

# Physiology of Folic Acid in Health and Disease

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**Abstract:** Folates are important cofactors in the transfer and utilization of one-carbon-groups and play a key role in the remethylation of methionine thus providing essential methyl groups for numerous biological reactions. Furthermore, folates donate one-carbon units in the process of DNA-biosynthesis with implications for the regulation of gene expression, transcription, chromatin structure, genomic repair and genomic stability.

As the role of folate deficiency in atherosclerotic cardiovascular disease, neurological and neuropsychiatric disorders, in congenital defects and carcinogenesis has become better understood, folate has been recognized as having great potential to prevent these many disorders through folate supplementation for the general population. Folate acts directly to produce antioxidant effects, interactions with enzyme endothelial NO synthase (eNOS) and effects on cofactor bioavailability of NO. Folate acts indirectly to lower homocysteine levels and insure optimal functioning of the methylation cycle. Folate metabolism provides an interesting example of gene-environmental interaction. A great part of the population, especially subgroups with higher demand, appears to have suboptimal folate intake, as determined through more sensitive parameters now widely determined. The available data strongly suggest that criteria for "folate deficiency" may have to be redefined.

## INTRODUCTION

Folic acid, a water soluble B vitamin, has recently gained considerable attention because of its presumed role in the pathogenesis of birth defects, cardiovascular disease, cancer and neuropsychiatric disorders. A substantial portion of the population appears to be deficient in folate, the most common vitamin deficiency in developed countries. Vitamin deficiencies were traditionally associated with clinical signs and symptoms and were rarely diagnosed in affluent, well nourished societies. With growing insight into molecular mechanisms it has become increasingly clear that moderate folate deficiencies are implicated in the process of chronic diseases that generate considerable costs but have good potential for prevention. More sensitive diagnostic measurements allow earlier and more precise diagnosis and a better estimation of true folate requirements for optimal health, and have thus prompted review of current recommendations for daily requirements.

Folates are essential cofactors in metabolic pathways that facilitate methylation reactions, as well as formation and transfer of "one-carbon units" to purines and pyrimidines in the biosynthesis of DNA. Proper function of the folate cycle depends upon normal function of involved enzymes, availability of vitamins and balanced requirements.

Impairment of folate metabolism, however, is associated with hypomethylation, hyperhomocysteinemia, DNA damage, impaired cell proliferation and malignancies.

With increased understanding of the fundamental reactions associated with folic acid and its bioactive derivatives, inexpensive safe supplementation with folate appears to be a promising strategy in prevention and treatment of a variety of disorders with implications for the greater part of our population.

## BIOCHEMISTRY

It was observed in the 1930s, that megaloblastic anemia could be successfully treated with liver and yeast extracts. The active ingredient was isolated from 4 tons of spinach leaves by Snell in 1941; the name folate was appropriately derived from the Latin *folium*, leaf. The term "folic acid" usually describes a group of compounds derived from 5,6,7,8-tetrahydropteroyl-*-*glutamate, more commonly referred to as tetrahydrofolate (THF). These derivatives from a common framework have distinctive metabolic characteristics.

THF is made up of a pteridine core ring system (2-amino-4-hydroxy-pteridine) that is linked to *p*-aminobenzoic acid (that is, pteric acid) by a methylene bridge from the C-6 position of the pyrazine ring, thus forming tetrahydroptericoic acid (Fig. 1). An additional link attaches glutamate to the *p*-aminobenzoic acid (with 1 to 9 residues). The pyrazine ring is fully reduced at the 5,6,7 and 8 positions and forms the biologically active form, referred to as 5,6,7,8-tetrahydrofolate (THF). Dihydrofolate (DHF) is formed through stepwise reduction of the 7 and 8 positions, using two molecules of NADPH. Several other biologically active compounds derive from this basic structure.

Mammals may synthesize the pteridine ring, but are unable to link it with the other compounds. Humans depend on dietary intake and synthesis of biologically active folate.

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## ORIGINAL FIGURE WILL BE PASTED HERE

**Fig. (1). Structure of 5,6,7,8-tetrahydropteroyl- $\gamma$ -glutamate (THF).**

Vital processes in folate disposition include conversion of dietary folylpolyglutamates to monoglutamates, intestinal resorption, receptor and carrier-mediated transport across cell membranes, cellular metabolism and export. Both receptor-mediated and carrier-mediated transport mechanisms are variably expressed in different tissues, thus partially explaining the differential effects of impaired metabolism. Multiple reactions are required to convert dietary folic acid into metabolically active folates. The greater part is attached to enzymes indicating tight metabolic regulation and limited availability of folates [1].

### METABOLISM

Natural dietary folic acid is a small, water soluble molecule occurring mainly as polyglutamate forms (preferably 5-methyl-THF and 10-formyl-THF) with  $\gamma$ -carboxyl ( $\gamma$ -amide) linked glutamate side chains. These molecules cannot cross cell membranes and therefore cannot be resorbed as such.

The glutamyl residues are cleaved by  $\gamma$ -glutamylcarboxy conjugase (E.C. 3.4.19.9) at the brush border membrane of the duodenum and jejunum, creating monoglutamate folate that is then easily resorbable in the proximal small intestine. The transmembranous transport at the enteral cell is specific and depends on pH [2]. The conjugase of the jejunal brush border membrane is a lysosomal exopeptidase, which cleaves the terminal  $\gamma$ -glutamate-residue [3]. A second enzyme with endopeptidase activity is located intracellularly in the jejunal mucosa [4]. High doses of folic acid (several mg) are resorbed almost quantitatively and exit the circulation within minutes for uptake in the liver and other tissues. After oral administration the maximum plasma

concentration is normally reached after 1-2 hours. Monoglutamates will be conjugated into polyglutamates intracellularly by  $\gamma$ -glutamate synthase (E.C. 6.3.2.17) and can be stored and metabolized in this configuration [5,6]. Furthermore, a gradient is consequently kept up that favours further uptake of monoglutamate into the cell. This is supported by folate pumps directed outwards that are directly linked to adenosine 5'-triphosphate (ATP) hydrolysis [7]. Maintenance of the transmembrane folate gradient thus depends on the net effect of these two processes [8]. In cell cultures, an equilibrium is soon reached between intra- and extracellular spaces, the intracellular pool consisting of 95% polyglutamates. The cell division rate is proportional to intracellular folate pools [5].

Within the cell, monoglutamate compounds are reconverted through hydrolysis, and can then pass the basolateral membrane and reach the liver through the portal circulation. Serum mainly carries 5-methyl-THF (~80%), which is bound unspecifically e.g. to albumin with low affinity. A minor fraction is, however, specifically attached to a folate-binding protein [9], whose metabolic significance remains unknown [10]. A further small fraction is constantly released through cell lysis. About a third is not bound and remains free.

Absorbed folate is cleared from the circulation within minutes and is taken up by various tissues and the liver, which is the most important storage organ. Hepatocytes store 10-20% of the folates, the greater part is released after metabolism, mainly into the bile. There, the folates participate in the enterohepatic circulation, with a daily turnover rate of approx. 90  $\mu$ g [11]. The folate concentration in bile is about the tenfold of serum levels. This means that bile is a readily available endogenous folate source of



excrete n-formiminoglutamate in urine following a histidine load. This test can thus be used to detect folate deficiency [23,24].

### ONE CARBON UNIT (C<sub>1</sub>-) TRANSFER AND DNA SYNTHESIS

DNA synthesis and cell proliferation require transfer of "one carbon groups"; this is a fundamental function of folates. All mammalian cells contain THF; however, most folate exists with various additional carbon groups attached. Carbon groups including methyl (-CH<sub>3</sub>), methylene (-CH<sub>2</sub>-), hydroxymethyl (-CH<sub>2</sub>OH) and formyl (-CHO) are termed "one-carbon groups" and are presented as attachments to THF. Carbon groups are passed on for biosyntheses of purines and pyrimidine (Fig. 3). 10-formyl-THF directly provides the carbon groups to be inserted into the C2 and C8 position of the purine ring for the biosynthesis of the purine building block of DNA (adenine, guanine). Conversion of 10-formyl-THF to THF and carbon dioxide is catalyzed by the liver-specific 10-formyl-THF dehydrogenase (E.C. 1.5.1.6), whereby excess C1-units are released and the THF pool is maintained [25].

The highly reactive 5,10-methylene-THF provides one carbon units (ultimately methylating thymidylate to deoxyuridylate (dUMP), generating deoxythymidinemonophosphate (dTMP), which is catalyzed by thymidylate synthase (E.C. 2.1.1.45). This reaction is a key event in the synthesis of pyrimidine and limits DNA synthesis. The product of this reaction is dihydrofolate (DHF), which is inactive in metabolism and must be regenerated to THF by dihydrofolate reductase (E.C. 1.5.1.3), consuming NADPH + H<sup>+</sup>. This enzyme plays an

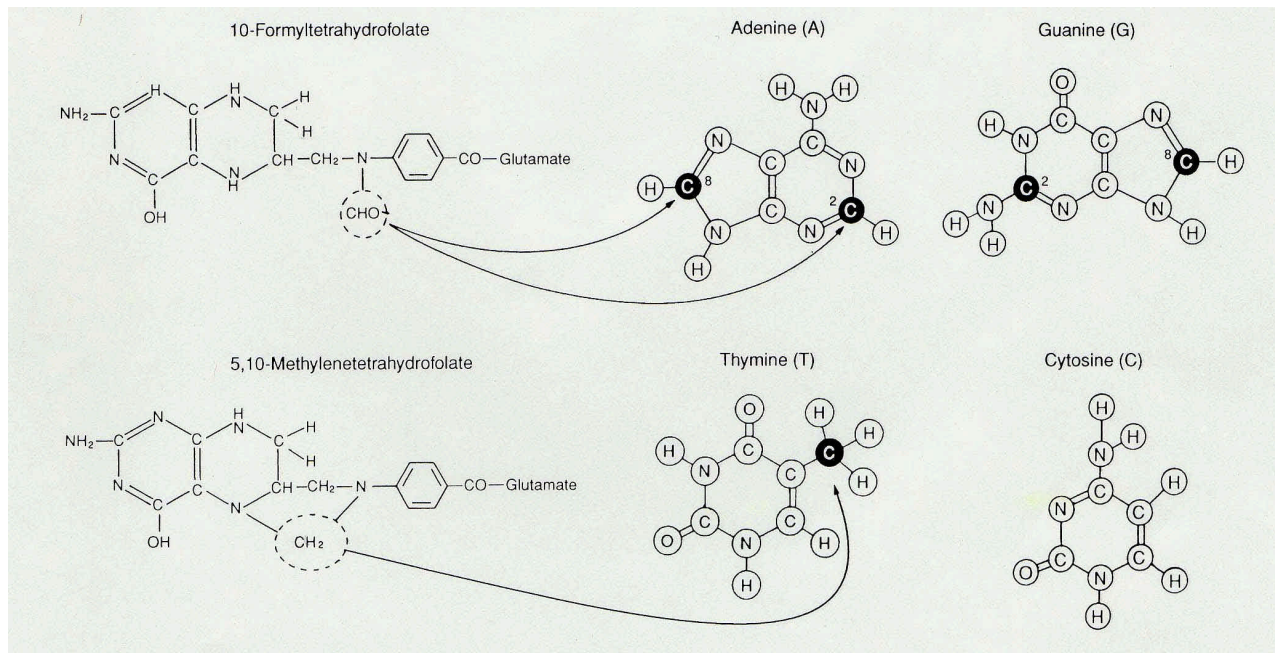
important role in folate homeostasis and has received much attention as the site of action in cancer treatment with such inhibitors as methotrexate [26]. This agent blocks the enzyme and thus availability of THF. Affinity of the drug for the enzyme is 10<sup>5</sup> greater than physiological DHF. Consequently, one-carbon metabolism is reduced and thus biosynthesis of purine and pyrimidine and ultimately of DNA, arresting cell proliferation.

These regenerated THF molecules can then accept another one-carbon group from either the amino acid serine or from 10-formyl-THF and thus participate in further biosynthetic reactions.

During the conversion of serine to glycine, a hydroxymethyl group is attached to THF, yielding 5,10-methylene-THF catalyzed by the vitamin-B<sub>6</sub>-dependent serine hydroxymethyl-transferase (EC 2.1.2.1).

The biosynthesis kinetics of purines and pyrimidine depend on the metabolism of folates demonstrating the fundamental role of the latter for the human organism. Reduced availability of folates leads to impairment of such cell functions as DNA replication, which is required for mitotic cell division during M-phase and depends upon precise disposition of the required elements. These reactions are nearly identical in eukaryotic and prokaryotic organisms and allow antibiotics and cytostatic drugs to inhibit growth and proliferation of cells and microorganisms. The therapeutic action of sulfonamides is due to competitive inhibition of p-aminobenzoic acid with inhibition of bacterial folate synthesis.

The purine bases are, unless used otherwise („salvage pathway“), oxidized to uric acid (via xanthine) and excreted.



**Fig. (3). Folates donate C<sub>1</sub>-units for insertion in DNA biosynthesis.** This figure is taken from a paper (page 225) by Scott, JM., Weir, DG. *Journal of Cardiovascular Risk* 1998; 5(4): 223 – 227. Lippincott Williams & Wilkins.[ISSN 1350-6277].

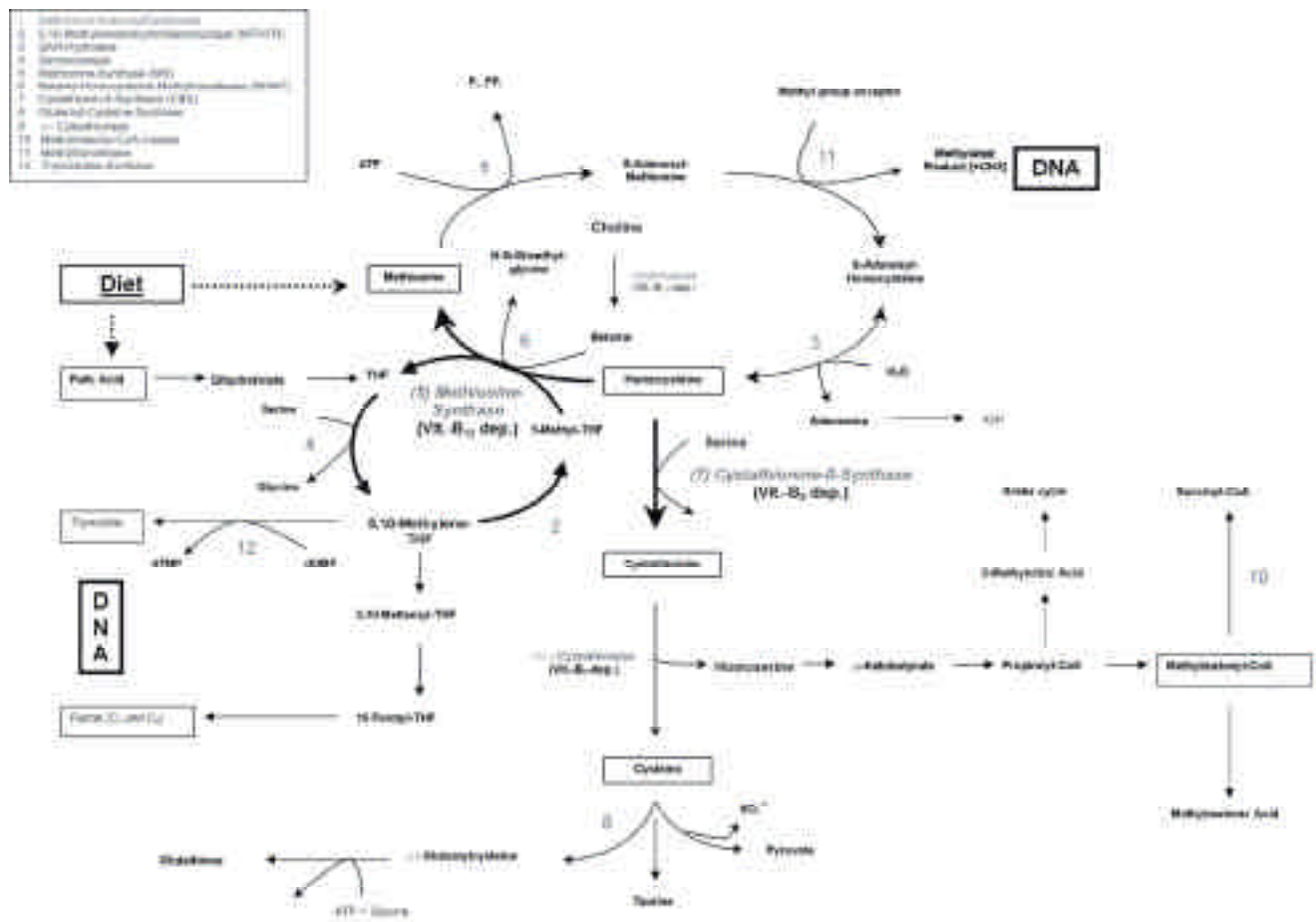


Fig. (4). Interaction of folate and homocysteine metabolisms with implications for methylation and synthesis of DNA.

Methylated xanthins are 1,3-dimethylxanthine (=theophylline) and 1,3,7-trimethylxanthine (caffeine), which are both associated with homocysteine concentration [27].

### Methylation cycle

Folate serves as substrate in the remethylation cycle converting homocysteine to methionine and S-adenosyl-methionine (SAM). SAM essentially provides methyl groups for more than a hundred important reactions including methylation of DNA. 5,10-methylene-THF is reduced to 5-methyl-THF in a  $NAD^+$ -dependent reaction by the enzyme 5,10-methylen-THF reductase (E.C. 1.5.1.20), thus channeling one-carbon-groups into the methylation cycle. This methyl group is utilized to remethylate homocysteine to methionine, catalyzed by cobalamin-dependent methionine synthase. SAM, the next derivative of methionine, acts as a high-energy methyl group donor to a wide range of compounds including DNA, proteins, neurotransmitters, hormones and phospholipids [28].

Remethylation to methionine thus depends upon disposition of folate and cobalamin. Inhibition or deficiency of folate slows the remethylation cycle and numerous methylation reactions are reduced due to lack of methyl

groups required for methyltransferases activity. DNA hypomethylation also occurs, a common feature of malignant cells [29,30].

Donation of methyl groups by SAM leads to formation of S-adenosyl-homocysteine (SAH), which is immediately hydrolysed to homocysteine. This conversion by SAH hydrolase (E.C. 3.3.1.1) is potentially reversible [31] and is indeed preferred in hyperhomocysteinemia, lowering the concentration of homocysteine. Increase of SAH inhibits the capacity of methyltransferases and may reduce their activity by 50 – 70% [32,33].

Another important enzyme involved in regulation of the folate cycle is 5,10-methylenetetrahydrofolate reductase (MTHFR), which irreversibly reduces 5,10-methylene-THF to 5-methyl-THF. Reduced MTHFR activity could severely affect folate distribution and cell functioning. MTHFR is a homodimer with a subunit Mr of 77kDa containing a catalytic and regulatory domain [34]. The binding of SAM results in allosteric inhibition of the enzyme, which can be reversed by SAH [35]. This inhibition of SAM acts synergistically with the activation of cystathionine synthase (E.C. 4.2.1.22) and is an important mechanism in the regulation of homocysteine metabolism [36]. A common thermolabile variant MTHFR 677C T was first described in

1988 [37], later identified as a point mutation in nucleotide 677 with C → T transition, resulting in exchange of valine for alanine [38]. The heterozygote (CT) variant is associated with a 30% reduction in enzyme activity and the homozygote mutation with a 70% reduction. Conversion of 5,10-methylene-THF to 5-methyl-THF thus is dramatically decreased. The TT mutation is associated with increased demand for folate and elevation of homocysteine, especially in the presence of suboptimal folate concentrations [39]. The TT variant leads to changes in intracellular folate distribution such as increase of formylated THF and the ratio of formyl/methyl-folates [40] in RBC, whereas only 5-methyl-THF is contained in the wild type variant [41].

This dysregulation of the folate cycle may lead to severe consequences in pregnancy. Down's syndrome [42] and neural tube defects (NTD) are associated with the MTHFR C677T polymorphism [43] and with low folate status [44]. Preconceptional folate intake, however, can dramatically reduce the incidence of newborns with NTD [45]. Reduced enzyme activity must also result in increased concentrations of 5,10-methylene-THF thus promoting conversion of dUMP into dTMP and biosyntheses of DNA, which may explain the protective role of the MTHFR polymorphism on colorectal malignancies [see below]

## THE FOLATE TRAP

The folate-trap hypothesis explains why cobalamin deficiency often results in functional folate deficiency. Conversion of 5,10-methylene-THF to 5-methyl-THF *in vivo* is practically irreversible [46]. 5-methyl-THF can only be reused when the methylation cycle is maintained through cobalamin-dependent remethylation generating THF. Thus continuous transfer of methyl groups depends upon 5-methyl-THF and cobalamin. This is the only known direct linkage of two vitamins in man. Folate and cobalamin depend on and utilize each other. In cobalamin deficiency, even when there are enough folates and 5-methyl-THF, there can be an intracellular deficiency of biologically active THF [47]. This is called the "folate trap". 5-methyl-THF is increasingly "trapped" because it can neither be converted to THF nor go back to 5,10-methylene-THF. When this happens, the important methylation cycle will be reduced, with implications for numerous reactions [48]. Cofactors of one carbon group transfer reactions are decreased and cell division rates are limited. Polyglutamate synthesis ceases, limiting the pool to monoglutamates which are not effectively retained by the cell. Importantly, methionine synthase activity is decreased due to insufficient cobalamin with secondary reduction of folate metabolism and reduced de-novo synthesis of purines and pyrimidines [49], further aggravating the situation. Lack of reduced folates is seen early in rapidly dividing and proliferating cells in hematopoiesis in bone marrow.

The clinician can not distinguish between folate and cobalamin deficiency as a cause of megaloblastic anemia. Treatment with cobalamin will terminate the inhibition rapidly and the blood picture will quickly normalize. If folate alone is given, however, it will only be converted to DHF and THF, which may be utilized for cell division but without

termination of the blockade. Therefore, in megaloblastic anemia, THF is only able to normalize reduced synthesis of thymidylate [50]. In any case, these folates must also enter the trap. Long term treatment with high doses of folate only mask the real cause of pernicious anemia due to vitamin-B<sub>12</sub>-deficiency. Serum folate will increase (accumulation of non regenerated 5-methyl-THF) with concomitant decrease of intracellular (RBC) folates. Intracellular storage is impaired because polyglutamate synthesis is inhibited.

Inhibition of the methylation cycle and impairment of cellular functions affect synthesis of myelin, the nerve sheath. Unrecognized long-term cobalamin deficiency thus can cause severe neurological damage [21]. Therapy with folate alone may let the damage progress to irreversibility because administration of 5-methyl-THF will not close the folate trap when both folate and cobalamin are deficient [51].

There has been discussion as to whether the folate (methyl-) trap can be regarded as a physiological reaction to the danger of methylgroup deficiency in protein malnutrition [52]. In malnutrition, lack of methionine will lead to lack of SAM and transfer of methyl-groups. Under this condition the conversion of 5-methyl-THF to THF and especially remethylation of homocysteine to methionine will be preferred. DNA biosynthesis and cell division are reduced at the same time and the requirement for methionine is low. With folate deficiency, priority will thus be given to saving the function of neurological (brain) tissue [52].

## NUTRITION

The total content of folates in the human body was determined by kinetic studies with labeled folate to be approx. 20 – 70 mg [53,54]. About one-half is stored in the liver [21] and is equally distributed between cytosol and mitochondria [55]. Good nutritional sources of folate are yeast, grain, meat, vegetables, dairy products, fruits and nuts. Even with plentiful calories and apparently balanced and sufficient nutrition, folate intake may be suboptimal even in developed countries.

This is due, in part, to modern handling and processing of food, especially grain products. When wheat is processed to flour, up to 90% of the oils, vitamins, minerals, phytochemicals and fibers are removed [56]. In addition, folate is labile and sensitive to exposure, heat and preparation food. Consequently, much of the folate content in food is lost in transport, storage, and finally, cooking. With modern processed food, it appears to be difficult for the majority of the population to meet important nutritional requirements without supplementation. Low intake, impaired resorption and increased demand may further predispose certain groups at risk for suboptimal folate intake such as the elderly, alcoholics, the sick, pregnant women and children.

Mean intake was reported as 277 – 297 µg for men between 19 and 30 years of age in the IOM report [57]. These data are in agreement with data compiled in Europe, where mean intake in adults was determined to average 291 µg/day for men (197 – 326 µg) and 247 µg (168 – 320 µg) for women) [58].

It would seem desirable to ensure a minimum intake of at least 350 µg/day, but it appears that only a minor fraction of the population actually achieves this goal through natural dietary intake. Intake of 600 – 650 µg/day or supplementation with approx. 200 – 350 µg/day decreases plasma homocysteine to normal values if elevated, suggesting this to be the optimal total intake [58].

## FOLATE DEFICIENCY

Folate deficiency is regarded as the most common vitamin deficiency in developed countries. Traditionally, folate deficiency was thought to be associated with overt clinical manifestations such as changes in blood count and cell morphology. Exclusive measurements of serum folate levels are not reliable as normal blood pictures are very frequently seen with low plasma folate concentrations. Most likely, one must distinguish between minimal intakes required to prevent overt clinical manifestation of deficiency, and intakes associated with optimal performance and health. Furthermore, requirements may depend on specific factors such as age, ethnic background and gene-environmental interactions. It is thus almost impossible to give absolute minimum recommendations in absolute values for the general public.

Elevated homocysteine levels are an early and sensitive indication of suboptimal folate status. Decrease of homocysteine after folate supplementation may further indicate suboptimal folate status regardless of plasma folate concentrations. Experience based on more than 2500 measurements at our institution correlates most total plasma homocysteine elevations (>12 µmol/L) with folate levels in the middle or lower third of the normal reference range.

With full folate stores first clinical signs of deficiency can be expected after at least 3 – 6 months of inadequate intake. Hypersegmented neutrophils may appear approx. 3 months after cessation of folate supply. The histidine loading test will be positive later and after months, megaloblastic anemia can be expected. An absolute minimum requirement of at least 50 µg / day of folate must be supplied to avoid acute signs of deficiency. Possible signs of chronic deficiency include cognitive dysfunction, dementia, insomnia, mood and other mental changes and depression [59,60].

As mentioned above, certain subgroups are at increased risk for folate deficiency due to higher demand. Folate deficiency is rarely found as an isolated vitamin deficiency but is usually associated with other deficits. Alcoholics, for example, may suffer from general malnutrition and transport defects for folates and thus commonly exhibit folate deficiencies. Patients with chronic inflammatory bowel diseases, which is a risk factor for malignant transformation in the intestinal tract [61], have higher folate demands [62]. Drugs such as sulfasalazine interfere with folic acid absorption and reduce bioavailability further aggravating folate deficiencies when patients on these drugs do not receive supplementation [63].

## BLOOD

Folate deficiency arrests cell division, as is best seen in a cell population with rapid turnover. The presence of abnormal red cell precursors (megaloblasts) in the bone marrow and anemia with raised mean corpuscular volume (macrocytic) in peripheral blood can indicate either folate or vitamin-B<sub>12</sub>-deficiency. Not all folate deficient subjects exhibit macrocytic anemia and diagnosis solely based on these features will miss a large number of patients with inadequate folate supply.

Reduced availability of 5,10-methylene-THF inhibits thymidylate synthase and therefore DNA biosynthesis. Because of the high rate of cell division in bone marrow folate deficiency will soon be evident in impaired hematopoiesis with appearance of abnormal red cell precursors (megaloblasts); this condition is known as megaloblastic anaemia. Most of the precursors become arrested at various stages of interphase [64]. Neutrophil hypersegmentation usually precedes macrocytosis and is therefore considered a more sensitive hematologic finding in folate deficiency [65]. In a classic self experiment 5 µg / day of folate led to hypersegmentation of neutrophils after only 7 weeks in an adult [66].

Oval macrocytosis with megaloblasts, hypersegmented neutrophils, leucopenia with right shift and thrombocytopenia may also be found in advanced stages [67]. The functional end result in impaired DNA synthesis due to folate deficiency is ineffective hematopoiesis affecting all cell lines. Up to 90% of precursor cells in bone marrow can be affected leading to pancytopenia. Destruction of RBCs increases serum bilirubin reflecting red cell lysis and breakdown of hemoglobin. Before neutropenia is apparent in advanced stages of folate deficiency, functional impairment of white (immune) cells may occur, favoring infections. A significantly higher infection rate with human papilloma virus (HPV) –16 was found in cervix tissue [68]. HPV-16 infections are a risk factor for dysplasia, a condition in cervical smears that can be reversed through folate supplementation [69,70]. A significant correlation between folate, homocysteine and IgG antibodies against *Chlamydia pneumoniae* was recently found in patients with coronary artery disease [71]. Impaired phagocytic and bactericidal properties of polynuclear leukocytes (neutrophils) have been described in folate deficiency *in vitro* and *in vivo* [72].

## DIAGNOSIS OF FOLATE DEFICIENCY

Folate concentrations are commonly determined in plasma, serum and RBCs. The folate content of circulating red blood cells is a better index of folate status and reflects general tissue supply [73,74]. The half-life of folate in red blood cells is approx. ~100 days [75] and thus can not react to short-term fluctuations. Because RBCs have a life-span of approx. 120 days, determination of RBC folate will not be a very sensitive indicator of developing folate deficiency. If concomitant vitamin-B<sub>12</sub> deficiency is present, 5-methyl-THF will increase ( folate trap) and be released from cells into serum as monoglutamate. That explains the finding of reduced RBC-folate and increased serum folate under this

condition. In short, measurement of plasma folate allows better estimation of dietary folate intake [73], whereas RBC folate reflects tissue distribution.

Plasma folate should be determined from a blood sampling taken after an overnight fast; fasting is not necessary prior to determination of RBC folate. RBC folate exceeds serum folate approx. thirtyfold under normal conditions. Higher concentrations are usually explained by intake of vitamin supplementation. Samples should be stored on ice immediately, or at least centrifuged and cooled, because hemolysis will release folate (and homocysteine) and may lead to false positive concentrations.

The most sensitive and specific test for cellular folate deficiency, which antedated sensitive and accurate homocysteine assays, was the deoxyuridine suppression test [54,76]. It is directed at the *de novo* thymidine synthesis pathway and 5,10-methylene-THF adequacy. Although discrimination of vitamin deficiency was better than determination of either methylmalonic acid or homocysteine [77], pathological results have been observed even with normal serum folate concentrations [78].

After a histidine loading test (oral intake of 15 or 20 g histidine) patients will excrete increased N-formiminoglutamate in the urine, which can be used diagnostically to detect folate deficiency [23,24]. Normally less than 30  $\mu\text{g} / \text{mL}$  (0.1 to 18 mg / day) are excreted. Deficiency of active folates will result in greatly increased loss (up to 2000 mg / day). Neither of these tests are now in common use. There is a very strong and inverse correlation between folate status and homocysteine. Homocysteine is sensitive for folate deficiency, but it does not distinguish between anemia due to folate or vitamin-B<sub>12</sub> deficiency, and may also be raised for other reasons such as renal failure or intake of certain drugs. Its diagnostic sensitivity to detect folate deficiency was determined to be ~91% [74]. Thus homocysteine is a sensitive determinant of folate [79] and is suitable for screening purposes. If measurable elevated homocysteine concentrations reflect folate deficiencies, this would mean that up to 5 – 7% of the general population and approx. 40% of patients with vascular disease are folate deficient [80,81]. It also suggests that currently accepted reference ranges for sufficient folate intake should be reconsidered.

Cystathionine is elevated in almost all patients with known folate deficiency [82] and it as well cannot distinguish between folate and vitamin-B<sub>12</sub> deficiency. Determination of this metabolite is therefore of limited use for detecting folate deficiencies.

#### **EFFECT OF FOLATE DEFICIENCY ON HYPOMETHYLATION, DNA DAMAGE AND CARCINOGENESIS**

Methylation of DNA is a key biologic event with implications for regulation of gene expression, transcription, chromatin structure, genomic repair and genomic stability [30,31]. Hypomethylation can be caused by folate deficiency, and results in reduced remethylation to

methionine with decreased provision of SAM, the most important methyl group donor in man. Furthermore, this deficiency will also increase homocysteine and SAH concentrations; this in turn inhibits methyltransferase activity in a dose-responsive relationship. In consequence, low folate supply will not only limit availability of SAM but also reduce activity of the enzymes catalyzing the methyl group transfer. Additionally, low deoxythymidylate and increased deoxyuridylates due to limited 5,10-methylene-THF will lead to misincorporation of uracil instead of thymine into DNA [64,83]. This in turn requires repairs to chromosomal strand breaks and cell damage with high risk of transformation into malignancy. It is for these reasons that folate deficiency can directly influence carcinogenesis [84] favoured by hypomethylation and impaired DNA-stability. This had been demonstrated *in vitro* in dose-response dependency at folate concentrations found physiologically (1 – 10 ng/mL) in plasma [85]. Low folate supply has been associated with carcinomas of the colorectum, the cervix, lungs, esophagus, pancreas [86] and breast [87].

Excessive DNA misincorporation of uracil constitutes a potentially mutagenic lesion requiring repair through excision by specialized enzymes (glycosylase). These repairs cause temporary strand breaks associated with defective replication. Even the repair mechanisms are defective in folate deficiency [88,89]. Simultaneous excision of multiple opposing strands will result in double strand breaks with further risk increase of instability. If double strand breaks remain unrepaired, there is cellular transformation and even more degeneration. Both phenomena, excessive uracil incorporation, and chromosomal strand breaks, are potentially reversible through folate supplementation [83].

A common finding in malignant cells is global or local hypomethylation, which constitutes constant change in neoplastic cells [31,90]. *In vivo* and *in vitro* studies of human cells showed chromosomal strand breaks, excessive misincorporation of uracil into DNA, and hypomethylation [91,92]. Another typical finding is formation of micronuclei; this strongly correlates with homocysteine and cobalamin levels [93]. Genomic stability is reduced to a minimum *in vitro*, when folate concentration is > 227 nmol/L in medium [91,92]. Interventional studies have demonstrated minimum hypomethylation, strand breaks, micronuclei formation and uracil misincorporation when RBC folate is > 700 nmol/L, cobalamin > 300 pmol/L and homocysteine levels < 5.5  $\mu\text{mol/L}$  [91]. Thus maximal genomic stability could be achieved through daily additional intake of only approx. 200 – 400  $\mu\text{g}$  of folate and > 2  $\mu\text{g}$  of cobalamin. In fact, a total intake of 700  $\mu\text{g}$  folic acid and 7  $\mu\text{g}$  cobalamin was effective in a placebo-controlled study to reach the maximum micronucleus index [91]. Smokers are known to have low serum and RBC folate [93,94] and morphologic changes in epithelial cells of the buccal mucosa [95]. This cannot be explained by lower dietary intake but rather by a local (tissue) deficiency [94,95]. Components of smoke such as organic nitrites and cyanates effectively convert folates into biologically inactive forms [96]. An inverse relation was seen for folate supply and prevalence of lung cancer in men [97]. Hypomethylation of the p53 tumor suppressor gene in lymphocyte DNA is associated with a twofold increase of risk for bronchial carcinoma in male smokers [98] and can

be due to functional folate deficiency [99]. Importantly, this phenomenon is thought to be reversible through folate supplementation [100]. The cytological findings in male smokers with metaplastic cells in sputum were considerably improved after 4 months of supplementation with folic acid and cobalamin [88].

It may be that DHF and 5-methyl-THF are responsible for inhibition of cell proliferation in carcinoma cells [85], explaining the protective effect on development of colorectal tumors associated with MTHFR C677T homozygous mutation (TT) that is twofold compared to heterozygote mutation or wild type [101]. The homozygous variant is associated with a 70% reduction in enzyme activity and accumulation of 5,10-methylene-THF [102]. Folate deficiency further inhibits genomic repair mechanisms due to oxidative and alkylation damage. In human colorectal adenocarcinoma tissue, antibodies against 5-methylcytidin were used to demonstrate quantitative and qualitative changes in DNA methylation and tissue morphology [103]. Folate deficiency-mediated cellular abnormalities were found in mucosal cells in the entire intestinal tract [103,104]. Animal studies have further shown protective effects of folate administration on the prevalence of colonic tumors, again in a dose-responsive fashion [105].

Patients with chronic inflammatory bowel diseases such as ulcerative colitis and Crohn's disease are at risk of developing cancer and have a higher demand for folates [106,107]. This is aggravated by administration of sulfasalazine [108]. Increased intake of folate is associated with a 60% reduction in tumor development in ulcerative colitis, but a 50% increase was found if chronic administration of sulfasalazine is not accompanied by folate supplementation [109]. The total risk reduction for detection of dysplasia or carcinoma was calculated as 18% for each 10 ng/mL increase in RBC folate [110] and was supported in two prospective studies [111,112].

Plasma folate must not necessarily differ between patients and controls, and the absolute level does not indicate low total concentrations [113]; thus, systemic determination of folate levels is a poor indicator for folate deficiency in tissue. Homocysteine indicates cellular deficiency more accurately [114] and correlates well with the risk of colorectal carcinoma, especially with alcohol intake [115]. Gender and alcohol appear to act as "effect modifiers".

The growing body of evidence from epidemiologic, animal, *in vitro* and *in vivo* studies points toward a realistic means of colonic cancer prevention [113,116-118], urging better folate supply in the general population. Low serum and RBC folate concentrations, and most particularly elevated homocysteine, were also found to correlate with the risk of invasive cervical carcinoma [69,70,119], and folate supplementation improved cervical dysplasia [68-70]. Low folate levels and megaloblastic changes were found in users of oral contraceptives as early as in the 1960s and were explained on the basis of interference with intestinal conjugase [120,121]. The protective effect of folate supplementation, however, appears limited to dysplasia, and cannot be expected in lesions such as carcinoma *in situ* or more advanced stages.

## CARDIOVASCULAR DISEASE

Several observational studies found a clear association between low folate intake and risk of coronary heart disease [122-124]. Because numerous mechanisms of homocysteine-mediated vascular damage have been suggested (mostly from *in vitro* experiments), almost all interventional studies focused primarily on the relationship between homocysteine levels and risk of coronary heart disease. Folate was only seen as a means of reducing homocysteine. Only recently has a beneficial effect on vessels independent of homocysteine been attributed to folate itself. Vascular endothelial dysfunction is a recognized key event in the etiology of atherosclerosis and is an accepted surrogate end point for clinical investigations in cardiovascular disease. Folic acid supplementation was demonstrated to improve endothelial dysfunction in asymptomatic subjects with hyperhomocysteinemia [125,126] as well as in hyperhomocysteinemic patients with established coronary heart disease (CHD) [127,128]. Interestingly, this beneficial effect was also observed in subjects without elevated homocysteine concentrations [129,130] suggesting a distinctive effect of folates on the endothelium independent of homocysteine. Further indirect evidence comes from the recent observation that therapy with the antifolate methotrexate may promote atherosclerosis [131]. In interventional studies, it is difficult to distinguish between the effects of folate administration and the homocysteine-lowering effect related to the vitamin increase. This is particularly the case when a study group is compared with a placebo group without any changes. In an attempt to separate the effects, our study group designed a study of CHD patients without a placebo group, in which the patients served as their own controls. All subjects received an identical dose of folate supplementation for 12 weeks in an on-off fashion and resistance vessel reactivity was repeatedly assessed by venous occlusion plethysmography. As expected all subjects had a 3.5fold increase in plasma folate, however, improvement of vessel reactivity was exclusively observed in individuals with a decrease in plasma homocysteine of > 2  $\mu\text{mol/L}$  [132]. In subjects with no significant changes in homocysteine levels, folate administration was associated with increased antioxidative capacity as determined by total antioxidant status (TAS), suggesting an antioxidative potential for folate [132]. Indeed, 5-methyl-THF was found to reduce superoxide generation *in vitro* and *in vivo* [128,133]. The antioxidative capacity of 5-methyl-THF was recently determined to be approx. 20fold lower than vitamin C, reducing superoxide generation and restoring NO synthesis [134]. Thus 5-methyl-THF may have direct effects on the enzymatic activity of NO synthase, which is supported by the identification of a pteridine-binding domain with similarities to the folate binding site of DHF reductase [135]. Protection against oxidative modifications of human low density lipoprotein (LDL) in a dose-responsive manner has recently been demonstrated, further supporting the hypothesis of direct and indirect antioxidant effect of folates [136]. In contrast, folate deficiency was found to result in increased lipid peroxidation in rats [137] and decrease of cellular antioxidant defense [138]. The nitric oxide (NO) system offers further potential mechanisms through which folates may effectively interact. NO-formation from L-arginine by eNOS requires several cofactors such as

tetrahydrobiopterin (BH<sub>4</sub>) [139]. It was suggested that folates may stimulate regeneration of BH<sub>4</sub> from the inactive oxidized quinoid dihydrobiopterin [140] thus increasing NO bioavailability *in vivo* [141] with a protective effect on vascular function.

## COMMENTS

With better understanding of the fundamental reactions associated with folate and its bioactive derivatives, it appears that cheap and safe supplementation of folate may be a promising tool in prevention and treatment of a variety of disorders. During the past decade, marginal folate deficiency has been increasingly viewed as an important public health issue. The US introduced food supplementation with 140 µg folic acid per 100 g in 1998 [142]. The primary intention focused on reducing the incidence of newborns with NTD [143-145]. Rapidly increasing interest and research activities led to the current assumption that low folate status and high homocysteine levels are risk factors for numerous degenerative conditions including cardiovascular diseases, neurodegenerative disorders and thromboembolic complications. The preventive potential of homocysteine-lowering through folate supplementation on cardiovascular mortality was calculated with up to 50,000 lives annually in the US alone [146], thus justifying the introduction of food supplementation by law. The cost-benefit ratio was calculated to be at least 1:24 to 1:29 [142,147]. Data from the Framingham study indicate a folate intake of less than 400 µg / day in 40% of the elderly [148]. Fortification has increased mean intake more than twofold between 1994 and 1998 [149,150] and reduced hyperhomocysteinemia by half [150], suggesting that the measure is very effective. The available data strongly suggest that criteria for "folate deficiency" may have to be redefined. Desirable folate intake is likely to depend on a variety of genetic, nutritional, environmental and ethnic factors, meaning that a more individual definition and understanding may be required. Data from large, prospective and randomized studies available in the near future will help to prove or refute the hypothesis that higher folate intake may indeed be very helpful in preventing disease. It is tempting to assume that we are traveling down a road that is not only very promising but also very safe.

## ABBREVIATIONS

NO	=	Nitric oxide
eNOS	=	Endothelial nitric oxide synthase
DNA	=	Desoxyribonucleic acid
THF	=	Tetrahydrofolate
DHF	=	Dihydrofolate
ATP	=	Adenosine 5`-triphosphate
RFC-1	=	Reduced folate carrier
dUMP	=	Deoxyuridylate

dTMP	=	Deoxythymidinemonophosphate
SAM	=	S-adenosyl-methionine
SAH	=	S-adenosyl-homocysteine
MTHFR	=	5,10-methylenetetrahydrofolate reductase
NTD	=	Neural tube defects
HPV -16	=	Human papilloma virus - 16
CHD	=	Coronary heart disease
TAS	=	Total antioxidant status
BH <sub>4</sub>	=	Tetrahydrobiopterin

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