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Molecular mechanisms underlying the potentially adverse effects of folate

Kyle C. Strickland, **Natalia I. Krupenko**, and **Sergey A. Krupenko***
Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425 USA

Abstract

The importance of proper consumption of dietary folate for human health has been highlighted by an extensive number of publications over several decades. Fortification of grain products with folic acid was initiated with the specific intent to prevent neural tube defects, and the scope of this endeavor is unique in that its target population (women of the periconceptional period) is many times smaller than the population it affects (everyone who ingests fortified grain products). Folate fortification has been wildly successful in terms of its goal; since its inception, the incidence of neural tube defects has markedly decreased. In the wake of this public health triumph, it is important to catalogue both the serendipitous benefits and potential side effects of folic acid supplementation. The vitamin is generally regarded as a harmless nutrient based on studies evaluating the safe upper limits of folate intake. In recent years, however, a concern has been raised with respect to a potential downside to folate supplementation; namely, its proposed ability to enhance proliferation of malignant tumors. The current review summarizes the available literature on the effects of folate supplementation and the molecular mechanisms by which high doses of folate may have negative consequences on human health, especially with regard to cancer.

Keywords

cancer; folate; folic acid; folate enzymes; metastasis; molecular mechanisms; dietary supplementation

Introduction

Folates are a group of coenzymes that function to carry single-carbon groups used in the biosynthesis of nucleotides and the metabolism of amino acids (schematically depicted in Fig. 1). The importance of folate first became evident after it was demonstrated as the active compound for correcting certain macrocytic anemias (reviewed in (1)). Since that time, folate deficiency has been implicated in other pathologies including neural tube defects (NTDs), homocysteinemia, cardiovascular disease, and cancer (1). Humans rely upon the

*To whom correspondence should be addressed: krupenko@musc.edu, Phone 843/792-0845.

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presence of folate in their diet because they are unable to synthesize the molecule *de novo*. Natural foods rich in folate include a variety of vegetables, beans and fruits, as well as beef liver. The fact that folate is necessary for cellular functions has been exploited by medical science, and antifolates are routinely prescribed for the treatment of cancers and rheumatologic disease (2). In contrast to humans, bacteria do not acquire folate from their environment but directly synthesize it from pteridine and *p*-aminobenzoic acid (PABA). Of note, this pathway is selectively targeted by sulfonamide antibiotics, which are structurally similar to PABA and competitively inhibit the enzyme, dihydropteroate synthetase.

Of particular importance, the connection between folate deficiency in early pregnancy and the fetal development of NTDs led to a public health initiative to fortify foods with folic acid. As a result, the United States Food and Drug Administration has mandated the addition of folic acid to cereals and grain products since 1998. This practice still remains a strong recommendation by the United States Preventive Services Task Force and also has been implemented in more than fifty countries worldwide (3, 4). This endeavor is unique in that the target population (women of the periconceptional period) is many times smaller than the population affected. The fortification resulted in a significant (36%) reduction in the incidence of NTDs as of 2006 (5), thus achieving its goal. Folate status can potentially benefit human health in many ways, and, since the inception of the mandatory folate fortification, there have been many studies that suggest a variety of coincidental health benefits for the general population. For example, in addition to preventing NTDs, preconceptional intake of folate has been associated with a significant reduction in the incidence of early spontaneous preterm births (6). As well, studies have underscored an inverse correlation between folate intake and diseases such as venous thrombosis, atherosclerosis, stroke, and even mood disorders (reviewed in (7)).

Folate controversies: is there an adverse effect of folate supplementation?

FA (pteroylmonoglutamate, vitamin B_9) is not a natural form of folate; it is a synthetic vitamer not found in significant amounts in fresh foods and non-fortified food products. There are distinct chemical differences between the reduced folates naturally present in the human diet and FA used in the fortification of food. A folate molecule consists of a pteridine ring conjugated to *p*-aminobenzoic acid that is modified by one or more glutamic acid residues. There are a variety of folate species, and each is distinguished by the presence and oxidation state of carbon attached to the N^5 and/or N^{10} positions of a tetrahydrofolate backbone. FA differs from natural folates in that (i) it contains a single glutamate residue and (ii) it is an oxidized and inactive form of the coenzyme. To become an active coenzyme, FA must be reduced twice: first to dihydrofolate and then to tetrahydrofolate (Fig. 1). Dihydrofolate reductase (DHFR) is the enzyme responsible for catalyzing both steps; however, it is a relatively slow enzyme in humans and appears incapable of completely converting large amount of FA to tetrahydrofolate (8). In contrast to natural folates, which are unstable and can readily degrade in food preparation and storage, FA is a stable compound (9). FA, being only a monoglutamate, also has excellent bioavailability (10). It should be noted that the combination of fortified foods and multivitamin supplements can result in a substantial accumulation of unmetabolized FA in cells (8). Moreover, this accumulation may be highly variable in individuals since up to five-fold differences in

DHFR activity have been reported in humans (8). The accumulation of intracellular FA is likely driven by increased levels of unmetabolized FA in blood, a phenomenon observed in numerous studies assessing the effects of FA intake (4, 11–15). Whether increased circulating FA is a risk factor for certain pathologies or whether it might have a beneficial effect is not clear at present. Even though the majority of the population in many countries is affected by the mandatory supplementation, there has been a lack of targeted efforts to investigate the broad effects of FA on human health to become aware about its possible side effects. Numerous reviews have discussed the possibilities of adverse effects of supplementation with FA, and each has been careful to advocate further research on the effect of supra-physiologic levels of FA on human health (4, 16–19).

Exemplifying this concern, there is a clinically important relationship between folate and vitamin B_{12} deficiency. Vitamin B_{12} (cobalamin) is a cofactor of methionine synthase, the enzyme catalyzing the regeneration of methionine from homocysteine and 5-methyltetrahydrofolate (5-MTHF). In instances of B_{12} deficiency, this enzyme is inactive leading to elevated homocysteine levels and the symptoms of megaloblastic anemia. Since this is the only reaction utilizing 5-MTHF in the cell, the absence of B_{12} causes the accumulation of 5-MTHF at the expense of other forms of folate. Direct experimental evidence for this phenomenon, commonly known as the “methylfolate trap,” has been obtained in a mouse model where disruption of the methionine synthase reductase gene (essential for methionine synthase activity) led to decreased plasma methionine and increased plasma homocysteine and tissue 5-MTHF (20). FA supplementation can correct the megaloblastic anemia caused by B_{12} deficiency, and this fact has raised the concern that folate may “mask” this easily detectable symptom and thus exacerbate the more toxic neurological sequelae of vitamin B_{12} deficiency, such as sub-acute degeneration of the spinal cord (1).

Other health concerns about folate supplementation have been raised in the literature. For example, some studies have suggested that high intake of FA may lead to cognitive dysfunction (21, 22). Also, a link between autism and folate has been suggested based upon the temporal correlation of the rise in autism diagnoses and FA fortification (23). While these potential negative effects are questionable, it would still be prudent to account for the possibility that FA supplementation may have no clear positive effect on diseases other than NTDs. For instance, recent meta-analyses failed to find a link between folate supplementation and the incidence of cardiovascular disease (24, 25). It is also important to consider that folate supplementation alone may not be a panacea for NTDs, and it is possible that the ability of folate to reduce the development of NTDs may be limited to certain genetic sub-populations (26). Moreover, a recent study in mice has demonstrated that depending on the genetic background, FA supplementation may even increase the incidence of NTDs (27).

Molecular mechanism underlying adverse cellular response to folate increase

Oral FA is considered non-toxic to humans: being a water-soluble vitamin, it is readily excreted in both sweat and urine (28). The upper limit for its ingestion has been set by the

Institute of Medicine Food and Nutrition Board as 1 mg/day, but this restriction seems arbitrary considering there are no definitive toxic effects of FA at a much higher daily dose (up to 15 mg/day) in healthy individuals (28). As mentioned above, FA is a stable molecule while most reduced folate coenzymes are unstable and can be chemically degraded through oxidation and breakage of the bond between pterine ring and glutamylated PABA moiety (reviewed in (9)). It should be taken into consideration that the majority of folate coenzymes are protein-bound since the concentration of folate-utilizing enzymes in the cell is higher than the total concentration of cellular folate, and such binding to proteins protects the cofactors from degradation (9). Interestingly, studies from our laboratory have demonstrated that excessive supplementation of cultured cells with 5-formyltetrahydrofolate (also known as folinic acid or leucovorin) dramatically (three-fold) increases the total intracellular level of reduced folates (29). Such a high total concentration could result in increased levels of free (non-bound) folate, which may then increase their degradation rate. However, even if folate degradation takes place at a significant rate, the products of this degradation are not toxic and can either be re-utilized in other biochemical reactions or excreted.

Other mechanisms of adverse effects of the vitamin have been suggested in the literature. Theoretically, increased blood levels of FA may interfere with cellular folate transport and metabolism, or regulatory functions of the coenzyme through competitive inhibition of binding of natural folates to enzymes and/or carrier proteins (30). High intake of FA may exert an antagonistic effect towards natural folates due to accumulation of dihydrofolate, which is known to inhibit thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR), leading to decreased levels of thymidylate and 5-MTHF (30, 31). Importantly, the shortage of thymidylate impairs DNA integrity and cellular division while the shortage of 5-MTHF decreases methionine biosynthesis thus affecting protein production and DNA methylation. As well, high doses of FA may simply saturate DHFR and potentially inhibit the entire folate metabolism (8). Thus, paradoxically, high doses of FA may induce effects similar to those produced by deficiency of the bioactive forms of folate. It has been also shown that folate-enriched diets are associated with reduced cytotoxicity of natural killer cells; immune cells thought to be a major line of defense against arising neoplasia (32). However, a recent study involving healthy individuals did not confirm an association between serum folate levels and the cytotoxic activity of natural killer cells (33), leaving the above mechanism an open question.

Folate and cancer

In addition to the mechanisms discussed above, FA could also produce an effect through the elevation of the reduced folate pool thus boosting folate metabolism and promoting proliferation, a phenomenon especially relevant to the pathogenesis of cancer. The first observation that FA promotes cancer proliferation dates back to the 1940s when studies by Sidney Farber and colleagues demonstrated that the administration of FA accelerated the progression of leukemia in children (34). The relationship between folate supplementation and cancer, however, is perhaps one of the most controversial subjects in the field. Early epidemiological studies have indicated that a low folate status or low folate intake increases, while high folate intake decreases, the risk of certain types of cancer in humans (35, 36). A list of potential mechanisms by which folate deficiency can promote carcinogenesis

includes: induction of DNA hypomethylation, secondary deficiency of choline, diminution in natural killer cell surveillance, increased chromosomal aberrations and fragility, uracil misincorporation in DNA, and facilitation of tumorigenic virus metabolism (37). While folate supplementation of normal cells appears to have a protective effect, it may also promote the progression of neoplastic lesions because rapidly dividing cells are critically dependent on an abundant supply of reduced folates to support *de novo* nucleotide biosynthesis and the methylation reactions necessary for cell division. This serves as the basis for treatment of cancer patients with antifolates, drugs that inhibit folate enzymes and thus prevent the *de novo* biosynthesis of nucleotides (38). Of note, later epidemiological studies have failed to provide a definite conclusion on the role of folate intake in mediating cancer risk (reviewed in (3, 16, 18, 19)). Part of the problem, however, lies in discriminating between the effect of folate on tumorigenesis or on undetected pre-neoplastic lesions, which are expected to be quite opposite. Interestingly, animal studies have demonstrated that the exposure to high levels of FA *in utero* may increase the risk of mammary tumors in the offspring (39) while folate depletion post-weaning may be protective against intestinal neoplasia (40), and these findings argue in favor of a direct effect of excess FA in promoting tumorigenesis. Though mechanisms underlying the observed phenomena have yet to be pinpointed, the effect in the former study was attributed to altered DNA methylation as a function of folate status.

The effect of folate on cancer initiation and progression is an extremely important public health issue because the mandatory fortification of grain food with FA has resulted in increased folate intake, raising the concern that it may increase the incidence of malignancies and cancer-related death (41). End-point effects of the vitamin could depend on its ingested form, synthetic FA versus natural (reduced) folate. For example, a recent randomized clinical trial indicated that supplementation with FA doubled the risk of prostate cancer while baseline dietary (natural) folate revealed a protective effect (42). Another example of this trend is the inverse correlation between the risk of pancreatic as well as colon cancer and the dietary (natural) folate intake while no effect was demonstrated for the FA supplemented diet (43). The dose of ingested folate clearly matters as well. Of note, in the above study on prostate cancer FA was given at the dose of 1 mg per day (44), which was 2.5-fold higher than the normally recommended daily allowance of 0.4 mg and was given on top of folate obtained from natural and fortified foods. The tumorigenic response to dietary folate may also depend on the cells/organs of origin and cancer type, but results on this matter are inconsistent (3, 45). Thus, while epidemiological studies of head and neck cancer, liver cancer and neuroblastomas mostly reported protective effects of folate (46–49), results of studies on colorectal, breast, prostate and lung cancers are far less conclusive (50–53). This inconsistency prompted the idea that effects of folate in tumorigenesis depend on the timing and duration of folate administration (18, 19, 54). These effects could be further modified by other factors such as age and the status of vitamins B_6 and B_{12} (4). Finally, it is likely that the relationship between the folate intake and cancer risk also depends on individual genotypic features including polymorphisms in folate enzymes (55–57).

In light of the controversy of the relationship between dietary folate and cancer risk, it is crucial to ascertain which molecular mechanisms are initiated/maintained by excessive

folate that could promote proliferation and tumorigenesis. The negative consequences of dietary folate deficiency at the cellular level include altered protein expression (58), decreased DNA repair capability and accumulation of DNA damage (59–61), increased chromosomal aberrations and fragility (62); events that ultimately reduce growth rate and impair cell division. Conversely, the abundance of folate coenzymes would be expected to prevent these negative events and promote proliferation. Indeed, direct experimental evidence of such dependence of proliferation from folate availability has been obtained in both cell culture and animal models. For example a recent study has shown that dietary folate restriction blocks prostate cancer progression in the TRAMP mice (63), a finding that is consistent with the idea that folate promotes cellular proliferation. It has to be seen whether a similar outcome can be reproduced in cancer patients. It should be pointed out that FA supplementation may also interfere with the action of antifolates by reducing their efficacy in the treatment of cancer, rheumatologic disease, and malaria (64–67).

Effects of folate on metastasis

The direct effects of folate on metastatic disease have yet to be thoroughly evaluated, but the treatment of metastatic tumors with antifolates has shown promising results (68), suggesting the involvement of folate metabolism in the metastatic processes. In agreement with this notion, a recent case report indicated that supplementation with large amounts of folate could accelerate metastasis in patients with hormone-resistant prostate cancer (69). In addition to its potential role in enhancing proliferation, folate may promote metastatic disease via its effects on cancer cell migration. The migratory ability of malignant cells is a key feature that distinguishes metastatic disease from benign solid tumors. The presence or absence of essential nutrients is an important component of the tumor microenvironment that contributes to cancer progression and metastasis. In this regard, extracellular folate status correlates with altered expression of genes responsible for cell adhesion, migration and invasion (70, 71). The ability of the cell to move and migrate depends upon reorganization of the cytoskeleton, a complex network of actin filaments, microtubules and intermediate filaments. The leading role in this process is played by actin due to its unique dynamic properties, associated with two interchangeable protein forms, globular (G-actin) and filamentous (F-actin). There appears to be a direct association between folate metabolism and actin; the disruption of the actin cytoskeleton reversibly increases the proportion of folate receptors on the cell surface and the rate of 5-MTHF delivery (72).

Rebuilding of actin filaments is regulated by multiple actin-binding proteins including the actin-depolymerizing factor cofilin, which appears to be the major calcium-independent regulator of this process. Cofilin is a small (19 kDa) ubiquitous protein that facilitates the turnover between filamentous and globular actin (73, 74). A study of cardiac development in folate receptor knockout mice has revealed dramatic alterations in components of the actin cytoskeleton network, including down-regulation of cofilin (75). This finding is also in line with a proteomic study that showed down-regulation of cofilin in rats fed a folate-deficient diet (76). In further support of the important role of folate in metastasis, it has been recently demonstrated that the folate withdrawal inhibits migration and invasion of cultured cells by a mechanism associated with cofilin-dependent alterations of the actin cytoskeleton (77). The role of folate in the promotion of metastasis could be associated with maintaining

methylation of the Rho family GTPases, a process required for their proper membrane association and activation of downstream targets controlling cytoskeleton dynamics. In support of this mechanism, treatment of cancer cells with the antifolate MTX led to a dramatic decrease of Ras methylation and its aberrant localization to the cytosol resulting in the decreased activation of MAPK/Akt (78). It has been also hypothesized that folate controls methylation of the cytoskeleton (79), which could be another mechanism to regulate migration. Curiously, the folate-metabolizing enzyme FTCD (formiminotransferase cyclodeaminase) has been shown to bind vimentin filaments perhaps controlling the assembly of intermediate filament cytoskeleton (80, 81). While such a connection may represent an important factor in epithelial-mesenchymal transition and the development of metastases, it is not clear whether the interaction of FTCD with vimentin is regulated by folate or not since the folate-dependent catalytic activity of the enzyme is not required for its function towards filaments (81).

The role of folate enzymes in tumorigenesis

An overview of the role of folate in cancer disease would be incomplete without consideration of the enzymes involved in the metabolism of the vitamin. Intracellular folate status can be defined by three major components: (i) dietary ingestion of the vitamin, (ii) its transport, and (iii) its metabolism by folate enzymes. Folate metabolism is a complex network of reactions which involves many forms of the coenzyme and numerous folate-metabolizing enzymes (simplistically depicted in Fig. 1) (82, 83). Alteration of their catalysis through activation, inhibition or expression changes could affect cellular function irrespective of folate supplementation. Folate enzymes have been implicated in tumorigenesis and cancer progression as well as in other diseases. Physiological functions of numerous folate enzymes have been evaluated by different approaches including the search for nucleotide polymorphisms as potential markers of NTDs, cardiovascular diseases and cancer (7).

Most of folate enzymes catalyze the reactions which benefit cellular proliferation. Accordingly, increased production of many folate-related proteins is often observed in tumors. The well-known examples are the folate receptor, which is highly expressed in cancer cells (84), and DHFR, which elevation in response to antifolate treatment is one of the main mechanisms of the resistance to these drugs (85). DHFR is required to convert dihydrofolate, produced in the reaction of the TMP biosynthesis, back to tetrahydrofolate, thus regenerating the active form of folate. The enzyme is elevated at the transition from G1 to S phase since TMP biosynthesis takes place during S phase while cells are preparing for division. This enzyme is also responsible for the incorporation of the dietary FA into the reduced folate pool through its conversion to dihydrofolate and then to tetrahydrofolate (Fig. 1). Of note, DHFR can be up-regulated upon increased folate consumption (86). Thus, elevated levels of FA in the cell may promote unnecessary proliferation by up-regulating the expression of DHFR thus increasing the production of tetrahydrofolate and the rate of nucleotide and methionine biosynthesis. Of note, the FA and DHFR-driven increase in dihydrofolate could inhibit MTHFR and 5-MTHF generation (31) thus diverting one-carbon groups towards nucleotide biosynthesis and enhancing proliferation. Furthermore, DHFR and another folate enzyme, thymidylate synthase (TS) have been shown to regulate

translation through the direct binding of several mRNAs including their own (85, 87). In this regard, TS has been shown to repress the translation of p53 mRNA, a process which might prevent the activation of this tumor suppressor (87). Thus, through such a mechanism, TS might function in an oncoprotein-like manner. Interestingly, elevated DHFR can antagonize the proliferation control exerted by another folate enzyme, ALDH1L1 (29).

Several folate enzymes involved in serine/glycine metabolism have been implicated in tumorigenesis and cancer progression as well. In a recent study, levels of SHMT2, MTHFD2 and MTHFD1L correlated with enhanced proliferation of cancer cells, and increased expression of these enzymes was associated with greater mortality in breast cancer patients (88). Another folate-related enzyme, glycine decarboxylase (GDC), was found to be elevated in tumor-initiating cells and is required for their growth and tumorigenic properties (89). Remarkably, the over-expression of GDC promotes cellular transformation, and aberrant activation of GDC correlates with poorer survival in lung cancer patients. In line with the above findings, the tumorigenic effects of GDC require catalytically active enzyme. GDC is a part of the multi-enzyme glycine degradation complex (89). The entire process utilizes glycine to donate one-carbon groups into folate pool and to produce energy, and it remains to be seen whether the above effects occur primarily through acceleration of folate metabolism or via another mechanism (such as increased energy production secondary to enhanced glycine utilization).

Two abundant folate-related enzymes with regulatory functions, ALDH1L1 and GNMT, fit the definition of type 2 tumor suppressors. ALDH1L1 (10-formyltetrahydrofolate dehydrogenase, FDH) controls the level of folate-bound one-carbon groups by removing them from the folate pool as CO_2, thus restricting folate-dependent biosynthetic reactions (90). The *ALDH1L1* gene is silenced in many human cancers through methylation of its promoter region (91), and its re-expression induces specific apoptotic cascades in cancer cells with JNKs and p53 as major downstream targets (92–94). GNMT (glycine-N-methyltransferase) controls the level of activated methyl groups by converting S-adenosylmethionine (SAM) and glycine to S-adenosylhomocysteine and sarcosine in a folate-dependent manner, thus limiting the methylation ability of the cell (95). GNMT is down-regulated in cancers as well and exerts an inhibitory effect on carcinogenesis (96, 97). A case-control study revealed that the enzyme has protective effects against prostate cancer (97). In agreement with these findings, GNMT knockout mice have a high tendency to develop hepatocellular carcinomas and demonstrate an increased amount of overall genomic methylation as well as promoter hypermethylation of tumor suppressors RASSF1A (inhibitor of oncoprotein Ras) and SOCS2 (inhibitors of JAK/STAT pathway) (98).

Conclusion

Folate-dependent reactions are vital for cellular division and homeostasis due to their involvement in nucleic acid biosynthesis, methylation reactions and amino acid metabolism. The widespread inception of FA supplements has been successful in the prevention of NTDs and may have additional benefits for the population as a whole. However, there is still controversy in the literature over the existence of both beneficial and harmful side effects of FA supplementation and it has become increasingly urgent to understand the long-term

effects of folate on human health. The precise molecular mechanisms by which folate influences cellular functioning are just beginning to emerge. It is likely that the most immediate downstream response to folate status would be alterations in nucleotide levels/ nucleic acid biosynthesis and methionine biosynthesis/SAM levels (schematically outlined in Fig. 1). Folate deficiency would lead to diminished production of nucleotides and SAM while the abundance of the vitamin might elevate their production. SAM is a universal methyl group donor in more than a hundred reactions in the cells including DNA, RNA and protein methylation, and the biosynthesis of numerous key metabolites such as polyamines, epinephrine, creatine, and phosphatidyl choline. However, the cellular response to these changes may produce versatile and not so easily predictable end-point effects. In relation to cancer, folate metabolism may play a particularly essential role in proliferation, tumorigenesis, and metastasis. Thus, it is important to discriminate between the mechanisms by which folate protects from or promotes tumorigenesis and metastasis. This knowledge will allow more specific dietary recommendations for different population groups to minimize any potential adverse effect of this vitamin.

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Abbreviations

5-MTHF	5-methyltetrahydrofolate
DHFR	dihydrofolate reductase
FA	folic acid
NTDs	neural tube defects

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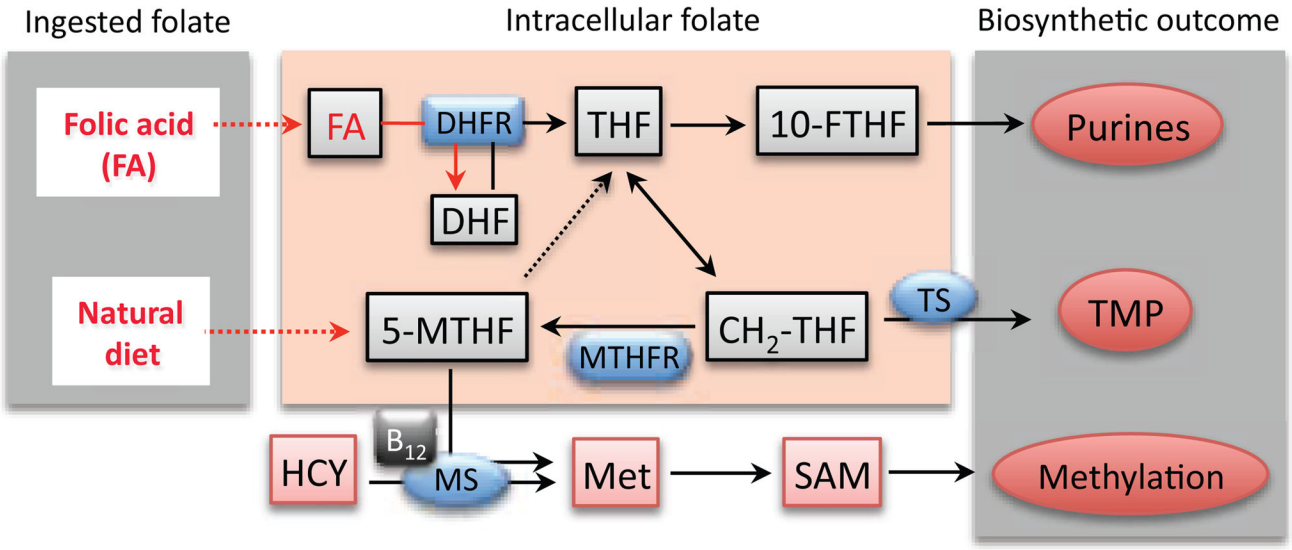

Figure 1. Folate metabolism

Folate is taken up by the cell in forms of folic acid (FA, supplements or fortified foods) or 5-MTHF (natural diet). In the cell, FA is sequentially converted to dihydrofolate (DHF) and then to the active form of the coenzyme, tetrahydrofolate (THF). Both reactions are catalyzed by DHFR (dihydrofolate reductase). Upon accepting a one-carbon group (comes either from serine, glycine, histidine or formate), THF is converted to other forms of folate. For simplicity, only folate coenzymes directly participating in the biosynthesis of nucleotides and methionine are shown (10-FTHF, 10-formyl-THF; CH_2-THF, 5,10-methylene-THF). HCY, homocysteine; SAM, S-adenosylmethionine; MS, methionine synthase; TS, thymidylate synthase; MTHFR, methylenetetrahydrofolate reductase. Reaction catalyzed by MS converts 5-MTHF to THF (indicated by dotted arrow) and requires vitamin B_{12}.