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Review

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Is folic acid supplementation to food benefit or risk for human health?

Abstract: The need for sufficient quantities of folic acid for normal embryogenesis and fetal development is well known. Women of childbearing age must be sure to have an adequate intake of folic acid periconceptionally prior to and during pregnancy. Folate plays an essential role in the biosynthesis of RNA and DNA, phospholipid and neurostransmitters synthesis, using S-adenosylmethionine (SAM) as the primary methyl group donor. In some countries, the knowledge that folic acid is preventable for the reduction of the risk for neural tube defects (NTD) and in lowering the high amount of homocysteine (Hcy) led to the recommendation that all women capable of becoming pregnant should consume 400 μg of folic acid daily. Later, in 1998, folic acid fortification of all enriched cereal grain product flour was implemented in some countries. In addition to careful monitoring of adverse effects, the studies are warranted in order to determine the potentially positive and deleterious effects of folic acid fortification and supplementation on the human health or human diseases. To further understand the metabolic action of folates we asked ourselves the question: Is the intake of a sufficient amount of this vitamin a benefit or risk for human health?

Keywords: deficiency; folates; food fortification; human health; polyamines.

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Introduction

Folic acid (FA), folacin (Vitamin B_9) or pteroylglutamic acid is a water-soluble vitamin. FA gets its name from the Latin word *folium* meaning 'leaf', since it is found in many leafy plants. The best folate sources in the diet are leafy green vegetables, as well as animal liver and kidney. Mammalian cells are devoid of the enzymatic capacity for folate biosynthesis and thus are absolutely dependent on folate uptake from exogenous dietary sources. Thus, the intestine plays a central role in regulating body folate homeostasis; similarly, the kidneys play a pivotal role in regulating body folate homeostasis by reabsorbing the filtered vitamin, thus preventing its loss in urine [1–4]. The primary deposits of folate are liver and kidney [5, 6].

The term folates refer to the various tetrahydrofolate derivatives naturally found in food, whereas FA refers to the oxidized synthetic compound used in dietary supplements and fortified food.

According to its structure, FA belongs to conjugated pteridines, consisting of a pteridine ring, paraaminobenzoic acid and glutamic acid. It appears in monoglutamate or polyglutamate forms. Only the monoglutamate form may be absorbed in intestinal wall [5, 6].

It is well established that adequate folate intake from the consumption of folate-rich foods is essential for health [2, 3]. The FA coenzymes are specifically concerned with biochemical reactions involving the transfer and utilization of the single carbon (C_1) moiety, such as methyl-, methylene-, methenyl-, formyl or formimino-groups to various substrates in a variety of enzymatic reactions that are intimately related to the synthesis of DNA, RNA, proteins and phospholipids (lecithin and sphingomyelin) and for the metabolism of several amino acids, including methionine, histidine, serine, glycine, and for some neurotransmitter synthesis (acetylcholine, epinephrine, melatonin) (Figure 1) [2, 3, 5, 6].

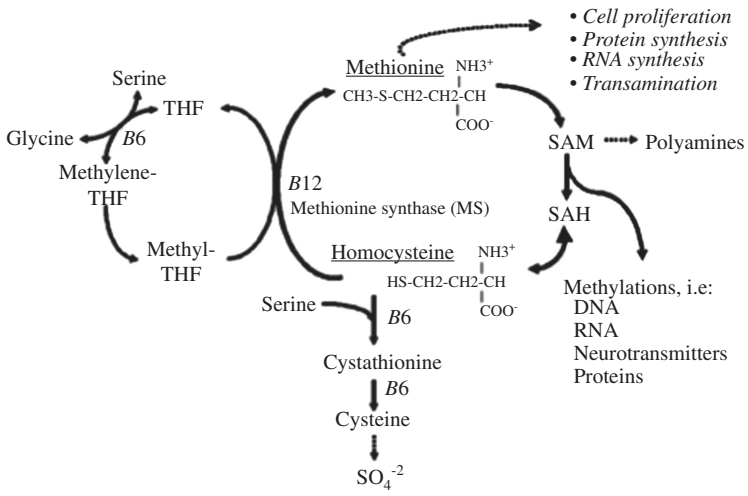

Figure 1 Folate has many functions in the body [3 – modified]. Copyright [2013, Medical faculty].

Within cells (principally liver cells), FA is reduced to dihydrofolate (DHF or H_2-folate) and then to tetrahydrofolate (THF or H_4-5, 6, 7, 8- tetrahydrofolic acid), by the action of folate and dihydrofolate reductase (DHFR), respectively [4]. THF is the form of folate that can enter the main folate metabolic cycle [2, 3, 5–7].

By regulating cellular S-adenosylmethionine (SAM or AdoMet), folates affect the important reactions in intermediary metabolism. SAM acts as a methyl donor for the dozens of different methyltransferases present in all cells. AdoMet is also used for polyamine spermidine (Spd) and spermine (Sp) biosynthesis (Figure 2) [8–10] .

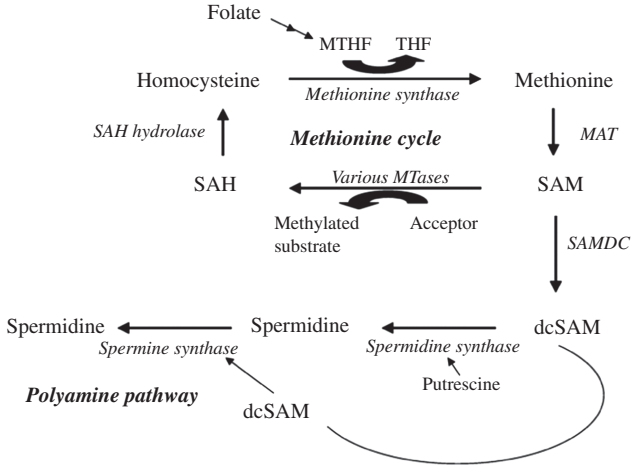

Figure 2 Folate and polyamines biosynthesis.
SAM is generated from methionine and ATP by MAT. dcSAM, decarbocylated SAM; MAT, methionine adenosyl transferase; MTases, methyltransferases; SAH, S-adenosylhomocysteine; SAM, S-adenosyl methionine; SAMDC, S-adenosyl methionine decarboxylase [8]. Copyright [2013, Medical faculty, Nis].

Dietary folate has a major impact on homocysteine (Hcy) levels, which may exert direct neurotoxic and prooxidative actions [11–13] with an inverse relationship between plasma folate and Hcy concentrations [14].

DNA methylation, catalyzed by DNA methyltransferases, is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromosomal modifications, and in the development of mutations, which can regulate tissue-specific expression of certain genes which is particularly important during embryogenesis [15–18]. The inheritance of information based on gene expression levels is known as epigenetics, as opposed to genetics, which refers to information transmitted on the basis of gene sequence [19–21]. DNA methyltransferases transfer methyl groups from SAM to cytosine and this regulates gene transcription. In humans, DNA methylation predominantly involves the covalent addition of a methyl group (CH_3) to the 59 position of cytosine that precedes a guanosine in the DNA sequence (the CpG dinucleotide). This is referred to as an epigenetic modification because it does not change the coding sequence of the DNA [22, 23].

Folate is one of many compounds of fruits and vegetables that could be acting as a cytoprotective agent. It has antioxidant properties, scavenging several reactive oxygen species *in vitro* and inhibiting lipid peroxidation. FA may decrease oxidative stress in human diseases, significantly lowering the levels of free radicals in liver, brain, and kidney, and increasing the content of SH-groups, the functional groups in glutathione (GSH), the important antioxidant [24–30].

The increased GSH production, due to the block of Hcy remethylation to methionine and potentiation of

transulfuration pathway, influences epigenetic processes, including DNA and histone methylation by limiting the availability of SAM, the cofactor utilized during epigenetic control of gene expression by DNA and histone methyltransferases [21, 27, 29].

In our recent investigations in mothers receiving folate supplementation, the significant malondialdehyde (MDA) amount decrease was observed in colostrum and mature human milk [30]. Our results are in agreement with the literature data suggesting that pharmacological doses of folate supplementation lowers plasma Hcy and serum MDA levels, probably by increasing glutathione [24, 30].

FA is able to induce angiogenesis and vasculogenesis [24, 26, 31], in part via a nitric oxide (NO)-dependent mechanism [31].

The random acylation of amino groups in wild-type pancreatic ribonuclease (RNase A) with FA is shown to decrease its catalytic activity dramatically, presumably because of the alteration to a key active-site residue, Lys41 [32, 33]. The recent literature data represent that conjugation to FA is known to enhance the uptake of molecules by human cells that overproduce folate receptors.

Metabolic manifestations of folate deficiency

FA deficiency is considered to be one of the most common nutritional deficiencies. A deficiency of FA may be caused by: deficient food supply, increased needs in pregnant women and nursing mothers, defects in utilization (in alcoholics or individuals with liver disease), malabsorption folate losses in hemodialysis and cancer patients. Also, the reasons for folate deficiency may be impaired functions of the enzymes or cofactors needed for the generation of active FA. In the case of folate deficiency, all of the reactions in one-carbon metabolism will be compromised to varying degrees. Folate deficiency results in the reduction of purine and pyrimidine biosynthesis and, consequently, RNA and DNA biosynthesis and cell division; reduction in the biosynthesis of phospholipids (lecithin and sphingomyelin) and neurotransmitter synthesis by using SAM as the primary methyl group donor. Metabolism of several amino acids (including methionine, gliding, serine, and histidine) and initiation of protein synthesis in the mitochondria are disturbed in the state via folate-dependent deficiency reactions [8, 9, 12, 13]. The fact that folates have the principal places in the regulation of cellular SAM, leads to impairments of methyltransferase reactions in response to a folate deficiency [17, 18]. Folate depletion alters DNA methylation; causing DNA instability and chromosome damage [34–37].

Another abnormality of folates depletion leads to hyperhomocyst (e) inemia. However, homocysteinemia with homocystinuria is a rare inborn error of metabolism, resulting from deficiency of enzyme cistationin-β-synthase, which participates in transulfuration pathway of Hcy [38–41].

Mechanisms of DNA instability in folate deficiency

DNA methylation is one of the most intensively studied epigenetic modifications in mammals. In normal cells, the methylation of DNA assures the proper regulation of gene expression and stable gene silencing. DNA methylation is associated with histone modifications and the interplay of these epigenetic modifications is crucial to regulate the functioning of the genome by changing chromatin architecture [42–46].

DNA hypomethylation

Folate deficiency affects DNA stability principally by two ways: DNA hypomethylation and uracil misincorporation [15–20, 45]. DNA-methylation produces 5-methylcytosine, a modified base that is found mostly at CpG sites in the genome. 5-methylcytosine regulates gene transcription and interferes with the binding of transcription factors or other DNA-binding proteins to block transcription. Alterations in promoter regions and repetitive DNA sequences are associated also with the regulation of non-coding RNAs expression, such as microRNAs that may play role in tumor suppression [46–48].

The extent of methylation of specific genes varies from tissue to tissue and changes during development. Genome sequence analysis reveals that a large fraction of all genes is AdoMet dependent methyltransferases. Undermethylation favors gene expression, whereas increased methylation is associated with gene silencing [44–49].

Uracil misincorporation

The second pathway, uracil misincorporation into DNA, is the other way through which folate may alter DNA synthesis and stability. Normally (at physiological condition), folate, as 5′10-ethylene THF, donates a methyl group to uracil, converting it to thymine, which is used

for DNA synthesis and repair. However, if folate is limiting, uracil misincorporation into DNA may occur leading to mutagenesis [15–20]. There is good evidence suggesting that excessive misincorporation of uracil into DNA not only leads to point mutations, but also results in single- and double-strand DNA breaks, chromosome breakage and micronucleus (MN) formation [43–46, 50, 51].

The sensitivity of human thymidylate synthesis to a folate deficiency was reported by Blount et al. [44] who observed the increased misincorporation of uracil into lymphocyte DNA, accompanied by an increased frequency of cellular micronuclei, a measure of DNA and chromosome damage [44]. Uracil misincorporation was markedly reduced by folate supplementation in folate-deficient subjects, which restored thymidylate synthesis [44, 47, 51]. As the cell attempts to repair itself, it breaks the DNA molecule to excise the uracil. If folate is continually limited, imbalances in deoxynucleotide triphosphates in the precursor pool occur. Uracil is misincorporated and repaired in what is termed 'a catastrophic repair cycle', which may lead to double-strand breaks, chromosomal damage and cancer [1–3]. Also, DNA hypomethylation potentially induces proto-oncogene expression, leading to cancer [43, 46, 47, 51].

Also, there is evidence that hypomethylation increases the susceptibility of DNA to nuclease attack, which can result in DNA strand breaks, thus disrupting DNA integrity. Hypomethylation and DNA strand breaks, resulting from folate deficiency, enhance the incorporation of tumorigenic viruses, such as human papilloma virus into human DNA [43, 46, 48].

Hyperhomocysteinemia in folate deficiency

Folates have the principal places in regulating cellular SAM [8–11, 52, 53]. The metabolic effect of folate deficiency is an elevation of blood Hcy, natural intermediate in the metabolism of SAM. The byproduct in the methylations, S-adenosylhomocysteine (SHcy), is located at a branch-point of metabolic pathways: either it is irreversibly degraded via the transsulphuration pathway to cysteine, a precursor of the synthesis of GSH, or it is recycled back to methionine via Hcy in the methyl cycle (Figure 1), also called the homocysteine-methionine cycle [38, 53, 54]. The homocysteine-methionine cycle is found in all tissues [39, 40]. The transsulfuration pathway of Hcy involves B_6-dependent enzymes, cystathionine-β-synthase and γ-cystathionase in cysteine and glutathione synthesis. Vitamin B_6 deficiency inhibits Hcy catabolism [55, 56].

The elevated plasma Hcy has been associated with significantly increased risk for cardiovascular, liver, kidney and neurological diseases. Hcy is an independent risk factor for pregnancy complications, birth defects, Down syndrome, cognitive impairment in the elderly, psychiatric disorders, neurodegenerative diseases and they may affect the development of some types of cancer [56–58].

Folate reduces levels of Hcy in the blood, and supports the physiologic functions of many body systems [58, 59]. Hyperhomocysteinemic individuals are characterized by raised plasma levels of asymmetric dimethylarginine (ADMA), a novel risk factor for teratogenesis. FA treatment reduces elevated plasma levels of ADMA in hyperhomocysteinemic subjects [60–62].

Another aspect of nutritional influences of folate-dependent action on one-carbon metabolism is pro-oxidative action with an inverse relationship between plasma folate and Hcy concentrations related to GSH synthesis, scavenging several reactive oxygen species *in vitro* and inhibiting lipid peroxidation. In folate deficiency, antioxidant properties are decreased. All of theses events are present due to decreased cysteine SH-groups content, the functional groups in GSH [63, 64].

Several studies suggest that Hcy may promote endothelial dysfunction [65–68]. Endothelium-dependent vasodilation possibly involves NO-related mechanisms [68]. A characteristic feature in patients with an inborn error of amino acid Hcy, homocysteinemia (homocystinuria), is premature vascular disease. About 50% of patients with homocysteinemia have thromboembolic events. High plasma Hcy levels are the risk factors for deep-vein thrombosis in the general population. The subjects with untreated homocystinuria are at greatly increased risk of atherothrombotic events. A characteristic feature in patients with homocysteinemia is premature vascular disease; about 50% of patients have thromboembolic events [65–68].

Elevated plasma total homocysteine (tHcy) and low folate levels are associated with reduced bone mass density (BMD) in women, but not in men. These findings suggest that tHcy may be a potential modifiable risk factor for osteoporosis in women. Hcy may play a role in the pathogenesis of osteoporotic fractures. In 2001, Khan et al. [69] showed in *in vitro* experiments that Hcy impairs cartilage calcification. THcy seems to be a predictor for hip fracture among elderly men and women [69–71].

Interactions between folates and vitamin B_{12} (cobalamin)

FA works in concert with vitamin B_{12} in one-carbon metabolism through methionine and folates cycles. FA

has a long history of use in conjunction with vitamin B_{12} for the treatment of macrocytic anemia. Recent research documents the critical nature of two nutrients, vitamin B_{12} and folate, in brain health and the maintenance of cognitive function, including memory [72–76]. Both vitamin B_{12} and folate deficiencies can cause peripheral neuropathy, memory loss, depression, personality changes, as well as psychosis. If not treated, mental and neurological changes can become permanent [77–79].

The principal mechanism whereby folate and cobalamin metabolism influences physiological function in human body is through the action of methionine synthase (MS) in methylation cycle [79–83] (Figure 1). MS activity is important for maintaining adequate levels of methionine and for preventing accumulation of Hcy. The mammalian forms of MS require cobalamin as a prosthetic factor in the form methylcobalamin (MeCbl) (Figure 1) [79–83]. The co-dependence of MS on folate and vitamin B_{12} provides a biochemical explanation why a single deficiency of either vitamin leads to the similar abnormalities [54, 84, 85]. Folates participation in the prevention the impairment of DNA synthesis is compromised when vitamin B_{12} concentration is low because MS activity is reduced [86].

Since MS is the only enzyme in mammalian cells that uses 5-methyltetrahydrofolate (5-CH_3THF), its deficient activity also results in the trapping of cellular folate as 5-CH_3THF, which becomes unavailable for other folate-dependent reactions involved in purine and pyrimidine biosynthesis, neurotransmitter and other single carbon transfer reactions [79, 86]. The polyamines spermine and spermidine are feedback regulators of MS both *in vivo* and *in vitro* [87].

Folates and polyamines

The naturally occurring polyamines (spermine, spermidine and putrescine), are found ubiquitously in mammalian systems.

Polyamines are involved in many different biological processes, such as regulation of gene expression and cell growth and differentiation, regulation of transcription, proliferation of both normal and transformed cells, ion channel regulation, and protein phosphorylation [88–102].

The principal precursor of spermidine and spermine is methionine (Figures 1 and 2). Folate has profound function for polyamine biosynthesis, altogether with vitamin B_{12} and vitamin B_6, participating in methionine metabolism [90–93, 103].

In the cells, polyamines, as polycations, interact electrostatically with negatively charged moieties, such as DNA, RNA, phospholipids, mucopolysacharides and proteins. Binding to DNA, polyamines play a significant role in stabilizing DNA, especially chromatin structure, during the cell cycle [89, 94–97].

In normal cells, polyamine levels are controlled by biosynthetic and catabolic enzymes (Figure 3) [99–102]. The biosynthetic enzymes are L-ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC), as well as aminopropyl transferase – spermidine- (Spd synthase) and spermine synthase (Sp synthase). SAMDC or AdoMetDC is responsible for decarboxylation of SAM [104]. Spd- and Sp synthase use decarboxylated SAM (dcSAM) as an aminopropyl donor, but are specific in respect to their acceptors, putrescine and spermidine, respectively [99–102]. The byproduct formed during the transfer of aminopropyl group to putrescine or spermidine is 5′-methylthioadenosine (5′-MTA). This nucleoside is rapidly converted back to methionine through the methylthioadenosine cycle, also called the methionine salvage pathway [8–11, 103, 105]. Both of decarboxylases, ODC and AdoMetDC, use active form of vitamin B_6, pyridoxal phosphate-B_6P.

The catabolic pathway of spermine and spermidine is a two-step process involving, in the first reaction, spermidine/spermine N′-acetyltransferase (SSAT), and in the second reaction, polyamine oxidase (PAO) (Figure 3) [106–108]. Acetylated polyamines are generally the preferred substrates of polyamine oxidase (APAO), a flavin containing amine oxidase, a peroxisomal enzyme present in all vertebrate tissues [109]. PAO may also use non-acetylated spermine and spermidine [110, 111].

Polyamine biosynthesis is up-regulated in actively growing cells, including cancer cells [112–117]. Therefore, polyamine concentration, as well as gene expression and activity of enzymes involved in polyamine biosynthesis, especially ornithine decarboxylase (ODC), are higher in cancer tissues than in normal surrounding tissues. Recent investigations revealed that increased polyamine availability enhances the capability of cancer cells to invade and metastasize to new tissues [118]. Polyamine depletion has been shown to inhibit cell proliferation and migration, whereas over-accumulation of polyamines induces apoptosis and cell transformation [111].

The polyamine-biosynthetic pathway represents an inviting target for the agents, inhibiting carcinogenesis and tumor growth [113].

The administration of FA to hepatectomized animals (30%) resulted in diminution of PAO activity in regenerating rat liver tissue after hepatectomy [119]. The supplementation of experimental animals with vitamin B_{12} alone or together with FA increased Spd and Sp levels in rat liver. At the same time, the supplementation of experimental

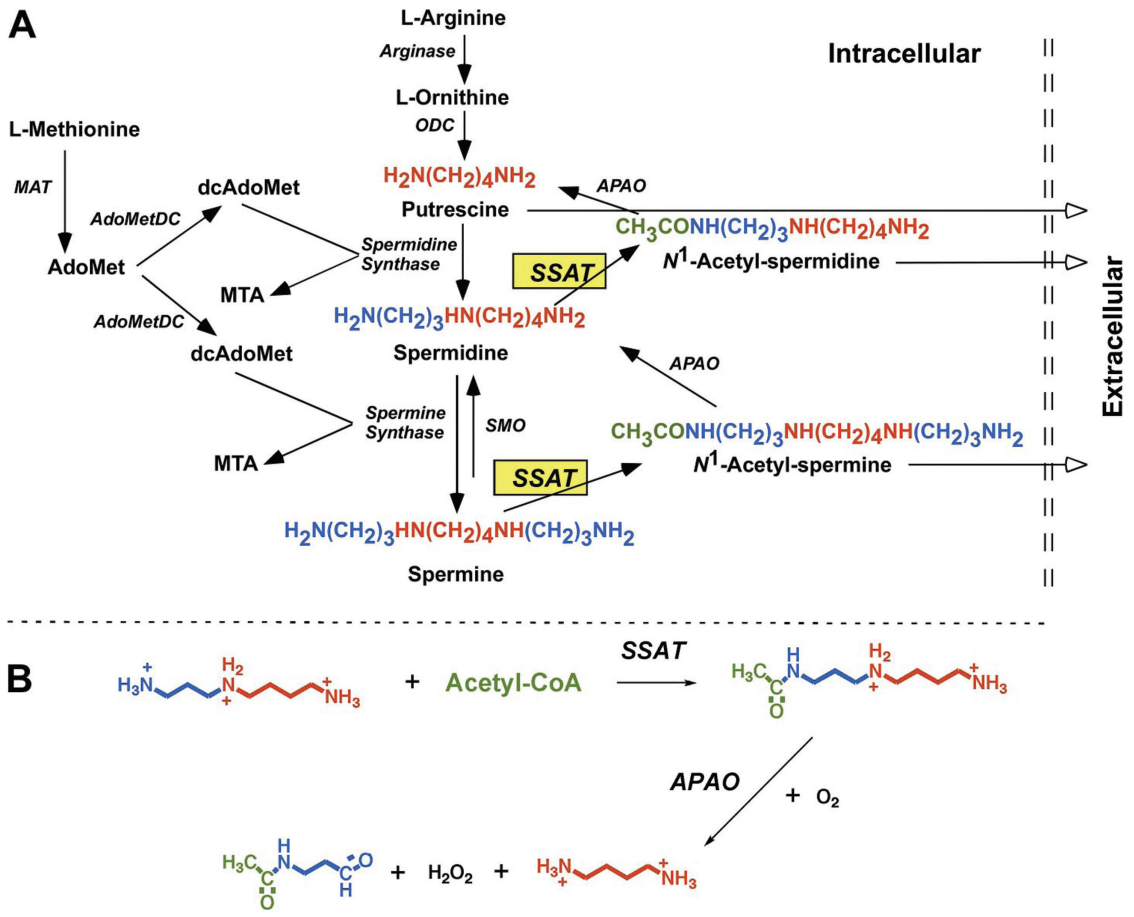

Figure 3 Role of folate through SAM in polyamine metabolism.
AdoMetDC, S-adenosylmethionine decarboxylase; APAO, acetylpolyamine oxidase: MAT, methionine adenosyltransferase; MTA, methylthioadenosine; ODC, L-ornithine decarboxylase; SAM, S-adenosyl methionine; SMO, spermine oxidase [106 – modified].

animals with vitamin B_{12} together with FA caused the decreasing of PAO activity. Our experimental results indicated the importance of FA and cobalamin in polyamine metabolism [120].

The spermidine N′-acetyltransferase was induced in the kidney after treatment with FA [121, 122]. 5-FU, the thymidylate synthase (TS) inhibitor, affected polyamine metabolism in colon carcinoma cells through the induction of the key catabolic enzyme SSAT [121, 122].

Folates and cancerogeneses

Many studies have shown that a diet high in folate (from fruits and leafy green vegetables) is associated with lower risks of many types of cancer [1–3, 123]. Some study point out that FA might prevent some cancers and might promote other neoplasias [123–125]. It has been hypothesized that early exposure to FA might prevent tumors through the provision of enough methyl groups to maintain proper methylation patterns and repair of DNA. In contrast, after the development of tumors, higher intake of FA might promote growth of existing tumors [126]. The timing of folate administration during cancer progression can modify outcomes. Folate administration prior to the existence of preneoplastic lesions can prevent tumor development, whereas provision of folate once early lesions are established appears to increase tumorigenesis [125, 126]. However, the presence of unmetabolized FA in blood is associated with decreased natural killer cytotoxicity. Since natural killer cells play a role in tumor cell destruction, this would suggest another way in which excess FA might promote existing premalignant and malignant lesions [126].

There are currently insufficient data to justify such an assessment and current evidence do not show an association between high FA intake and cancer risk, but neither do they confidently exclude [126, 127].

Folic acid antagonists – antifolates

In neoplastic cells, where DNA replication and cell division occur at an accelerated rate, the interruption of folate metabolism causes ineffective DNA synthesis, resulting in inhibition of tumor growth. Indeed, this has been the basis for cancer chemotherapy, using antifolate agents methotrexate (MTX) and 5-FU [123]. The antifolate agents are one group of antimetabolites and structural analogues of normal biochemical compounds. As competitive inhibitors, they compete with the naturally substrate for the active site of an enzyme and block the formation of undesirable metabolic products in the body [128]. Antifolates exert their action by disturbing the nucleic acid metabolism of cancer cells, including its synthesis, methylation and stability [129, 130].

Enzyme DHFR catalyzes tetrahydrofolate regeneration by the reduction of dihydrofolate, using NADPH as a cofactor. DHFR inhibition causes disruption of purine and thymidylate biosynthesis and DNA replication, leading to cell death. Therefore, DHFR has been an attractive target for chemotherapy of many diseases, including cancer [131–134].

The FA antagonists, MTX and aminopterin, close structural analogs of FA, as antitumor agents have found clinical application in the treatment of malignant diseases, especially in the treatment of leukemia in childhood [135]. These antifolates are extremely potent competitive inhibitors of DHFR and TS and, because of that, inhibit the synthesis of RNA and DNA [135].

MTX, a classical antifolate, is one of the most widely used and studied anti-cancer agents [128], including acute lymphoblastic leukemia, lymphoma, osteosarcoma, breast cancer, and head and neck cancer [128]. MTX is a potent and selective inhibitor of DFHR, which results in inhibition of DNA synthesis [132], due to thymidine starvation. Also, MTX is an indirect inhibitor of enzyme MS [132]. It might also induce depletion of intracellular-reduced folate coenzymes, reducing their transport through the folate receptor (FRα) and/or competing with folates for the reduced folate carrier (RFC) [136]. DHFR binds MTX about 100 times better than dihydrofolate. The development of drug resistance to MTX appears in the prolonged chemotherapy. Tumor cells that acquired MTX resistance have been found to have an increased number of DNA gene copies, encoding enzyme DHFR. The amplified DHFR genes in MTX-resistance cells produce a markedly increased number of DHFR copies, exceeding the amount of MTX, which can be delivered to cell, and thereby allow tumor cell DNA synthesis and tumor regrowth occurs [136].

The application of MTX disturbs the metabolism of polyamines in rapidly growing tissues. Inhibition of PAO, induced by MTX, in regenerating rat liver tissue, is probably the consequence of the inhibition of nucleic acids and protein synthesis [137, 138]. More than 90% of MTX, a classical antifolate, is cleared by the kidneys. So, MTX can be safely administered to patients with normal renal function. MTX-induced renal dysfunction leads to delayed drug elimination [132–134].

The enzyme, TS is considered an important target for the development of new anticancer agents. MTX and 5-FU were amongst the earliest anti-cancer drugs developed and both partly act through inhibition of TS [133, 134]. Among novel antifolates is pemetrexed which primarily targets TS as well as pralatrexate which blocks DHFR, and displays enhanced transport and cellular retention properties [135, 138].

Many human cancer cells lines and primary tumors have an absolute need for methionine. Disruption of the cell folate cycle by new generation antifolate drugs exert their action disturbing the nucleic acid metabolism of cancer cells, including its synthesis, methylation and stability. Melanoma cells are highly dependent on methionine and have the high activity of the methionine cycle, which permits the methylation of specific genes and activation of different survival pathways. Blockage of the methionine cycle by the new antifolate, [3-O-(3, 4, 5- trimethoxybenzoyl)-(-)-epicatechin, TMECG], tea catechin, is an effective therapy for melanoma [139, 140].

The specific activity of TMECG on melanoma methionine cycle was confirmed, not only in melanoma cells in culture, but it was also effective in an animal model, where it inhibited growth and metastasis of preformed tumors. This compound was successfully synthesized from the commercially available catechin. TMECG binds efficiently to human DHFR and down-regulates folate cycle gene expression in melanoma cells. Disruption of the folate cycle by TMECG is a plausible explanation for its observed biological effects and suggests that, like other antifolate compounds, TMECG could be of clinical value in cancer therapy [140].

Clinical manifestation of folates deficiency

The impaired folate-dependent intracellular metabolism can lead to several key pathologies, including megaloblastic anemia, homocysteinemia, cardiovascular disease, embryonic defects, in particular neural tube

defects (NTDs), congenital heart defects, and possibly, cancer [59].

Megaloblastic anemia

Folates are vitamins essential to the development of the hematopoesis [1–5]. Consequently, the reduction of erythrocytes maturation developed in folate deficiency, causes the appearance of megaloblastic anemia [135, 138, 141].

Megaloblastic anemia is macrocytic anemia due to folate and cobalamin (vitamin B_{12}) deficiency [142]. Megaloblasts are large, abnormal, nucleated cells that are precursors of erythrocytes; in a folate deficiency, they accumulate and are found in the bone marrow (Figure 4). These cells arise as a result of a failure of the red cell precursors to divide normally due to impaired DNA synthesis during the process of hematopoesis and the production of erythrocytes. There are also decreased numbers of white cells and platelets [142, 143]. FA supplementation prevents this type of anemia.

Neural tube defects (NTDs)

Experimental studies, epidemiological data and clinical trials showed the necessity of sufficient quantities of FA (at the time of conception and early pregnancy) for normal embryogenesis and fetal development for the prevention of neural tube defects and other neurological malformations [59, 144]. Alterations in DNA methylation result in changes of gene expression and can have important consequences for embryogenesis [14, 15, 145, 146]. Folate metabolism must adapt during pregnancy to multiple fetal and maternal physiologic influences that change throughout gestation. Increasing folate intake, leading to increased concentrations of folate coenzymes in tissues, may overcome an unidentified metabolic defect in the production of proteins and/or DNA or regulation of gene expression at the time of neural tube development and closure [135, 146, 147]. The literature data have established that low or inadequate folate status may contribute to congenital malformations (spina bifida and anencephaly), important factors of fetal and infant mortality [148].

FA prevents neural tube defects in newborns, so women of childbearing age must be sure to have an adequate intake prior to and during pregnancy. All women of child-bearing age should consume 0.4 mg per day of FA. Also, women, wanting to become pregnant, must take supplements daily before and until the 12th week of pregnancy. The research has shown that a daily supplement of recommended dose reduces the chance of NTDs [147–149].

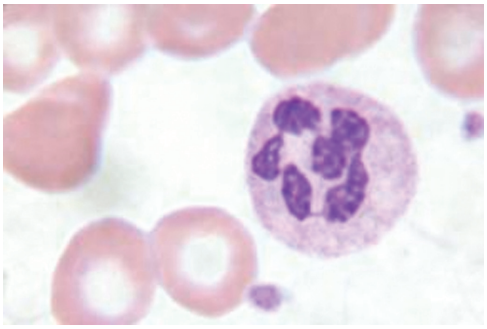

Figure 4 Peripheral bloods smear from a patient with megaloblastic anemia.
Notice that the RBCs are large (macrocytic anemia) and oval shaped, and the poly is hypersegmented serious organ failure can occur in individuals with megaloblastosis [135].

Metabolic effects of folate on physiological functions of cardiovascular system, liver and kidney

Folates and cardiovascular system

Folate deficiency is an independent risk factor for atherosclerotic vascular disease in the coronary, cerebral, and peripheral vessels and for arterial and venous thromboembolism [150]. Plasma concentrations of folate were positively associated with the risk of myocardial infarction. FA ability to lower Hcy suggests it might have a positive influence on cardiovascular disease [38, 54, 58, 151, 152]. Also, the potential mechanisms by which FA reduces the risk of cardiovascular diseases include antioxidant actions, or direct interactions with endothelial NO synthase [152]. Folates interact with the endothelial enzyme NO synthase (eNOS) and exert effects on the cofactor bioavailability and, thus, on peroxynitrite formation. Hyperhomocysteinemic individuals are characterized by raised plasma levels of ADMA, an endogenous NO synthase inhibitor, a novel marker of risk for endothelial dysfunction in a variety of cardiovascular diseases [60–62, 153].

Studies are now in progress to establish whether supplementation with FA will reduce cardiovascular risk [38]. ‘Homocysteine Theory’ stated that Hcy, is toxic for the vascular wall. In the years to follow, it became clear from both retrospective and prospective studies that also

mildly elevated total Hcy levels are independently associated with an increased risk for vascular diseases [59, 65, 67, 151–153].

The place of folates in liver metabolism

Liver tissue serves as storage for FA. Folates play essential functions in hepatocytes metabolism [9, 53]. The liver has the central role of methionine metabolism. Under normal conditions, up to 85% of all methylation reactions and as much as 48% of methionine metabolism occur in the liver, which indicates the crucial importance of this organ in the regulation of blood methionine [154].

Folates, by regulating cellular SAM, are a control switch that regulates essential hepatic functions such as regeneration, differentiation, and the sensitivity of this organ to injury. In folate deficiency, all forms of folate are reduced within cells, impairing the growth and maturation of hepatocytes. In normal liver, the majority of SAM is used in methylation reactions, since the SAM decarboxylation pathway, followed by aminopropylation (the pathway needed for spermidine and spermine biosynthesis) requires <10% of the available SAM. In hepatocytes, SAM levels are related to hepatocytes function. Patients with liver cirrhosis have decreased hepatic GSH levels [155, 156]. Intracellular ROS (superoxide, hydrogen peroxide, the hydroxyl radical and the peroxynitrite), due to their high metabolic activity, are generated continuously in cells during folates depletion. SAMs protective actions in the liver include increased GSH levels and antioxidative properties of folates [155–158].

Lower synthesis of AdoMet, due to folate deficiency, might contribute to hepatocyte dedifferentiation with consequent increases in regeneration and malignant transformation. Reduced tissue AdoMet may facilitate the synthesis and release of tumor necrosis factor and other cytokines [9, 53, 154–160].

Folates and kidneys

The kidneys play a pivotal role in regulating body folate homeostasis by reabsorbing the filtered vitamin, thus preventing its losses in the urine [161]. Administration of pharmacological amounts of FA to rats results in renal cell injury and subsequent hypertrophy and hyperplasia [162]. FA induces dose-dependent nephrotoxicity in mice and rats, with the rapid appearance of FA crystals within renal tubules and subsequent acute tubular necrosis, followed by epithelial regeneration [163].

In rats, single intravenous doses of FA induce damage to renal tubular epithelium, deposition of FA in tubular lumen, increase in wet kidney weight, oliguria and interstitial connective tissue proliferation [164, 165]. There is a predictable relationship between the dosage of FA and the abnormalities of morphologic features and function in kidney injury [164]. FA-induced nephropathy is associated with increased Angiopoietin-1 (Ang-1) protein expression in renal epithelia and arteries. Ang-1 overexpression *in vivo* produces large, numerous, highly branched vessels [165].

In patients with renal failure, hyperhomocysteinemia is a common feature. Hcy has been implicated as a pathogenetic factor in the development of arterial disease in a number of *in vitro* and experimental animal studies that have demonstrated an association between hyperhomocysteinemia and endothelial cell damage [166]. The proposed underlying pathophysiological mechanisms for this phenomenon include reduced renal elimination of Hcy and impaired nonrenal disposal, possibly because of inhibition of crucial enzymes in the methionine-homocysteine metabolism by the uremic milieu [167]. Hyperhomocysteinemia is a common finding in dialysis-dependent end-stage renal disease patients [168].

Folates, brain and the peripheral nervous system

Folates are essential to the development of the nervous system, playing important roles regulating neurogenesis. Folates are present in relatively high concentrations within the brain. Cerebrospinal fluid level is about three times of that in the blood. FA is poorly transported to the brain and rapidly cleared from the central nervous system [73–77, 81, 169, 170].

The incidence of FA deficiency is high in patients with various psychiatric disorders, including depression, dementia and schizophrenia [171, 172]. In epileptics on anticonvulsants, folate deficiency often occurs because anticonvulsants inhibit folate absorption. In these patients folate deficiency is often associated with psychiatric symptoms. These findings underscore the importance of folate metabolism in neuronal homeostasis [170]. Folate is involved in the synthesis of acetylcholine, norepinephrine, melatonin, histamine, through SAM methylation pathways. Folate deficiency has a greater effect on choline and acetylcholine metabolism in the peripheral nervous system than in the brain and this effect escalates with age [173].

Folate deficiency could have diverse effects on the neurochemistry of schizophrenia, as folate functions as a single carbon donor in the synthesis of glycine from serine (Figure 5) [171–179].

FA functions primarily as a methyl-group donor required for many methylation reactions. Reduction in the methylation cycle has multiple effects on nerve cells, since the interruption of the methylation cycle causes neuropathy.

Disturbance of the methylation cycle can also happen in vitamin B_{12} deficiency due to reduced activity MS, the vitamin B_{12}-dependent enzyme. In this deficiency, blockage of the methylation cycle causes the folate cofactors in the cell to become trapped as 5-methyltetrahydrofolate; this process produces a pseudo folate deficiency in such cells, preventing cell division and giving rise to an anemia identical to that seen in folate deficiency [63, 73, 75, 77, 171, 172, 174, 175]. There is a risk that if FA is given to people who have undiagnosed deficiency of vitamin B_{12} because it may lead to neurological damage. Vitamin B_{12} deficiency produces both an anemia identical to that of folate deficiency, but also causes irreversible damage to the central and peripheral nervous systems [175, 176].

The neurotoxin effects of high blood levels of Hcy may also play a role in the neurological and psychiatric disturbances that are associated with folate and vitamin B_{12} deficiency [180–182]. High blood levels of Hcy is an independent risk factor for central and peripheral nervous system diseases, including stroke, several neurodegenerative conditions, Alzheimer's disease and Parkinson's disease, as well as to cognitive decline and schizophrenia [73–78, 175, 176–179].

Folate deprivation, which leads to homocysteinemia, decreased the reduced form of GSH, indicating a depletion of oxidative buffering capacity, the reason for neurodegeneration [23–26, 63, 64, 181, 182].

Many children on the autism-spectrum are manifesting symptoms due to deficiencies in folate in the brain. This is important because folate is critical for the proper brain function and the deficiencies have been associated with learning, language, and cognitive imbalances, as well as propensity for seizures. Imbalances in methylation lead to cognitive problems, attention, focusing, language and social interaction issues. Both folate supplements and methyl-B_{12} therapy work hand in hand to improve brain function in autism [181, 182].

Folates and reproductive functions

There is increasing evidence that nutritional factors are important in reproduction and thus in spermatogenesis,

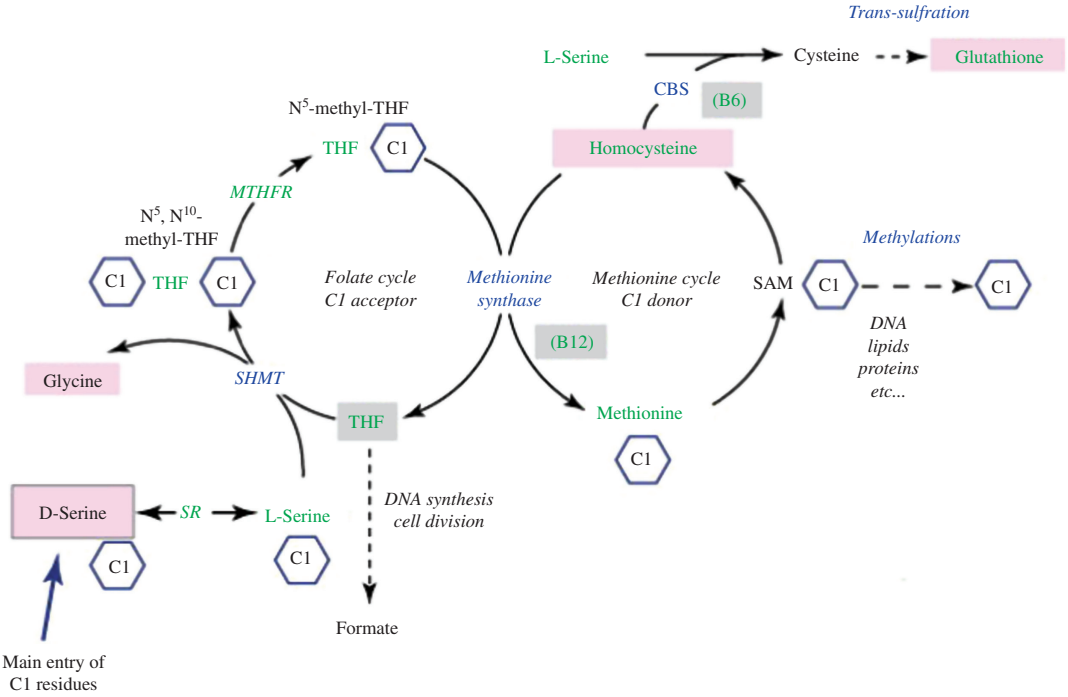

Figure 5 Folate functions as a single carbon donor in the synthesis of glycine from serine in folate cycle.
B6, pyridoxal'5-phosphate; B12, cobalamin; BHMT, betaine homocysteine methyltransferase; CBS, cystathione βsynthase; MTHFR, 5, 10-methylene-THF reductase; N5-methyl-THF, N5-methyltetrafolate; SAM, S-adenosylmethionine; THF, tetrahydrofolate [179 – modified].

as well [183–185]. Folates are necessary for normal fertility in men and women, as it is important during cell division and growth periods, such as infancy and pregnancy. In males, folate is necessary for spermatogenesis [20, 185]. Low folate in seminal plasma is associated with increased sperm DNA damage [183–185]. Given those epigenetic signals, such as DNA methylation and histone modifications are crucial for the proper functioning of the genome. Phenotypic differences in sperm production (quantitative as well as qualitative traits) at both inter- and intra-individual levels may also be due to an epigenetic variation. The authors reported significant variations for six genes at both intra- and inter-individual levels, concluding that epigenetic variations may contribute to the variable semen phenotype. There are indications that both FA and cobalamin supplementation may increase fertility [50, 72, 76, 186–188].

Folates have the principal places in affection of gene expression by regulating cellular SAM. Within the folate pathway, methylenetetrahydrofolate reductase (MTHFR) reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-CH_3THF), a methyl donor for remethylation of Hcy to methionine, the precursor of SAM (Figure 5). In adult male mice, MTHFR levels are highest in the testis. These findings, in conjunction with recent clinical evidence, suggest an important role of MTHFR in spermatogenesis [189, 190].

Severe MTHFR deficiency in male mice results in abnormal spermatogenesis and infertility. Deprivation of folates disturbs the Hcy pathway, involved in human subfertility [186–188].

In the recent years, there is an increased interest in the role of antioxidant properties of folates and other antioxidants – α-tocopherol (vitamin E), ascorbic acid (vitamin C) and the retinoid (vitamin A), as modulators of fertility outcome. Oxidative stress is associated with sperm quality and infertility. Abnormalities in the male genome characterized by damaged sperm DNA may be indicative of male subfertility regardless of normal routine semen parameters. Folate has antioxidant properties, scavenging several reactive oxygen species and inhibiting lipid peroxidation by increasing the content of SH-groups, the functional groups in glutathione [188, 191].

Folate deficiency states produce an elevated Hcy concentration and impair the remethylation cycle of Hcy, disturbing the biosynthesis of phospholipids, proteins, DNA and RNA. These processes are essential in spermatogenesis. Wang and Fenech [189] have established that total normal sperm count increases after combined zinc sulfate and FA treatment in both subfertile and fertile men [191, 192]. Low folate concentration in seminal plasma is associated with more sperm DNA damage in fertile men and is detrimental for sperm DNA stability. DNA methylation is important for proper spermatogenesis and male fertility. There are indications that both FA and cobalamin supplementation may increase fertility [186–188].

Deleterious effects of folate supplementation and food fortification on human health

Folate is critical for nucleotide synthesis and methylation reactions and has been associated with a number of health benefits [1–3, 123–127, 193–195]. In doses typically administered for therapeutic purposes, FA is considered non-toxic. There is an accumulation of many good data about the positive effects ranging from the established reduction of neural tube defects, cardiovascular disease and, possibly, dementia, Alzheimer disease and certain types of cancer. It seems to be a 'wonder drug' that is not only inexpensive, but also safe for use as a chemopreventive agent [123–127, 195–197]. FA and folates may act as effective antioxidants *in vivo*. Their activities may become important in view of nutritional supplementation and fortification of food with FA [23, 198].

When fortification is introduced, several hundred thousand people were exposed to an increased intake of FA [148, 149]. The intake of FA from fortified food (100–200 μg/d) together with the use of nutritional supplements, multivitamin preparation, lead to body state of folate oversupplementation. This practice of chronic intake of high doses of FA may lead to the adverse toxic effects of FA [198–200]. Today there is a little knowledge about the potential safety and physiologic consequences of chronic intake of such high doses of FA. At the present, a few reports, however, have raised questions about the safety and physiologic consequences of chronic intake of such high doses of FA [19–21, 123–127, 198–201].

One of the major risks associated with excessive intake of FA is the development of cancer [28, 34, 198]. However, recent human studies have suggested that overdoses of FA (people supplementation and food fortification) may promote the progression of already existing, undiagnosed, preneoplastic and neoplastic lesions [133, 134, 136–138]. However, clinical investigation suggests a decreased colorectal cancer risk in subjects with low folate status [137, 138]. It was concluded that there currently were insufficient data to support the concerns that FA fortification

promoted cancer. This possibility of a detrimental component to the role of folate in carcinogenesis could have implications in the ongoing debate in food science, concerning mandatory folate fortification of foods [193–199].

Consumption of FA in excess of 400 μg per day among older adults resulted in significantly faster rate of cognitive decline than supplement non-users. The studies suggest that high FA intake could cause serious cognitive consequences in the elderly as vitamin B_{12} deficiency [85, 200, 201].

Conclusion

Periconception maternal intake of FA reduces the risk of neural tube defects and, possibly, other birth defects. Therefore, FA supplementation periconceptionally has been recommended to women since the early 1990s. In several countries food fortification with FA has been implemented in order to increase the average serum folate concentration. Until now, most attention has been paid to the beneficial effects of folate and the detrimental effects of tHcy. Fortification of cereal grain flour products with FA, and oversupplementation, may disturb the physiological function of many organs like the brain, cardiovascular system, liver, kidney and for human reproduction as well. Excessive intake of FA is the reason for the development of cancers. In line with this, there is an ongoing discussion about possible adverse effects of excessive folate. The food safety agencies in a number of countries, considering mandatory FA fortification, have reconsidered both beneficial and adverse effects of FAs.

Folate plays a central role in the reaction of methylation and regulation of concentration of SAM. This activity is compromised in folate deficiency. As a result, polyamine, spermidine and spermine biosynthesis is deprived. In the opposed direction, in a situation of high amount of FA, biosynthesis of polyamines increases which may explain the mutagenic activity in oversupplementation of FA. There is a need for better investigations of the relationships between the high presence of FA in human tissues and the amounts of polyamines related to malignant processes in human body.

Also, antifolates (FA antagonists) are widely used in therapy of malignant diseases. Interruption of folate metabolism causes ineffective DNA synthesis, resulting in inhibition of tumor growth.

On the basis of our knowledge about metabolic functions of folate, it may be concluded that FA supplementation is unwonted, except for recommended doses for women periconceptionally and during pregnancy, as well as the recommended doses for some diseases. Also, in health, it is the best to supply these needs by fresh vegetables and fruit dietary intake.

Future directions

Today, it is very important to evaluate the beneficial effects of different levels of FA administration on the human health or in human diseases. Having in mind the importance of folates in the control of intermediary metabolism and the significance for normal human physiology, we suggest that, for maintaining homeostasis in metabolic reactions, the cells need an adequate amount of this vitamin. If there is deprivation or oversupplementation of FA in the regulatory network, defects appear. However, a large knowledge gap remains in understanding its mechanisms and why abnormally very high folate levels may be detrimental for physiological function of many organs like the brain, liver, and kidney and for human reproduction and the cardiovascular system as well.

For future studies, considering the relation of polyamines and rapidly proliferating tissues (especially cancers), there is a need for better investigations of the relationships between high presence of FA and polyamine metabolism related to malignant processes in the human body. There are many questions in folate biochemistry which remain to be resolved.

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