi, Beckwith-Wiedemann*, and *Silver-Russell* syndromes) [14]. Numerous scientific research has also shown that abnormal DNA methylation can contribute to the initiation of the carcinogenesis process [19, 31, 40, 44, 48]. The research indicates that abnormalities in the DNA methylation profile may also be associated with the occurrence of cardiovascular diseases [17, 28, 36, 73], metabolic disorders (including obesity and type 2 diabetes) [10, 35, 50, 61] and schizophrenia [47, 55, 62] or depression [5, 9, 34]. It has also been assumed that the abnormal course of DNA methylation may play a significant role in the development of *Alzheimer's* disease pathogenesis [59, 64, 71] and liver diseases [12, 43, 67]. However, due to the inconsistent results of scientific analyzes carried out in this field, it is not possible to formulate clear conclusions on this subject. Understanding the relationship between the selected nutrients and the course of the DNA methylation process may be significant both in the context of controlling the rate of body aging, and in the prevention and treatment of diseases which pathomechanism is associated with DNA hypo- and/or hypermethylation [3, 10, 17, 19, 26].

The aim of the present study is to discuss the relationship between methyl nutrients and the DNA methylation process in the course of selected diseases in adults, based on a review of the current literature.

DNA METHYLATION

DNA methylation is a reversible, post-replication, enzymatic modification of DNA that plays a crucial role in transcriptional gene silencing. It occurs primarily during the S phase of the cell cycle. Methylation is a process of covalently attaching methyl groups ($-CH_3$) onto nitrogen bases of nucleotides – in particular to cytosine, but less often to adenine – and forming products of which the most frequent are C^5-methylcytosine (m^5C), and sometimes also N^4-methylcytosine (m^5C), and N^6-methyladenosine (m^6A) [16, 33, 42].

The DNA methylation process requires DNA methyltransferases (DNMTs) family enzymes such as adenine-specific and cytosine-specific DNA methyltransferases, which have an ability to transfer methyl groups between individual structures in the body [17, 19, 26, 33, 36, 40, 42]. Among DNMTs, DNA methyltransferase 1 (DNMT1) plays the most crucial role in regulating the DNA methylation process. This enzyme is responsible for copying the DNA methylation pattern to the newly synthesized DNA strands during the replication process. It also has the ability to repair errors that appear in the DNA methylation pattern. On the other hand, the DNA-methyltransferase 3-like (DNMT3L) participates indirectly in the DNA methylation process, and despite lacking catalytic properties, has the ability to stimulate activity of DNMT3a and DNMT3b methyltransferases, which are responsible for *de novo* methylation [22, 33, 36, 40, 42]. The primary role of DNMTs is to catalyze the additional reactions of methyl groups derived from the donor S-adenosyl-L-methionine (SAM), to the C^5 carbon of the cytosine pyrimidine ring, or to the amine group of adenine (N^6) or cytosine (N^4) [22, 26, 33]. This process leads to the formation of methylated DNA and S-adenosyl-L-homocysteine (SAH) [17, 26, 33, 39, 42]. The conversion reaction of SAM to SAH is a complex biochemical process known as one-carbon metabolism [16].

Methylation is a targeted process that mainly occurs on cytosines contained in CpG islands. CpG islands are short stretches of DNA (about 1000 bp) characterized by a high content of specific sequences of 5`-CpG-3` dinucleotides (cytosine-phosphate group-guanine) [17, 26, 33, 36, 39, 42]. The islands occur mainly within or in close proximity to the promoter regions that are the gene origin and are essential for cell functioning. About 70% of CpG dinucleotides in the human body have a methyl group attached to cytosine (CG – cytosine-guanine), and as a result the genes encoded by this sequence are not accessible for cellular transcription systems. There is an inverse relationship between the transcriptional activity of certain genes and the degree of methylation in the promoter regions – the higher the methylation level, the weaker the gene expression. On both DNA strands, the methylation at CpG sites occurs in a mostly symmetrical way and only a small number of CpG sequences undergo asymmetrical methylation [17, 26, 33, 39, 42]. The structure that results from the interaction of transcription factors with promoter genes is a open chromatin – a highly acetylated chromatin containing CpG islands [26, 33, 42]. About half of all human genes contain CpG islands, and these are called housekeeping genes, as well as tissue-specific genes. It has been considered that transcriptionally inactive sequences are most often methylated, while transcriptionally active genes are hypomethylated [17, 26, 33, 42].

Increasing the frequency of CpG islands methylation in the promoter regions leads to silencing of a certain gene expression due to chromatin condensation resulting from a decrease in histone acetylation. Consequently, this phenomenon leads to blocking or disturbing synthesis of the encoded products (mainly proteins) by a certain gene. The exact mechanism that determines this relationship is not yet fully understood. One of the proposed models assumes that this process involves methyl-CpG binding proteins (MBPs) that bind to the methylated DNA, with the participation of methyl-CpG binding domains (MBDs). The MBPs interact with chromatin remodeling complexes and with a histone deacetylase. These modifications result in histone H3 lysine 9 methylation, followed by the deacetylation of histones, causing a change in the chromatin structure from the active, relaxed form (euchromatin) to its concentrated form (heterochromatin), which is inaccessible to transcription factors. On the other hand, histone protein acetylation of lysine, which occurs at histone H4, leads to partial decondensation of chromatin, making it more available to transcription factors, and causing an increase in the gene expression level. This is due to the fact that the condensation and decondensation of chromatin take place in a dynamic way, because both forms can intertwine with each other to either allow or block gene transcription. Therefore, it has been indicated that gene expression can be modified both by DNA methylation and by acetylation or deacetylation of histones. Histones are transformed with the participation of histone deacetylases (HDACs) and histone acetyltransferases (HATs). However, it is important to note that due to the possibility of 5-methylcytosine deamination to thymine, epigenetic changes can be transformed into permanent mutations [16, 19, 22, 40, 42].

Methylation is a reversible process that is independent of DNA, because when necessary genes can be reactivated. However, the exact mechanism determining the course of this process has not yet been fully understood. It has been recognized that DNA demethylation can take place through enzymatic processes, and in a passive way. The enzymatic process occurs independently of the cell division cycle and under the influence of demethylases, stimulating the detachment of methyl groups from DNA, including the ten-eleven translocation proteins (TETs). The passive way occurs due to a lack of methylation of the newly synthesized DNA chain by DNMTs during replication [26, 33, 40, 42].

The DNA methylation pattern changes during the body's development, growth, and aging. Nevertheless, all properly functioning cells have

a stable DNA methylation pattern, which is specific to each cell type [3, 13, 26, 33, 36, 42]. Some of the changes in the DNA methylation pattern tend to be a physiological adaptation to changing environmental conditions, however, some changes may be related to a developing disease or intensification of the cell aging processes in the human body [20, 33, 44]. Regulatory mechanisms, such as DNA demethylation, chromatin structure modification, and active transcription, should prevent those changes in the DNA methylation pattern. If the functioning of these mechanisms is disturbed, an uncontrolled DNA methylation process can occur. This consists of a lowering of the genome methylation level, or the attachment of *de novo* methyl groups, the main consequence of which can be the disturbance of DNA integrity and stability. Changes in the DNA methylation pattern include, but are not limited to, hypo- and hypermethylation as well as m^5C transition to thymine. 5-methylcytosine formed as a result of cytosine (C) methylation in the DNA strand can undergo spontaneous deamination to thymine (T), resulting in the C → T transition. Due to the fact that DNA thymine glycosylase exhibits low repair activity that is additionally inhibited by DNMTs, this mutation is often not repaired, which can lead to its fixation. It has been shown that the DNA methylation pattern in the course of selected chronic diseases can differ from that which occurs under physiological conditions [26, 32].

MECHANISMS CONDITIONING THE INFLUENCE OF METHYL NUTRIENTS ON THE COURSE OF DNA METHYLATION

It is believed that diet can influence the course of DNA methylation as a result of the interaction of at least three different mechanisms [19, 26]. It should be emphasized that exact explanation of the biochemical mechanisms that determine the dependencies described below is not fully possible because in most of the scientific analyzes, the authors relied mainly on hypotheses and general assumptions. Therefore, it is necessary to conduct further research in this area in order to draw clear conclusions [26].

The first and most often-described mechanism is the optimal supply of methyl nutrients in one-carbon metabolism, which enables the proper course of the DNA methylation process. An adequate supply of methyl nutrients determines the production of SAM, which is the primary donor of methyl groups in the DNA methylation process. Methyl nutrients include vitamins, the most important of which are: folates (vitamin B_9), riboflavin (vitamin B_2), cobalamin (vitamin B_{12}), pyridoxine (vitamin B_6), choline (vitamin B_4), as well as amino acids such as methionine and betaine. These substances play the role of both substrates and cofactors in transformations related to one-carbon metabolism [16, 17, 26, 39, 40]. Methyl nutrient deficiencies in the body can lead to disturbances in SAM synthesis, and thus to the formation of abnormalities in various biochemical pathways in the DNA methylation process [16, 26, 40]. The analysis conducted by *Inoue-Choi* et al. [24] showed that the concentration of choline, methionine and SAH in the subjects' blood serum was positively correlated with the concentration of SAM in their blood. The authors emphasized the significant role of controlling the concentration of methyl nutrients in the blood serum in the context of preventing the occurrence of DNA methylation disorders resulting from insufficient SAM content in the body.

Another mechanism that can determine the course of the DNA methylation process is the influence of nutrients on the change in the activity of enzymes related to the regulation of the DNA methylation process, especially with regard to one-carbon metabolism [16, 19, 26, 40]. It has been indicated that a deficiency of methyl nutrients can indirectly interfere with the activity of DNA methyltransferases as a result of the insufficient supply of SAM. Moreover, it is presumed that some nutrients can play the role of DNMTs inhibitors in the human body [19, 26].

It has been assumed that another mechanism explaining the discussed dependence can also be the indirect influence of nutrients on the process of DNA demethylation [26]. This is because individual publications describe vitamin C as potentially affecting the process of DNA demethylation by supporting the reactions associated with TETs proteins [41, 69]. It has also been indicated that disturbances in the regulation of transformations related to TETs may also result from insufficient supply of methyl nutrients [66]. However, it should be emphasized that the hypotheses explaining the influence of diet on the process of DNA demethylation are still inconsistent [26, 41, 66, 69].

The DNA methylation process includes the interconnected cycles of folate and methionine, in which individual nutrients play both the role of substrates and cofactors of biochemical reactions (Fig. 1) [39]. Folates are found in many food products. Their rich sources include green leafy and cruciferous vegetables, legume seeds, avocados, liver, beets and eggs [39, 40]. Dietary folates are metabolized to tetrahydrofolate (THF), which through numerous changes is converted to 5-methyl-THF under the influence of, among other things, serine hydroxymethyltransferase (SHMT) and vitamin B_6, as well as methylenetetrahydrofolate reductase (MTHFR) and vitamin B_2 [16, 17, 39, 40]. Vitamin B_6 is mainly found in fish, meat, offal, as well as in legume seeds, nuts and seeds. Wholegrain cereal products are also valuable sources of this vitamin.

Whereas the basic nutritional sources of vitamin B_2 are milk and its products, eggs and offal [40]. 5-methyl-THF transfers the methyl group to the methionine cycle as a result of homocysteine remethylation to methionine [16, 17, 39, 40]. This reaction requires the participation of vitamin B_{12}, which is present only in animal products – meat, fish, eggs, dairy products and offal [39, 40]. The richest sources of methionine in food are primarily protein-rich products, e.g. meat, fish, eggs, dairy products, soybeans and Brazil nuts [39]. 5-methyl-THF can be converted back to THF and reused in the above DNA methylation process. Moreover, homocysteine can also receive a methyl group, which is necessary for the production of methionine, as a result of the conversion of betaine to dimethylglycine (DMG) taking place under the influence of betaine homocysteine methyltransferase (BHMT) [16, 26, 39, 40]. Betaine can originate from both food and the process of endogenous choline oxidation. Foods such as quinoa, wheat germ, bran, beetroot, spinach and seafood are a rich source of betaine in the diet [39]. The main food sources of choline are egg yolk, offal, wheat germ, soybeans, meat and fish [39, 40]. The methionine formed from homocysteine is then converted to SAM, from which, with the participation of DNMTs, methylated DNA (in the form of 5-methylcytosine) and SAH are formed. Then, under the influence of SAH hydrolase, SAH is hydrolyzed to adenosine and homocysteine. Subsequently, homocysteine can be reused in the methionine cycle or be broken down to cystathionine and cysteine by trans-sulfuration, which is dependent on β-cystathionine synthase (CBS) and vitamin B_6 [16, 17, 39, 40]. Cysteine can then be used for the synthesis of body proteins or be transformed, for example, into taurine and glutathione. Glutathione shows strong antioxidant properties [16, 40].

Upon the transfer of methyl groups, SAM is converted to SAH, which competes with SAM for influence over the DNA methyltransferases. SAH acts as a DNMT inhibitor, therefore its excess in the body can disturb the course of reactions catalyzed by this group of enzymes [16, 26]. It has been shown that changes in SAM and SAH levels resulting from a methyl-deficient diet for a period of over 18 weeks led to irreversible changes in DNA methylation in the liver cells of F344 male rats [54]. Moreover, it has also been found that a moderate increase of homocysteine levels in the blood serum is associated with an increase in SAH levels, with no changes in SAM levels, while the increased SAH levels are associated with the occurrence of DNA hypomethylation [70]. Maintaining the balance between SAM and SAH, which relies on stimulating SAM synthesis and supporting SAH removal, seems to be crucial in ensuring the proper DNMT activity. Nevertheless, due

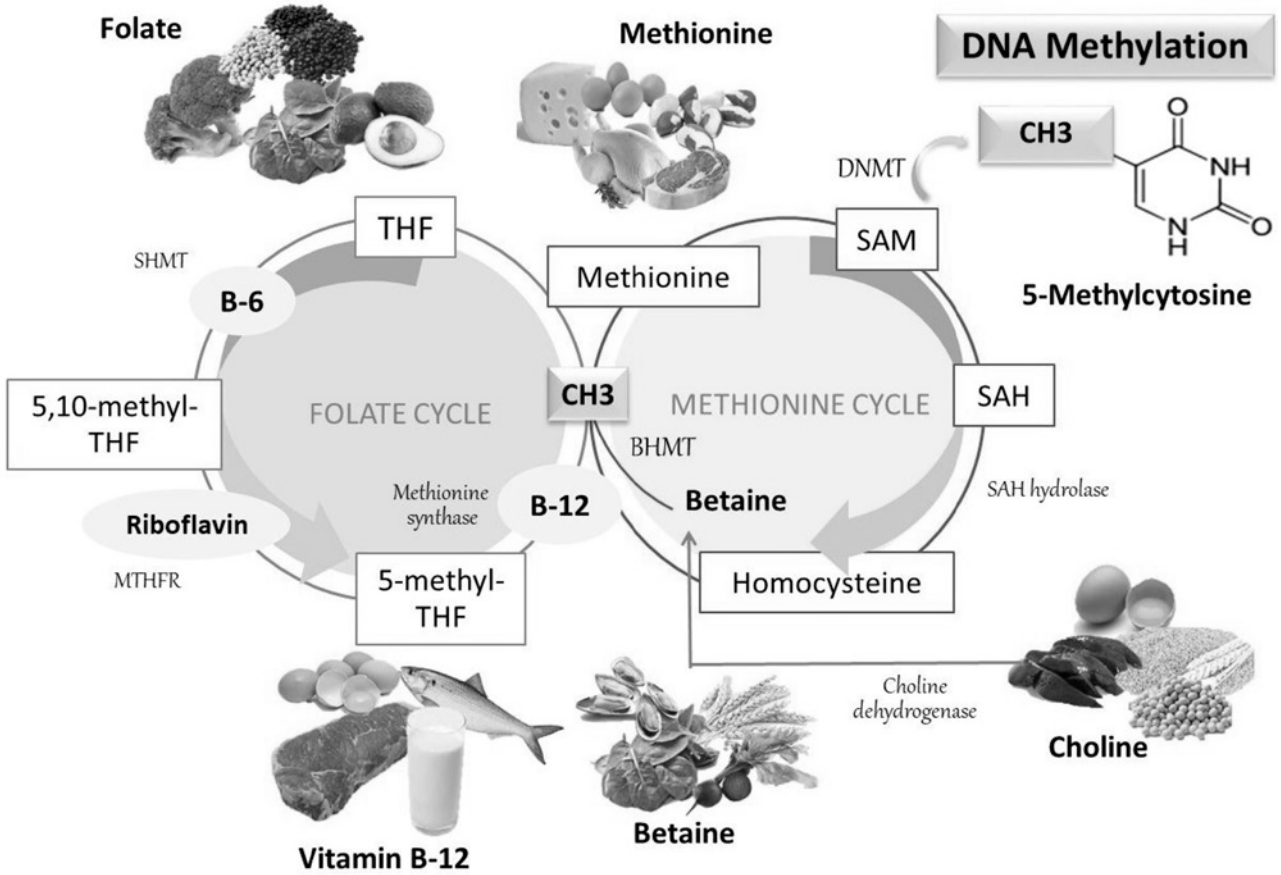

Figure 1. Nutrients involved in one-carbon metabolism [39]

to the complexity of SAM and SAH transformations in the human body, it is not possible to formulate unambiguous conclusions indicating that the SAM to SAH ratio could be a common indicator determining the risk of DNA hypo- or hypermethylation in the human body [26].

EFFECT OF METHYL NUTRIENTS ON THE PROCESS OF DNA METHYLATION IN THE COURSE OF SELECTED DISEASES IN ADULTS

The relationship between methyl nutrients and the course of the DNA methylation process has been described in several animal studies [11, 27, 54]. *Pogribny* et al. [54] found that in F344 male rats, which were fed a methyl-low diet for 9 weeks (low in methionine, choline and folates), SAH did not change but SAM content decreased by 70% compared to rats on a control diet. Thus, a significant imbalance between SAM and SAH was demonstrated in the organisms of the tested animals. In addition, it was also observed that the number of unmethylated CpG sites in liver cells increased by 60% as a result of the methyl-deficient diet. Interestingly, the reintroduction of a diet with the correct methyl nutrient content made it possible to reverse previously induced DNA hypomethylation only in the rats that were fed a deficient diet for 9 weeks. For animals that were exposed to methyl nutrient deficiencies for 18, 24, or 36 weeks, reintroducing these nutrients did not influence the normalization of the DNA methylation levels. It is worth noting, however, that the presence of glutathione S-transferase (GST), which is associated with the process of hepatocarcinogenesis, was confirmed in the liver tissue of the studied rats even after 9 weeks of using a methyl-low diet. In a study by *Kalani* et al. [27] for 6 weeks C57BL/6J mice were fed a diet containing increased methionine content (1.2%) and a reduced amount of folate (0.08 mg/kg body weight), vitamin B_6 (0.01 mg/kg body weight), and vitamin B_{12} (10.4 mg/kg body weight). Based on analysis, it was shown that the C^5-methylcytosine levels in the brain of the tested mice had increased as a result of the applied diet. Moreover, in mice on a methionine-rich diet and deficient in folates and vitamins B_6 and B_{12}, a decrease in the expression of the netrin-1 protein was observed as a result of the increase in the methylation level in the promoter region of the netrin-1 gene, which resulted in the occurrence of long-term memory disorders in the tested animals. Interestingly, the mice's long-term memory improved when netrin was readministered. The authors indicated that, based on the results obtained in the study, it should be assumed that a methyl-deficient diet can contribute to the development of learning and memory disorders. The authors also stated that the decrease in netrin-1 expression due to hypermethylation of its gene can, presumably, be associated with impairment or loss of long-term memory. In a study by *Cordero* et al. [11], *Wistar* rats were fed one of three diets: a Western-type diet (defined as increasing the risk of obesity), a Western-type diet additionally supplemented with methyl nutrients (choline, betaine, vitamin B_{12} and folic acid), and a control group. It was shown that after 8 weeks, the rats in both of the intervention groups gained body weight and increased their body fat. Interestingly, the intensification of fatty liver was observed only in the animals on the Western-type diet. In rats that were additionally supplemented with methyl nutrients in addition to the Western-type diet, the fatty liver was reduced. The authors indicated that methyl nutrients can have a potentially protective effect against the accumulation of excess fat tissue caused by a high-fat diet, however, the effect is probably limited to only the liver. *Cordero* et al. also suggested that the potential mechanism determining this link might, to some extent, be related to the influence of methyl nutrients on the course of the DNA methylation process in liver cells. However, it was emphasized that in the rats from both intervention groups there were some changes in the DNA methylation profile.

Analysis of the overall relationship between methyl nutrients and the course of the DNA methylation process has also been described in human studies [3, 8, 38, 51]. A systematic review conducted by *Amenyah* et al. [3] demonstrated a relationship between methyl nutrients that are specific to one-carbon metabolism, and the DNA methylation process. In a study by *Li* et al. [38] involving the 6-year observation of 5,687 women aged 65-79, it was found that a diet that is rich in vitamin B – which is a methyl donor in the DNA methylation process (along with folate, vitamin B_2, B_6 and B_{12}) – can modify long-term changes in body weight and body fat. This happens through the effects of these nutrients on DNA methylation determined by the rs752579 variant of the sterol regulatory element binding factor 1 (SREBF1) gene. However, not all scientific analyses in this area have obtained similar conclusions. In a study by *Perng* et al. [51], conducted on 987 healthy people aged 45-84, no correlation was found between the supply of methyl nutrients in the diet and the DNA methylation level in the tested subjects. However, it was found that a higher body mass index (BMI) in the subjects was associated with a higher degree of methylation of the long interspersed nucleotide element-1 (LINE-1). In another analysis by *Chamberlain* et al. [8] conducted on 5,168 healthy adults, no correlation was found between the methyl nutrients content in the diet and the CpG island methylation level in the tested subjects.

Nevertheless, most scientific studies have focused on the assessment of the effect of individual methyl nutrients on the course of DNA methylation [7, 18, 45, 58]. The compounds most often reported in this context were folate and vitamin B_{12}. Folates are the primary methyl-group donors in the DNA methylation process. In comparison, appropriate amounts of vitamin B_{12} are required to remethylate homocysteine to methionine because vitamin B_{12} acts as a cofactor in this reaction. Therefore, it has been assumed that a deficiency of both of these components can lead to disturbances in the course of changes related to SAM and SAH in the body [16]. In an analysis by *Hirsch* et al. [18], conducted on a group of healthy people, it was found that higher folic acid levels in the blood serum (>45 nmol/L) was associated with an increased content of SAM and SAH in the erythrocytes of the tested subjects. This is not, however, related to the SAM-to-SAH ratio, and the CpG island methylation level in the promoter region of the extracellular superoxide dismutase (EC-SOD) gene. Neither was a correlation found between the vitamin B_{12} levels in the blood serum and the SAM or SAH content in the erythrocytes of the tested subjects. Alternatively, *Pufulete* et al. [58] showed that the folic acid levels both in blood serum and in erythrocytes were inversely correlated with the plasma homocysteine levels ($r = -0.573$, $p < 0.001$ and $r = -0.307$, $p = 0.01$, respectively), as well as with the presence of DNA hypomethylation in the colon mucosa ($r = -0.311$, $p = 0.01$ and $r = -0.356$, $p = 0.03$). When gender, age, BMI, smoking, and genotype were taken into account, however, it was found that this relationship was much weaker ($p = 0.07$ and $p = 0.08$). On the other hand, in an analysis by *Morris* et al. [45] conducted on 1,858 people ≥60, it was found that the concentration of 5-methyl-THF in the body was associated with better results of tests assessing cognitive functions in tested subjects with no vitamin B_{12} deficiency in the blood serum. A study by *Bednarska-Makaruk* et al. [7] carried out on a similar age group, showed that patients with dementia had lower folic acid levels ($p = 0.002$) and 5-methyl-THF ($p = 0.005$) in the blood than those without memory impairment. Moreover, a positive correlation was also observed between the concentration of folic acid in the blood serum and the DNA methylation level in the tested subjects ($p = 0.013$).

A deficiency of folic acid and vitamin B_{12} in the body can also be a risk factor for the development of certain types of cancer [4, 53, 57]. An analysis by *Piyathilake* et al. [53] of 315 women who tested positive for human papilloma virus (HPV) type 16, who were diagnosed with cervical intraepithelial neoplasia (CIN), stage CIN2+ or ≤CIN1, showed that in women with a higher degree of HPV 16 DNA methylation and a higher concentration of both folic acid and vitamin B_{12} in the plasma, the probability of CIN2+ was lower by 75% ($p < 0.01$) and 60% ($p = 0.02$), respectively. The authors suggested that maintaining the appropriate methyl donors content in the body, including in particular folic acid and vitamin B_{12}, can play a critical role in ensuring a high degree of CpG islands methylation, among other things, within the promoters of the HPV E6 gene, which are associated with a higher probability of being diagnosed with CIN2+. On the other hand, a study by *Badigi* et al. [4] found that women with higher blood homocysteine levels were more likely to be diagnosed with CIN2+ (OR = 1.86, $p = 0.005$). In contrast, the higher folic acid levels in the plasma were a significant determinant of lower blood homocysteine levels in the tested subjects (OR = 0.4, $p = 0.0002$). In women with homocysteinemia, a lower degree of methylation of LINE-1 of peripheral blood mononuclear cells (PBMCs) (OR = 2.3, $p = 0.0007$) was observed significantly more frequently (PBMCs are a potential biomarker of CIN2+ development). The authors emphasized that maintaining the appropriate content of folic acid in the body can have a potentially beneficial effect on reducing homocysteine blood levels and increasing the degree of PBMC LINE-1 methylation, which could, consequently contribute to the reduction of the risk of developing CIN2+. In another analysis by *Pufulete* et al. [57] it was showed that patients diagnosed with colorectal cancer had a 26% lower folic acid content in the body (95% CI: 6-44%, $p = 0.01$) and a 21% lower vitamin B_{12} level in the blood (95% CI: -38-1%, $p = 0.06$), compared to a control group of people without colorectal disease. Moreover, it was also found that high folic acid levels in the blood were associated with a reduced risk of developing colorectal cancer ($p = 0.01$). While the DNA hypomethylation of colon cells and leukocytes was associated with an increased risk of colorectal adenoma ($p = 0.02$ and $p = 0.01$, respectively).

Apart from the assessment of the relationship between the total content of folic acid and vitamin B_{12} in the body and DNA methylation, scientific research also analyzed the impact of the supply of these components in the diet on the course of the discussed process of DNA methylation [1, 29, 52, 63]. In a study by *Agodi* et al. [1] carried out on 177 healthy, non-pregnant women, it was shown that subjects whose fruit consumption was lower than the median value for the whole group (<201 g/day) were 3.7 times more likely to develop LINE- 1 hypomethylation, compared to women with fruit consumption higher than the median (OR = 3.7, 95% CI: 1.4-9.5). Similarly, the female subjects whose dietary folate intake was 3.6 times lower than the demand, were more likely to develop LINE-1 hypomethylation compared to women with normal dietary content of folates in their diet (OR = 3.6, 95% CI: 1.1-12.1). The authors have indicated

that low fruit consumption and too low dietary folate content may possibly increase the risk of cancer development because of LINE-1 hypomethylation. Notwithstanding, in the study by *Pirouzpanah* et al. [52], an inverse relationship was found between the folate supply in the diet and the intensification of the hypermethylation process in the promoter regions of retinoic acid receptor beta (RARB) gene, such as in female patients <48 years of age and breast cancer gene 1 (BRCA1), and in tested subjects >48 years old age. It should be emphasized that with regard to the vitamin B_{12} content in their diet, this relationship was significant only with the RARB gene, and only in female patients aged <48 years old. The authors indicated that an insufficient supply of folate and vitamin B_{12} in the diet can contribute to the increased likelihood of breast cancer development as a result of hypermethylation of the promoter regions of the discussed genes. However, this effect is probably dependent on the age of the female patients. In a study by *Kawakita* et al. [29], covering over 12.5 years of observation, it was shown that higher intake of folate in the diet and higher consumption of folic acid-fortified products were associated with a reduced risk of head and neck cancer. However, this effect was dose-dependent (for folate intake in the diet: quartile 1 vs. 4: HR = 0.35, 95% CI: 0.18-0.67, and for the consumption of folic acid-fortified products: quartile 1 vs. 4: HR = 0.49, 95% CI: 0.3-0.82). The authors suggest that the above results are presumably related to the role of folates in regulating the DNA methylation process, but this relationship was not analyzed in the study. Interestingly, in a study by *Steluti* et al. [63], no correlation was found between dietary folate supply and the level of DNA methylation, even in people who consumed folic acid-fortified flours.

One of the best-studied issues in the context of the discussed topic is the analysis of the effect of supplementation with selected methyl nutrients, including folic acid and vitamin B_{12}, on the DNA methylation level and the risk of developing diseases associated with disturbances in this process [3, 6, 21, 25, 30]. In a randomized, double-blind study by *Kok* et al. [30] conducted on healthy people aged 65-75 with moderately elevated levels of homocysteine in the blood serum, it was shown that 2-year supplementation with folic acid at a dose of 400 μg/day and vitamin B_{12} at a dose of 500 μg/day contributed to the occurrence of changes in the DNA methylation profile of several genes, including genes related to neurological functions and the process of carcinogenesis. Nonetheless, in a study by *Bea* et al. [6] conducted on 408 healthy, postmenopausal women, a dependence between periods of folic acid supplementation and its concentration in erythrocytes was observed, compared to the DNA methylation level. It was shown that in women with a higher concentration of folic acid in erythrocytes (compared to the lower concentration of this parameter), the average DNA methylation level was higher in the period before supplementation (5.12 vs. 4.99%, p = 0.05), but decreased in the post-supplementation period (4.95 vs. 5.16%, p = 0.03). The authors emphasized that the increased concentration of folic acid in erythrocytes as a result of supplementation can affect the DNA methylation levels in leukocytes in postmenopausal women. However, not all scientific analyses have obtained similar results on the effect of supplementation with selected methyl nutrients on the course of the DNA methylation process. In a randomized, double-blind study conducted by *Jung* et al. [25] it was found that a 3-year supplementation with folic acid at a dose of 800 μg/day did not affect the DNA methylation level in a population of healthy women and men aged 50-70 with moderate hyperhomocysteinemia. Moreover, in the study by *Hübner* et al. [21] no relationship was found between the 1-year supplementation of B vitamins (500 μg of folic acid, 500 μg of vitamin B_{12} and 50 mg of vitamin B_6 per day), vitamin D (1,200 IU/day) and calcium (456 mg/day) on the SAM and SAH levels in the blood serum and the LINE-1 methylation level, in a group of people aged 46-88. Nevertheless, in a meta-analysis conducted by *Amenyah* et al. [3] it was found that despite many methodological inaccuracies, it could be concluded that supplementation with folic acid, both alone and in combination with vitamin B_{12}, can increase the DNA methylation level. However, a need for further analyses on this topic was emphasized, to obtain data enabling the formulation of unambiguous conclusions in this area.

A study by *Li* et al. [37] conducted on obese C57BL/6J mice showed that a 10-week, fat-rich diet, combined with folic acid supplementation, contributed to the reduction of body fat and blood glucose levels, and improved insulin sensitivity in the tested mice. The animals that were fed only with a high-fat diet showed increased weight gain compared to mice in the control group. In addition, it was shown that animals on a high-fat diet without additional folic acid supplementation developed type 2 diabetes and insulin resistance. The authors also indicated that the observed differences between the intervention groups resulted from the fact that folic acid supplementation contributed to the reduction of DNA methylation within adipose tissue of adenylate cyclase type 3 (ADCY3) gene, and rap guanine nucleotide exchange factor 4 (RAPGEF4) gene, which have the ability to influence adipose tissue metabolism and regulate insulin sensitivity in the body. Conversely, *Park* et al. [49] found in their analysis that the changes in DNA methylation patterns as a result of folic acid supplementation were different in women aged 18-35, and depended on their body

weight. As a result of folic acid supplementation at a dose of 800 μg/day for 8 weeks, serum folic acid levels increased both in women with normal body weight (from 38.36 ± 2.5 to 71.41 ± 3.03 nmol/L), and those with obesity (from 27.12 ± 3.09 to 56.85 ± 3.9 nmol/L). However, in the obese women, this level was significantly lower than in the women with normal body weight, before and after the introduction of folic acid supplementation in both groups. Nevertheless, the change in serum folic acid levels as a result of the introduced supplementation was greater in the obese women than those with normal body weight (109.6% vs. 86.2%). Moreover, it was indicated that the number of changes occurring in DNA methylation as a result of folic acid supplementation was higher in the obese women, compared to those with normal body weight (99 vs. 56 CpG stretches). The authors suggested that the need to modify the recommendations for folic acid supplementation doses depending on women's body weight should be considered.

There have also been studies that analyzed the effect of folic acid supplementation on the risk of developing cancer, and the course of cancer, in relation to disturbances in the DNA methylation process [2, 15, 29, 46, 56, 60]. In a randomized study by *O'Reilly* et al. [46], which included post-polypectomy patients using folic acid supplementation at a dose of 600 μg/day for 6 months, an increase in folic acid levels was observed that was determined directly in colonocytes, as well as a decrease in the DNA methylation level compared to a placebo group. The authors suggested that folic acid supplementation in patients at risk of developing colorectal cancer can affect the folic acid supplementation deficiency directly in the colonocytes, which presumably reduces the risk of initiating the process of carcinogenesis due to DNA hypomethylation. A randomized study by *Pufulete* et al. [56] found that DNA hypomethylation caused by insufficient folate intake in the diet can be normalized as a result of the implementation of folic acid supplementation. The same study showed that folic acid supplementation for 10 weeks at a dose of 400 μg/day in patients with colorectal adenoma had the following effects: 81% increased concentration of folic acid in blood serum (95% CI: 57-104%, $p < 0.001$ vs. placebo), 57% increased concentration of folic acid in erythrocytes (95% CI: 40-74%, $p < 0.001$ vs. placebo), 12% decreased serum homocysteine levels (95% CI: 4-20%, $p < 0.001$ vs. placebo), 31% increased methylation in leukocytes (95% CI: 16-47%, $p = 0.05$ vs. placebo), and 25% increased methylation in the colon mucosa (95% CI: 11-39%, $p = 0.09$ vs. placebo). Interestingly, not all studies have formulated similar conclusions about these dependencies. In the previously mentioned study by *Kawakita* et al. [29], no evidence of the relationship between folic acid supplementation and the risk of developing head and neck cancer was found. Moreover, a randomized, double-blind study [2] of a 10-week folic acid supplementation at a dose of 400 μg/day in patients with colorectal adenoma, with no deficiency of folic acid and vitamin B_{12} in the body, found no effect on the improvement of methylation of promotor regions of the estrogen receptor 1 (ESR1) and human *MutL* homolog 1 (MLH1) genes. ESR1 and MLH1 genes can undergo hypermethylation in the course of colon tumors. Surprising conclusions were formulated in the study by *Farias* et al. [15], carried out using human colonosphere formation in a colon cancer cell line grown in vitro. It was shown that long-term increased folic acid intake from both folic acid supplements and folic acid-fortified food may lead to abnormalities in DNA methylation patterns, especially those related to DNMT activity. It was suggested that this relationship can cause initiation of carcinogenesis, or the progression of pre-existing neoplastic tumors. The authors have emphasized the need for further analyses on the impact of excessive supplementation with methyl nutrients on the risk of development or progression of cancerous tumors. A meta-analysis conducted by *Qiang* et al. [60] found that each increase in dietary folate content by 100 μg/day was associated with a 12% reduction in the risk of development of esophageal cancer. Moreover, each increase of B_6 vitamin intake in a diet by 1 mg/day contributed to the reduction of the risk of esophageal cancer development by 16%. Interestingly, it was shown, however, that each increase of B_{12} vitamin intake in a diet by 1 μg/day was associated with a 2% increase in esophageal cancer risk, particularly in the United States and Europe, which could indicate both geographic and histological differences in this regard. The authors concluded that the increased intake of dietary one-carbon metabolism-related B vitamins could be a protective factor in the risk of developing esophageal cancer, with the exception of vitamin B_{12}, the supply of which in the diet should be controlled.

There are not many studies analyzing the effects of choline and betaine on the course of DNA methylation in adults [23, 63]. Both of these components play a crucial role in one-carbon metabolism. Betaine is involved in the process of converting homocysteine to methionine, and can be obtained from diet or as a result of choline oxidation in endogenous processes [16]. In the study by *Steluti* et al. [63], it was found that of all methyl nutrients, only the supply of betaine in the diet had an effect on modifying the DNA methylation level. *Imbard* et al. [23] conducted a study on 109 healthy volunteers, analyzing the relationship between the concentration of choline and betaine in the blood serum, folate, SAM, SAH and homocysteine levels in the blood, and DNA methylation levels. Based on the obtained data, a strong positive correlation

was found between the choline levels and folic acid levels in the blood. A strong positive correlation was also found between choline and SAM and SAH levels in blood serum, although in the multivariate linear regression model no correlation was found between blood choline levels and the SAM-to-SAH ratio. Moreover, it was pointed out that the serum betaine levels were positively correlated with folic acid levels and negatively with the blood homocysteine levels. Interestingly, no statistically significant relationship between the analyzed indicators' levels in the blood serum and DNA methylation levels was confirmed. Nevertheless, the authors emphasized that there is a need to extend the scope of the scientific research evaluating the effect of methyl nutrients on the course of the DNA methylation process by analyzing the role of choline and betaine in this regard. Several studies have also assessed the effect of dietary choline and betaine intake on the risk of developing various types of cancer [65, 68, 72]. One such analysis was a two-stage case-control study by *Zhang* et al. [72], with the participation of 807 women diagnosed with breast cancer, and 807 healthy women qualified for the control group. Based on the obtained data, an inverse relationship was found between the choline and betaine content in the diet and the risk of breast cancer development. The adjusted odds ratio (OR) for the highest quartile intake of the analyzed nutrients, in comparison with their lowest amount in the diet, was, respectively: 0.4 for choline (95% CI: 0.28-0.57, $p < 0.001$), 0.58 for betaine (95% CI: 0.42-0.8, $p < 0.001$), and 0.38 for choline and betaine combined (95% CI: 0.27-0.53, $p < 0.001$). Interestingly, it has also been found that the described relationship was statistically significant only in subjects with low dietary folate intake (<242 μg/day). *Zhang* et al. [72] emphasized the importance of the role of appropriate choline and betaine content in the diet, with regards to reducing the risk of breast cancer development in women. However, the potentially beneficial effects can also depend on the possible occurrence of folic acid deficiency in the body. Another study, by *Ying* et al. [68], analyzed the effect of choline and betaine intake in the diet on the risk of developing lung cancer, depending on smoking. Higher betaine content in the diet was statistically significantly associated with a reduced risk of lung cancer in smokers, with the protective effect being more pronounced in current smokers than in ex-smokers. Similar relationships were also observed with regard to choline content in the diet, however, the effect was less pronounced. The authors indicated that based on their results, it can be assumed that increased betaine intake in the smokers' diet, as well as choline (but to a lesser extent), can be a protective element in the risk of lung cancer development, through some kind of mitigation of the adverse effects of smoking. Meta-analysis conducted by *Sun* et al. [65] found that choline and betaine intake in a diet may reduce the risk of developing neoplastic diseases, however, the authors emphasized the need for further analyses in this area due to the small number of studies conducted so far. It was shown that the relative risk (RR) of cancer occurrence for the lowest and the highest ranges was, respectively: 0.82 (95% CI: 0.7-0.97) for the choline intake, 0.86 (95% CI: 0.76-0.97) for betaine in the diet, and 0.6 (95% CI: 0.4-0.9) for the total choline and betaine in the diet. It was determined that increasing the choline and betaine content by 100 mg/day contributed to the reduction of risk of developing cancer by 11% (0.89, 95% CI: 0.87-0.92).

CONCLUSIONS

Despite significant scientific progress in the field of nutriepigenetics, it is not possible to draw unambiguous conclusions about the relationship between methyl nutrients and the DNA methylation process in the course of selected diseases in adults. There are few randomized studies of high methodological quality that have analyzed this relationship in humans. Due to the fact that some of studies conducted so far were performed on animals and others on human cell lines, the comparison of these results is difficult. It is necessary to conduct further analyses on this topic, in order to obtain data enabling the formulation of unambiguous conclusions in this area.

Based on the research results examined above, it should be assumed, however, that both folic acid and vitamin B_{12} levels in the body, as well as folate and vitamin B_{12} intake in the diet of healthy people, can affect their DNA methylation level to some extent. In contrast, the data on the effect of excessive vitamin B_{12} content on the risk of developing cancer is inconsistent. Moreover, it has also been indicated that the control of the folic acid content, and to a lesser extent also of vitamin B_{12} in the body, can be particularly significant in patients at risk of developing cancer.

Research results on the effect of the supplementation of methyl nutrients on the DNA methylation process is inconclusive. Some studies have shown that folic acid supplementation, both alone and in combination with vitamin B_{12}, can increase the DNA methylation level. Nevertheless, many analyses have not confirmed this effect, or even an increase in the risk of developing cancer as a result of such diets.

There are few studies assessing the effect of choline and betaine on the course of DNA methylation in adults. On the basis of the cited studies, however, it should be concluded that the appropriate intake of both betaine and choline in the diet can have a beneficial effect not only on the overall improvement of the DNA methylation profile, but also to some

extent, on reducing the risk of developing cancer, and this effect may depend on the folic acid content in the body. Nevertheless, there is a need to extend the scope of scientific research evaluating the effect of methyl nutrients on the course of DNA methylation based on analyses of betaine and choline effect in this context, due to the small number of valuable studies carried out on this topic so far.

It should be emphasized, however, that the exact explanation of the biochemical mechanisms that determine the dependencies described above is not fully possible because in most of the scientific analyses, the authors relied mainly on hypotheses and general assumptions. More research is needed in this area in order to draw clear conclusions and fully understand these relationships. This could contribute to obtaining data that could be used to formulate guidelines for recommended intakes of methyl nutrients for dietary amounts, and supplementation. This recommendation could be valuable both in the context of prevention and treatment of selected diseases in adults, including cancer diseases in particular.

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