Folate and Human Development

Edited by

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Preface

Neural tube defects (NTDs) are a complex developmental trait in which several genes, interacting with environmental factors, create the phenotype. In the United States, the rate of NTDs has been reported to range from 4 to 10 per 10,000 live births. Currently, in the United States, the two most common types of NTDs, anencephaly and spina bifida, which occurs in varying degrees of severity, affect approximately 4000 pregnancies *per annum*, resulting in the birth of 2500 to 3000 children manifesting one or the other of these conditions. Nevertheless, it has been proposed that 50–70% of the defects comprising this constellation of conditions could be prevented with daily intake of 400 μg of folic acid throughout the periconceptional period. In this regard, The Centers for Disease Control and Prevention (CDC) recommended, in 1991, that women who have experienced a pregnancy affected by NTDs who are planning a new pregnancy consume 400 μg of folic acid daily beginning at least one month prior to conception and continuing through the first three months of pregnancy. In addition, in 1992, the US Public Health Service (USPHS) recommended that all women of reproductive age consume 400 μg of folic acid *per diem*. In 1996, the US Food and Drug Administration authorized and then required (1998) that enriched grain products be supplemented with folate. Also in 1998, the Institute of Medicine (IOM) recommended that all women of childbearing potential consume 400 μg of synthetic folic acid per day from fortified foods and/or a supplement in addition folate obtained from a varied diet.

These actions resulted in a significant decrease in the incidence of NTDs despite the 1998 report that only 29% of US women complied with the USPHS and IOM recommendations. Thus, in women receiving prenatal care, the rate decreased more than 19% (from 3.78/10,000 live births to 3.05/10,000) while in women receiving prenatal care only during the third trimester or none at all, the rate decreased approximately 13% (from 5.34/10,000 live births to 4.65/10,000).

The greatest need for folate occurs during pregnancy. During development, as the number of rapidly dividing cells increases, the requirement for folate increases. The situation is complicated by decreased absorption and increased clearance of folate during pregnancy. By the third trimester, the requirement for folate has almost doubled. In addition to NTDs, periconceptional folic acid supplementation reduces the occurrence of several human congenital malformations including craniofacial and heart defects. In the United States, because normal diets seldom supply the 400 μg *per diem* of folate required during pregnancy, 20–25% of otherwise normal pregnancies are associated with low-serum folate levels. During July and August 1998, a survey was conducted to assess knowledge of the benefits of adequate folic acid consumption among women of childbearing age in the United States. The results were compared to those obtained from a similar survey conducted in 1995. The 1998 findings revealed that only 7% of women knew that folic acid should be taken prior to pregnancy to reduce the risk of NTDs. It is to be noted that, although recommendations regarding folic acid consumption were issued by health authorities in a number of countries in the early 1990s, assessment of periconceptional intake of folic acid revealed a disappointingly low level of compliance. Therefore, it appears that, regardless of food fortification policies, continued promotion of the benefits of folic acid supplementation to optimize the folate status of women of child-bearing age will be required in most countries. Indeed, although periconceptional folate supplementation has been encouraged in the United Kingdom since the early 1990s, no concurrent decline in NTD pregnancies has been observed by regional congenital anomaly registries. Conceivably, additional nutritional inadequacies also may be involved. However, these have not been extensively researched. In this regard, it is of interest to note that, in California between 1989 and 1991, the interaction between maternal preconceptional dietary and supplemental zinc intake and the risk of NTDs was investigated in a population-based case-control study. Four hundred and thirty (430) NTD-affected fetuses/infants and 429 randomly selected, non-malformed infants comprised the case and control populations. The preconceptional use of vitamins, minerals, and food supplements was reported by the mothers who completed a 98-item food frequency questionnaire. Phytate intake, a dietary constituent known to interfere with zinc absorption, appeared to negatively impact the zinc/NTD association. It was observed that increased servings of animal products, which are the most bioavailable food source of zinc, were associated with a reduced risk for NTDs. Risk estimates for zinc intake changed little after controlling for multiple

sociodemographic factors and total folate intake, but were attenuated after controlling for nutrients highly correlated with dietary sources of zinc. The analyses indicated that risk of NTDs in fetuses and infants decreased with increasing maternal preconceptional zinc intake. However, it remains unclear whether increased zinc intake or other nutrients or combinations of nutrients that may be highly correlated with dietary zinc intake are causally associated with reduced NTD risk.

Homocysteine status also appears to play a role in NTDs. Homocysteine, a sulfur-containing amino acid is generated through the demethylation of methionine. It is metabolized via three principal routes. The predominant pathway is selected by physiological need. Thus, homocysteine can be metabolized to cysteine by transsulfuration or remethylated to methionine or hydrolyzed to α-ketobutyrate, ammonia, and H_2S. Regulation of the plasma level of homocysteine is dependent on nutrient uptake, especially uptake of folate and vitamins B_6 (pyridoxine) and B_{12} (cobalamin). In addition, its metabolism is affected by genetic individuality. Excess levels of homocysteine are thrombophilic and damage the vascular endothelium. In adult populations, total plasma homocysteine (tHcy) is an established clinical risk factor for coronary artery disease as well as other arterial and vasoocclusive diseases. These vascular effects appear to be related to its role as a teratogen in the pathogenesis of NTDs and other developmental defects since genetic variants resulting in hyperhomocysteinemia are associated with NTDs. Thus, genetic variation in folate metabolic genes is expected to contribute to the risk of NTDs.

The observation that homocysteine and vitamin B_{12} levels are independent predictors of NTD risk suggested that the gene encoding methionine synthase might play a role in the induction of NTDs. Methionine synthase catalyzes the vitamin B_{12}-dependent conversion of homocysteine and 5-methyltetrahydrofolate to methionine and tetrahydrofolate. However, tests of an association between specific methionine synthase alleles and NTDs indicated that inherited variations in the gene do not contribute to NTD risk, at least not in the population studied. However, impairment of folate and vitamin B_{12} metabolism has been observed in families with NTDs. Therefore, it is conceivable that genetic variants/mutants of enzymes in the homocysteine remethylation pathway might act as predisposing factors contributing to NTD risk. The first polymorphism discovered that was associated with increased NTD risk was the 677C–>T mutation (A222V) in methylenetetrahydrofolate reductase (MTHFR). It is to be noted that this variant also has been associated with increased risk of nonsyndromic orofacial clefts. A polymorphism, 66A–>G (I22M), in

the gene that encodes the enzyme, methionine synthase reductase (MTRR), that activates vitamin B_{12}-dependent methionine synthase, also has been reported. This mutation has an allelic frequency of 0.51 and increases the risk of NTDs when vitamin B_{12} status is low. In addition, in the presence of the 677C–>T mutant MTHFR genotype, the 66A–>G (I22M) MTRR mutant increases the risk of NTDs under conditions of adequate vitamin B_{12}. When the genotype and B_{12} status of 56 children with spina bifida and 58 mothers of spina bifida children were compared to control groups consisting of 97 children and 89 mothers, the spina bifida cases and associated mothers were approximately twice as likely to possess the homozygous 66A–>G (I22M) MTRR mutant genotype than the control groups. But, the difference was not statistically significant. However, the risk for homozygous 66A–>G (I22M) MTRR mutant genotype mothers with low B_{12} status to deliver a child with spina bifida increased approximately fivefold [odds ratio (OR) = 4.8, 95%; CI = 1.5–15.8], while the OR for spina bifida in children with this combination was 2.5 (95%; CI = 0.63–9.7). On a background of combined MTHFR and MTRR homozygous mutant genotypes, children had a fourfold increase in risk (OR = 4.1, 95%; CI = 1.0–16.4) of manifesting spina bifida while mothers had a threefold increase in risk (OR = 2.9, 95%; CI = 0.58–14.8) in delivering a child with this condition. Clearly, the interaction between vitamin B_{12} deficiency and the mutant MTHFR and MTRR genotypes indicates a multifactorial induction of NTDs. However, the mechanism is complex and unresolved. Furthermore, the MTHFR 677C–>T and MTRR 66A–>G mutations are each associated with increased risk of Down syndrome. In the presence of both mutations, the risk is even greater.

It is well known that drugs and other chemicals can induce birth defects in humans. For example, pharmaceuticals such as valproic acid and other antiepileptic drugs that interfere with folate metabolism can induce NTDs. Therefore, a question worth considering is: Is low folate status during pregnancy a factor that increases or contributes to an increase in the risk of induction of NTDs and other birth defects from exposure to ambient levels of environmental xenobiotics? Unfortunately, this is a public health issue for which we have little information. Therefore, to gain further insight into the etiology of NTDs as well as all other birth defects that affect so many for their lifetime, continued research on the role of folate and other nutrients is imperative. The editors believe that the research data gathered by the contributors to this work are a step in the right direction.

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Animal Models of Folate-Related Neural Tube Defects

Nicholas D. E. Greene and Andrew J. Copp

INTRODUCTION

As in many areas of human developmental biology, the use of animal model systems may be of considerable benefit in attempting to understand the role of folate in prevention of neural tube defects (NTD). At present, direct study of prenatal human development is limited because of ethical considerations and the paucity of embryonic material available. Model systems provide several advantages, including the ability to study early stages of embryonic development in terms of morphology, biochemistry, and molecular biology. Potential teratogenic and therapeutic agents can be tested in a controlled environment to examine their effects on developmental processes and their relationship to folate metabolism. In addition, use of in vitro embryo culture methods enables the effect of such agents to be tested in the absence of potentially confounding maternal factors.

Increasingly, the animal model of choice is the mouse, although several others, notably the rat and chick, have been used, each with its own advantages. Studies of the mouse benefit from the vast amount of genetic data that has accumulated in recent years. Among the vertebrates, the mouse is second only to human in our understanding of its genetics. Hence, the mouse genome can now be manipulated by gene targeting and transgenic approaches, providing powerful tools for understanding the function of genes that may be involved in all aspects of developmental biology. Moreover, advances in mouse genetics have enabled the identification of the defective genes in many of the naturally occurring mutant strains of mice that have previously been identified as providing particularly useful models for human diseases. Added to this is our increasing ability to study, in the mouse, the many key

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genes of early development originally identified in invertebrate model systems such as *Drosophila melanogaster* and *Caenorhabditis elegans.* Overall, therefore, the mouse provides an excellent model system for studies of mammalian development with a view to extrapolation to human developmental disease.

When considering folate-related defects specifically, the use of animal models circumvents three major problems that would hamper the study of genetic or environmental factors directly in humans:

1. *Limitation of the availability of early embryonic material*. The availability of naturally occurring or targeted mutant mouse strains provides the opportunity to study the effects of single or multiple known genes at early stages of development when human embryonic material is scarce. Where abnormalities of folate metabolism are detected, they can be correlated with both the developmental defect and the gene whose product is abnormal. Similarly, teratogen-induced defects can be reproducibly generated to allow analysis of embryos at different developmental stages.
2. *Variability in environmental factors*. The occurrence of NTD is determined by both genetic and environmental factors *(1,2)*. Studies of the role of folate in human development are frequently complicated by the presence of many coexisting variables such as maternal age, diet, folate status, vitamin B_{12} levels, and exposure to potential teratogens *(3,4)*.

   The ability to maintain mice under more strictly defined conditions (e.g., with uniform diet and maternal age) reduces this inherent variability and facilitates analysis of particular etiological factors. Moreover, the relatively large size of mouse litters enables comparisons among wild-type, heterozygous, and homozygous mutant embryos within a single maternal environment. This is particularly useful when studying gene–environment interactions.
3. *Variability in genetic background.* The incidence of NTD is known to be affected by multiple genetic factors *(3)*, although very few of these genes have yet been identified in humans. In the mouse, NTD can result from single-gene defects, but with a major influence of the genetic background. Such background effects can probably be attributed to the action of "modifier" genes that are polymorphic between different mouse strains or human populations. In humans, the C677T polymorphism of methylene tetrahydrofolate reductase (MTHFR) is associated with an increased risk of NTD in some populations but not in others *(5–8)*. Although the apparent population differences may have partly resulted from environmental factors or from differences in study methodology *(9)*, the effect of the MTHFR polymorphism is probably also modified by other genes that differ between populations. Experimental variability associated with genetic background effects can be avoided in mouse studies by the use of the many genetically defined inbred strains that are now available. These strains permit the analysis of single-gene or teratogenic effects in the absence of additional genetic variation and also facilitate the search for modifier genes that may predispose to, or protect individuals from, NTD.

The following sections review our current understanding of the role of folate in relation to NTD and other developmental defects, as revealed by the analysis of mouse models.

GENETIC MODELS ASSOCIATED WITH FOLATE METABOLISM

There are now more than 60 mouse genetic models in which NTD have been described as part of the mutant phenotype *(10,11)*. The models can be categorized into (1) those characterized by the persistent presence of an open neural tube (i.e., comparable to the human NTD anencephaly and meningomyelocele) and (2) those in which the neural tube closes but develops abnormally in various ways. The relevance of this second group of defects to human NTD is unclear and, here, we concentrate on the first group as examples of defective neural tube closure. In particular, we focus on those models in which gene mutations cause defects where folate metabolism is implicated either in the pathogenetic mechanism or in providing protection against development of the NTD (Table 1).

In addition to mouse strains, for which the nature of the mutant gene is known, there are a number of naturally occurring strains in which the mutant gene has not yet been identified. Characterization of these genes should give further information about the pathways with which folic acid interacts and will provide further candidate genes to be analyzed in human NTD cases.

Axial Defects

In the *axial defects* (*Axd*) mutant mouse, open spinal NTD and tail flexion defects are observed in presumed homozygous mice among the offspring of heterozygote crosses *(12)*. The mutation does not affect mean litter size or resorption rate, suggesting that *Axd* does not cause lethality. Tail defects are also observed in some heterozygotes and seem likely to result from delayed closure of the low spinal neural tube *(12)*. As in several other mouse models, genetic background has a significant effect on the penetrance of the defect, with approximately half of the heterozygotes displaying a tail phenotype on the most susceptible background *(12,13)*.

In terms of folate-preventable NTD, the *Axd* mouse does not provide an ideal model, as there is no effect of maternal supplementation with folinic acid or vitamin B_{12} *(14)*. In contrast, maternal methionine supplementation is reported to reduce the incidence of caudal NTD among the offspring of heterozygous crosses by approx 40% *(13,14)*. This may indicate an abnormality in a folate-related pathway because 5-methyl tetrahydrofolate is the one-carbon donor for conversion of homocysteine to methionine by methionine synthase (Fig. 1, reaction 1). However, a simple methionine

Table 1
Summary of Genetic Mouse Models for Folate-Related NTD and Associated Defects

Mutant	Origin	NTD?	Gene	Folate-responsive?
Axial defects	Natural	Yes	ND[a]	No (methionine yes)
Cart1	Targeted	Yes	*Cart1*	Yes, intraperitoneally
Crooked tail	Natural	Yes	ND	Yes, diet
Curly tail	Natural	Yes	ND	No (inositol yes)
Folbp1	Targeted	Unknown[b]	*Folbp1*	Yes, diet
Folbp2	Targeted	No	*Folbp2*	ND
Splotch	Rad.[c]	Yes	*Pax3*	Yes, intraperitoneally and in vitro
Curly tail/splotch	Natural/rad.	Yes	*–/Pax3*	ND
Undulated/patch	Natural	Yes	*Pax1/PDGFRα*	ND
Splotch/NF1	Rad./targeted	Yes	*Pax3/NF1*	ND
Alx4/Cart1	Targeted	Yes	*Alx4/Cart1*	ND

[a]ND = not determined.
[b]Embryos die prior to completion of neural tube closure; *see* text for references.
[c]Rad. = radiation induced.

deficiency caused by a defect in methionine synthase appears unlikely, as plasma methionine levels are normal in *Axd* heterozygous females. A defect in homozygous embryos cannot be excluded, although this does not appear to have been tested *(14)*.

Although methionine treatment has a protective effect against caudal NTD in *Axd* mice, the effect on neural tube closure appears to differ at more cranial levels because a few methionine-treated embryos develop exencephaly *(13)*. As the genotype of embryos was not determined in these studies, it is unclear whether *Axd* mutants exhibit an increased susceptibility to cranial NTD. Alternatively, methionine may have had a deleterious effect on cranial neural tube closure in the *Axd* study, as also noted in *splotch* mice *(15)*.

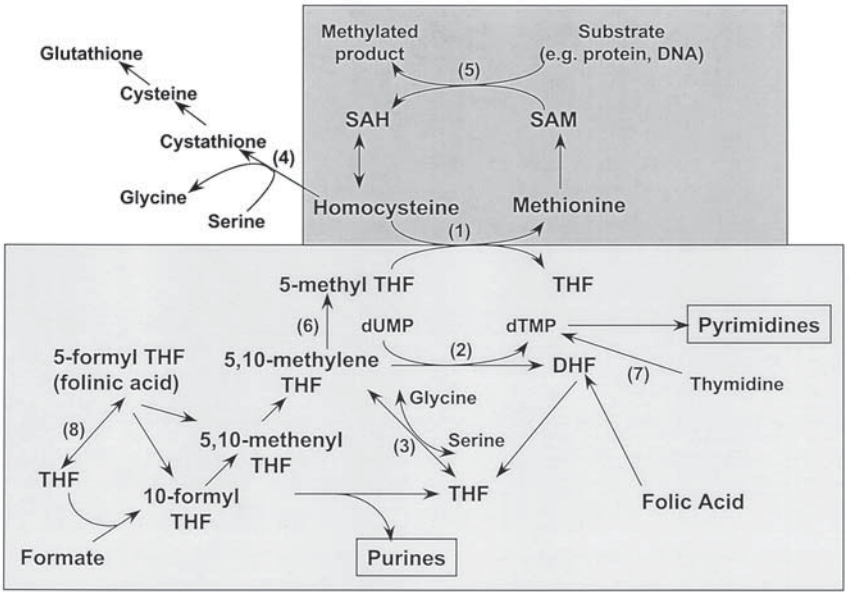

Fig. 1. The main pathways of folate, methionine, and homocysteine metabolism. The dark shaded box encloses the "methionine–homocysteine cycle" and the light shaded box encloses the "folate cycle." Enzymatic reactions referred to in the text are indicated by numbers in parentheses. Abbreviations: DHF, dihydrofolate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; SAH, *s*-adenosyl homocysteine; SAM, *s*-adenosyl methionine; THF, tetrahydrofolate. Modified from ref. *118*.

CART1 KNOCKOUT

Gene targeting has been used to generate mice with a null mutation in *Cart1*, a homeobox-containing gene that encodes the transcription factor, cartilage homeoprotein 1 *(16,17)*. Homozygous *Cart1* mutant mice develop cranial NTD and die shortly after birth. The penetrance is influenced by genetic background with a maximum NTD incidence of 100% on a 129/SvEv strain background *(17)*. Cranial NTD result from failure of closure at the prospective forebrain/midbrain boundary, so called Closure 2. Failure of neural tube closure is thought to be the result of a reduction in the number of mesenchymal cells in the forebrain of homozygous mutant embryos at embryonic day 9 (E9). This deficit appears to result from an increase in cell death and correlates with the forebrain-specific expression of *Cart1* in the cranial mesenchyme. *Cart1* expression is absent from the E9 midbrain mesenchyme, which is histologically normal. Interestingly, however, the neural folds do not close in the midbrain, raising the possibility either of undetec-

ted, low-level *Cart1* expression in the midbrain or of an influence from neighboring brain regions.

The deleterious effect of the *Cart1* mutation is greatly reduced by folic acid supplementation. Treatment by intraperitoneal injection during the first half of gestation reduced the incidence of NTD by approx 60% *(17)*, although all the rescued mice died shortly after birth, indicating that there are residual defects that are not prevented by folic acid. Because treated litters were not examined during development, it is unclear whether folic acid treatment corrects the deficiency in forebrain mesenchyme. Folic acid may act to correct an abnormality of folate metabolism caused by loss of *Cart1* function. Alternatively, the protective effect may be unrelated to the underlying defect. For example, if *Cart1* deficiency causes mesenchymal cells to die, NTD could be prevented if folic acid serves to stimulate proliferation of the remaining mesenchymal cells.

Crooked Tail

Heterozygous *crooked tail* (*Cd*) mutant mice exhibit characteristic tail defects. Homozygotes display a range of phenotypes that include early embryonic lethality and exencephaly, whereas remaining mice are small in size and have vertebral skeletal defects *(18,19)*. The risk of exencephaly is approximately twice as high in females as in males, mimicking the female preponderance among anencephalics seen in humans *(20)*. The *Cd* gene has been mapped to mouse chromosome 6 but remains to be identified *(19)*.

Prenatal dietary supplementation with folic acid causes a reduction in the percentage of affected *Cd* embryos and provides the first model for a dietary response to folic acid *(19)*. A reduction in the risk of exencephaly has been reported for folate-controlled diets containing 0 mg/kg (folate free) and 10 mg/kg compared with a baseline 4 mg/kg folic acid treatment. One possible explanation for this surprising result is that at 0 mg/kg, the majority of exencephalic embryos die early in gestation, yielding a low NTD frequency, whereas at 4 mg/kg, most embryos are rescued from early lethality but still develop exencephaly, yielding an apparently higher NTD frequency. Because parallel data were not reported for control embryos, it is not clear whether *Cd* homozygotes are particularly prone to die in folic-acid-deficient conditions. *Cd* may provide a useful model for NTD in humans and it will be of interest to determine whether there is an underlying defect in folate uptake or metabolism. In addition, because embryos in the initial study were collected at E12.5–14.5, it is important to test whether "rescued" embryos are viable postnatally and whether less severe defects may persist after folate treatment.

Folic-Acid-Binding Protein Knockout

Knockout mice have been generated for folic-acid-binding proteins, folbp1 and folbp2, the murine homologs of human folate receptors α and β, respectively *(9,21,22)*. Folbp1 is a high-affinity integral membrane receptor responsible for transport of folate into the cytoplasm, whereas folbp2 is a low-affinity, glycosylphosphatidylinositol (GPI)-anchored receptor *(23)*. The genes encoding both proteins are expressed embryonically, suggesting that they could be involved in mediating the effect of folic acid during development *(24)*.

Homozygous null embryos for folbp1 are severely growth retarded, fail to complete axial rotation, and die *in utero*, whereas folbp2 null mice are viable *(22)*. The specific effect of folbp1 absence on neural tube closure cannot be determined from this study, as null embryos do not develop to a morphological stage at which the neural tube should have closed. It is clear, however, that folbp1 is essential for normal development, probably to maintain sufficient cytoplasmic folate levels to meet metabolic requirements. The uptake of folate may also be suboptimal, but sufficient for normal development, in folbp2 mutants and folbp1 and folbp2 heterozygotes. For example, although mice of these genotypes are apparently phenotypically normal, nonpregnant mice do show an abnormal response to folate deficiency. A folic-acid-deficient diet causes an increase in plasma homocysteine in wild-type mice, but this increase is significantly greater in mutants. Because elevated homocysteine is a risk factor for NTD, the offspring of such mice could be susceptible to develop NTD, perhaps in the presence of additional genetic or environmental factors. Therefore, it may be revealing to generate compound mutants of folbp knockouts with other folate-related mouse mutants.

Oral supplementation of heterozygous folbp1 females with folinic acid prior to and during pregnancy led to the survival to late gestation of some homozygous offspring *(22)*. Litters were not collected until E18, when nonviable embryos would have been resorbed, so the proportion of rescued embryos could not be measured. Neither was the viability of rescued mice determined. Although the surviving folbp1 null homozygotes in this study do not exhibit NTD, it is not clear whether this represents the prevention of NTD by folinic acid or prolongation of survival, enabling otherwise normal neural tube closure to progress to completion.

Splotch

Splotch mutant mice (*Sp*) are so called owing to the characteristic white belly spot of heterozygotes, which results from a neural-crest-related pig-

mentation defect *(25)*. Homozygous mutants exhibit a range of neural-crest-related abnormalities, limb muscle defects, and neural tube defects, the latter comprising both exencephaly and spina bifida.

A proportion of homozygous embryos die around E14 as a result of heart defects *(25,26)*. The *Sp* and Sp^{2H} mutant alleles cause similar phenotypes and encode defective copies of the *Pax3* gene *(27–29)*. Pax3 is a transcription factor containing both paired-box and homeobox DNA-binding motifs. It is expressed in the dorsal neural tube, migrating neural crest, and dermomyotomal cells *(30–32)*.

Mutations in the human *PAX3* gene are found in Waardenburg syndromes types I and III in which there is a characteristic pigmentation defect *(33,34)*. The occurrence of isolated NTD in heterozygous Waardenburg patients and in a suspected homozygous case shows that the *splotch* mouse may provide a useful model for NTD *(35,36)*. However, *PAX3* mutations do not appear to contribute directly to a large proportion of human NTD *(37,38)*. Interestingly however, it has recently been shown that the PAX3 protein may be implicated in human DiGeorge syndrome, through binding to the candidate HIRA protein *(39)*. This emphasizes the general principle that genes may be involved in human developmental defects in ways other than by direct mutation. In this case, misregulation of PAX3 binding may contribute to the neural crest phenotype of DiGeorge syndrome.

Splotch provides the first mouse model in which NTD are preventable by folic acid in association with a demonstrable abnormality of embryonic folate metabolism *(15)*. Abnormal folate metabolism was detected in whole-embryo culture using the deoxyuridine (dU) suppression test. Incorporation of [^{3}H]thymidine into DNA is suppressed by exogenous dUMP owing to the activation of thymidylate synthase that catalyzes the *de novo* synthesis of dTMP (Fig. 1, reaction 2) from dUMP and 5,10-methylene tetrahydrofolate (5,10-MeTHF). The degree of suppression by dUMP is diminished if folate metabolism is compromised, because the supply of 5,10-MeTHF then becomes limiting. For instance, the application of folate cycle inhibitors leads to diminished dU suppression in mouse embryos *(15)*. Two abnormalities of the dU test are observed in homozygous *splotch* embryos *(15)*. First, in the absence of exogenous dUMP there is an increased incorporation of [^{3}H]thymidine in *splotch* homozygotes compared with wild-type embryos. Heterozygotes exhibit an intermediate level of [^{3}H]thymidine incorporation. Second, the extent of suppression is significantly diminished in homozygotes compared with heterozygotes and wild-type embryos. These observations indicate that the supply of 5,10-MeTHF is insufficient to meet the developmental requirements of *splotch* embryos.

Treatment with folic acid reduces the incidence of cranial and spinal NTD both in embryo culture and following maternal treatment by intraperitoneal injection *(15)*. There is also a corresponding normalisation of the excessive [^{3}H]thymidine incorporation in *splotch* embryos following culture in the presence of folic acid. Therefore, in this model, prevention of NTD by folic acid appears to be associated with the correction of an underlying abnormality of folate metabolism *(15,40)*.

A proportion of heterozygous *splotch* embryos, that normally complete cranial tube closure successfully, exhibits cranial NTD following methionine treatment in embryo culture. This apparent increase in the penetrance of the *splotch* defect is also associated with a further increase in the incorporation of thymidine, suggesting that methionine exacerbates the underlying folate abnormality *(15)*. As described earlier, *Axd* mutants also exhibit occasional exencephaly following maternal methionine treatment, although methionine was curative with respect to the spinal defect *(13)*. Methionine may, therefore, have a general inhibitory effect on cranial neural tube closure, which then causes NTD in predisposed embryos. Such an effect could be mediated through suppression of the folate cycle. For example, the presence of excess methionine inhibits synthesis of thymidylate *(41)*, which is required for DNA synthesis.

COMPLEX GENETIC MODELS

Although a number of single-gene defects cause NTD in mutant mouse strains, the apparently spontaneous occurrence of most human NTD suggests that the majority are likely to arise as multigenic traits: for instance, when two or more independently segregating, mutant alleles are inherited together, each individually has only a low probability of inducing NTD. As the number of genetically defined mutant mouse strains increases, it is becoming possible to test the combined effect of multiple-gene defects by cross-breeding mice that carry different combinations of mutations.

Patch/Undulated

The *Pax1* transcription factor gene is mutated in *undulated* mice *(42)*, whereas the platelet-derived growth factor receptor α (*Pdgfrα*) gene is deleted in *patch* mice *(43)*. Double-mutant mice (*un/un Ph*/+) develop spina bifida occulta, a novel skeletal phenotype that is absent in both single-gene mutants *(44)*, although *Ph* homozygotes are reported to have occasional NTD *(45)*. The effect of folate supplementation has not been tested in these compound mutants.

Splotch/Curly Tail

Spinal NTD arise in *splotch* and *curly tail* mice as a result, respectively, of mutations in *Pax3* and an as-yet unidentified gene mapping to the distal region of chromosome 4 *(28,46)*. The phenotype of double mutants indicates that the mutations interact to increase the risk of spinal NTD *(47)*. In double heterozygotes, there is a 10% incidence of tail defects, although single heterozygotes for *splotch* or *curly tail* are unaffected *(25,26,48)*. Embryos of genotype *Sp/+,ct/ct* exhibit a significantly higher incidence of spinal defects, spina bifida, and tail defects.

Although the increased risk of NTD suggests that there is a modifier effect in the genetic sense, neither the *splotch* nor *curly tail* defect seems to be worsened at the developmental level in double mutants. NTD in *curly tail* arise from delayed closure of the posterior neuropore (PNP) that results from excessive ventral curvature of the caudal part of the embryo *(49,50)*. In double mutants, there is no apparent increase in the mean ventral curvature, suggesting that the *curly tail* defect is not directly affected. In *splotch*, NTD are accompanied by neural-crest abnormalities that cause small or absent dorsal root ganglia *(25)*. However, double mutants exhibit no abnormalities of dorsal root ganglia suggesting that *splotch* heterozygotes have not been pushed toward the homozygous phenotype by the presence of the *curly tail* mutation *(47)*. Therefore, the *Pax3* and *curly tail* mutations summate to produce NTD, an example of digenic inheritance analogous to that described for *undulated/patch* mice.

Neural tube defects in *splotch* are preventable by folic acid *(15)*, whereas NTD in *curly tail* mutant mice do not respond to folic acid *(4)* but are preventable by another vitamin, inositol *(51)*, with no abnormalities of folate metabolism *(15)*. It will be interesting to determine whether the NTD that arise as a result of interaction of the two mutations are preventable by folic acid or inositol or whether both agents are required. Such data may indicate whether combined folic acid and inositol supplementation should be considered for human trials.

Splotch/NF1

A genetic interaction has also been demonstrated between *Pax3* (*splotch*) and *NF1* mutations. *NF1* is a tumor suppressor gene that encodes neurofibromin, a protein with homology to the GTPase-activating protein family, which downregulates RAS activity. Mutations in *NF1* are associated with neurofibromatosis in humans *(52)*. In *NF1* knockout mice, there is a low incidence of exencephaly, which appears to principally affect females

(53). Heterozygotes are morphologically normal but do have behavioral abnormalities associated with learning deficits *(54)*.

NF1/splotch double mutants were generated by mating *splotch* and *NF1* null heterozygotes and then intercrossing the doubly heterozygous offspring. Among the offspring of this F2 generation, 100% of embryos homozygous for the *splotch* mutation developed NTD comprising exencephaly and/or spina bifida. Double homozygous embryos did not survive beyond E11.5 and all of the embryos at that stage had severe exencephaly and spina bifida, suggesting a possible interaction between the mutations. Moreover, among compound heterozygotes examined at E11.5, there was an approx 10% incidence of NTD, which do not occur in single heterozygotes for either mutation. Therefore, it is proposed that NF1 is a modifier gene for splotch *(53)*. The effect of folic acid therapy has not been tested in these double mutants (Table 1).

Alx4/Cart1

Homozygous *Alx4* knockout mice exhibit preaxial polydactyly, a ventral body-wall defect and delayed formation of the parietal bone *(55)*. Like *Cart1* mutants, heterozygotes are unaffected. *Alx4* and *Cart1* both encode transcription factors of the family that contain a paired-type homeodomain without a paired domain *(16,56)* and are both expressed predominantly in mesenchymal cells.

Although the mutant phenotypes are nonoverlapping, the production of compound mutants reveals interactions between the genes. The *Cart1* mutation exacerbates the polydactyly phenotype of the *Alx4* mutant in a dose-dependent manner *(57)*. Moreover, the craniofacial phenotype of *Cart1* homozygotes is modified by the presence of the *Alx4* mutation in heterozygous or homozygous form, giving rise to a cleft face, a defect that may reflect suppression of "Closure 3," the most rostral site of neural tube closure *(58)*. In addition, craniofacial abnormalities not observed in either single mutant also occur in $Alx4^{-/-}$, $Cart1^{+/-}$ double mutants. Double homozygotes also exhibit a split sternum that is not present in either single mutant *(57)*. Therefore, the gene mutations each modify the effect of the other and they also interact to cause novel phenotypes. The effect of folate supplementation on *Alx4* defects has not been evaluated. If defects are not responsive, it will be particularly interesting to test the effect of folate treatment in *Alx4/Cart1* double mutants. Folate treatment may prevent the novel phenotype caused by the interaction of the two mutations. In addition, where *Cart1* acts as a modifier of the *Alx4* phenotype, it will be interesting to determine whether folate treatment can have a protective effect through a folate-responsive modifier gene as opposed to an action directly on the primary defect.

GENETIC BACKGROUND EFFECTS

As in humans, genetic polymorphisms affect the incidence of NTD in mice. This is indicated by the strain-dependent variation in penetrance of NTD caused by gene mutations such as *Cart1 (17)* and teratogens such as valproic acid *(59,60)*. In several cases, folate metabolism has been implicated in the pathogenetic mechanism or as a protective agent.

One mechanism for the genetically determined susceptibility to neural tube defects is suggested by the observation that the initial cranial site of closure (Closure 2) varies among inbred strains of mice *(61)*. A more rostral location of this closure site confers a greater susceptibility to cranial NTD *(62)*. For instance, the mutation in the *Pax3* gene carried by the *splotch* (Sp^{2H}) allele causes a high incidence of cranial NTD when transferred, by breeding, onto a strain background with a rostral site of Closure 2. In contrast, the incidence is reduced on a strain background with a more caudal closure site *(62)*. A control backcross that does not alter the site of Closure 2 does not affect the incidence of cranial NTD. The correlation of the closure site with NTD risk also holds for defects induced by teratogens such as valproic acid *(59,63)* and hyperthermia *(64)*.

A strain-dependent variation in penetrance of a genetic mutation suggests the presence of modifier genes that are polymorphic or differentially expressed between strains. The ability to breed mutations onto different inbred genetic backgrounds facilitates the identification of such modifiers, either by backcross and haplotype analysis or by direct comparison of differential gene expression between the strains. A backcross approach was used to map a modifier gene for the *curly tail* defect, *mct1*, to chromosome 17 *(65)*. Differential expression of *gas5* between strains has been proposed to indicate its potential role as a modifier for susceptibility to heat-induced defects *(66)*.

FOLATE-INSENSITIVE GENETIC MODELS

Although clinical trials clearly demonstrate that folic acid supplementation reduces the incidence of NTD, there remains a significant proportion of human NTD [approx 30% in the Medical Research Council {MRC} trial *(67)*] that do not appear to respond to folate. It is therefore important to consider additional, folate-insensitive, mouse models in order to understand the pathology and possible prevention of this group of defects.

Curly Tail

Homozygous *curly tail* embryos exhibit a low-frequency of exencephaly in addition to a higher incidence of spinal NTD and tail flexion defects that

result from delayed closure of the posterior neural tube *(48,50)*. NTD show partial penetrance, with variable expressivity on different genetic backgrounds *(46)*. In this mutant, the defect underlying the development of spinal NTD has been identified as a reduction in cell proliferation in the ventral tissues, hindgut, and notochord *(68)*. The consequent growth imbalance between the ventral tissues and the neural plate causes excessive ventral curvature of the caudal region of the embryo that mechanically opposes closure of the posterior neuropore *(49)*.

Although several features of *curly tail* resemble spinal NTD in humans *(69)* [e.g., the female preponderance among exencephalics *(70,71)*], there is no preventive effect of folic acid or other folate metabolites including methionine *(4,72)*. Therefore, *curly tail* may represent a useful model for folate-insensitive NTD *(40)*. Neural tube closure in *curly tail* has proven to be sensitive to the level of a different vitamin, inositol. *Curly tail* embryos are particularly sensitive to inositol deficiency and develop a high incidence of exencephaly when cultured in medium lacking inositol *(73)*. In addition, inositol supplementation in vivo or in vitro reduces the incidence of spina bifida resulting from normalization of posterior neuropore closure *(51)*. The effect of inositol is mediated through the inositol/lipid cycle and involves activation of protein kinase C *(51)*, the exact isoforms of which are currently under investigation. It is proposed that inositol supplementation may also prevent some cases of human NTD that do not respond to folic acid, although the prediction has not yet been tested in a formal clinical study.

ENVIRONMENTAL MODELS

A number of environmental factors have been associated with folate-preventable NTD. Animal models provide the opportunity to analyze the effect of such factors against a uniform genetic background, enabling isolation of the factor under analysis.

Folate Deficiency

Human studies indicate that maternal folate deficiency is a risk factor for NTD *(74)*, although it is thought unlikely that folate supplementation reduces the incidence of NTD by simply correcting a dietary deficiency *(75–77)*. Similarly, folate deficiency is not sufficient to cause NTD in mice either *in utero* or in whole-embryo culture.

The effect of maternal folate deficiency on embryonic development has been studied using amino-acid-based diets in which the folate level was strictly controlled *(78)*. At levels below a 906-nmol/kg diet, there was a high proportion of resorptions and the embryos that did develop were smaller

than controls, but otherwise developed normally, without NTD. Folate deficiency has also been induced in rat whole-embryo culture using medium consisting of rat serum that had been extensively dialyzed to remove small molecules and then supplemented with glucose, amino acids, and vitamins *(79,80)*. Absence of folic acid from the medium causes generalized growth retardation but does not cause NTD. In contrast, deficiency of inositol, a vitamin that is capable of preventing NTD in a folate-insensitive mouse model *(51)*, causes NTD in mouse and rats *(73,79)*. It is possible that embryos may have a sufficient pool of intracellular folate to counteract the period of deficiency. Alternatively, some folate may remain in the dialyzed serum because of sequestration by binding proteins. Whatever the explanation, this study indicates that mouse embryos are relatively resistant to short-term deficiency of folate in terms of neural tube closure.

Folate Cycle Metabolites

Homocysteine

Elevated level of homocysteine in maternal serum is a risk factor for NTD in humans, suggesting that excess homocysteine may itself be deleterious *(81–83)*. Exposure of chick embryos to D,L-homocysteine or L-homocysteine thiolactone causes NTD, ventricular septal defects, and ventral closure defects *(84)*. In contrast, homocysteine treatment of cultured rat embryos does not cause NTD, although it is toxic at high doses *(85)*. These toxic effects can be reduced by concurrent treatment with 5-methyl tetrahydrofolate (THF) and serine that may act to promote the metabolic removal of homocysteine. Serine is the one carbon donor for formation of 5,10-methylene THF (Fig. 1, reaction 3), a precursor of 5-methyl THF that, in turn, is the carbon unit donor for remethylation of homocysteine by methionine synthase (Fig. 1, reaction 1). Serine is also required for removal of homocysteine by transulfuration, generating cystathionine (Fig. 1, reaction 4). However, the enzyme cystathione β synthase, which catalyses this reaction, may not be present in the neurulation-stage embryo *(86)*.

Conversely, the harmful effect of homocysteine may result from indirect inhibition of thymidylate synthase, the enzyme that catalyzes the production of dTMP. Increased demand for 5-methyl THF by methionine synthase would reduce the level of 5,10-methylene THF, a substrate for dTMP production (Fig. 1, reaction 2). Moreover, diversion of serine to the transulfuration pathway would also limit its availability for synthesis of 5,10-methylene THF. In support of this idea, cell culture studies suggest that excess homocysteine leads to inhibition of thymidylate synthase *(41)*.

A third possibility is that raised levels of *s*-adenosyl homocysteine (SAH) that may occur under conditions of homocysteine excess could inhibit critical methylation reactions (Fig. 1, reaction 5) in the embryo. Further studies in animal model systems should address these possibilities. It is important to note, however, that the levels of homocysteine that have proven toxic in vitro are significantly higher than those measured in human maternal serum or amniotic fluid. This suggests that in the human cases, raised homocysteine alone is unlikely to be teratogenic but could be a marker of NTD risk or could interact with other genetic or environmental factors to contribute to the development of defects.

Methionine

In different experimental situations, methionine supplementation can be beneficial or harmful. In embryo culture studies, using suboptimal culture conditions based on cow serum *(87,88)*, human serum *(85)*, or the presence of antibodies to the visceral yolk sac *(89)*, embryos exhibit growth retardation and abnormalities including cranial NTD. In each of these cases, methionine supplementation has an ameliorating effect. The relationship to folate metabolism *per se* appears to be indirect, as in the latter two cases, folinic acid or 5-methyl THF, do not have the same effect.

As described earlier, methionine increases the frequency of NTD in *splotch* mice in which folate metabolism is compromised *(15)*. The mechanism of methionine action in *splotch* embryos remains unclear but could result from methionine inhibition of thymidylate synthase *(89)*. This would limit the availability of dTMP and exacerbate the excess requirement for dTMP in *splotch* embryos. Alternatively, there could be increased flux through the methionine cycle with possible accumulation of deleterious intermediates, including homocysteine, as discussed earlier, or *s*-adenosyl methionine, which may hamper the remethylation of homocysteine via inhibition of MTHFR (Fig. 1, reaction 6) *(90)*.

Folate Cycle Antagonists

In humans, there is an increased risk of fetal loss and malformations in the offspring following treatment during pregnancy with folate cycle antagonists, including 5-fluorouracil and methotrexate *(91,92)*. Defects include cleft lip and palate, craniofacial malformations, and limb abnormalities, as well as NTD.

5-Fluorouracil: Inhibitor of Thymidylate Synthase

In mice, maternal treatment with 5-fluorouracil (5-FU) also causes limb and craniofacial defects in the embryo *(93,94)*. In these studies, the effect on

neural tube closure was not determined, as treatment was from E10 or E14, after the period of closure. Because 5-FU inhibits thymidylate synthase (Fig. 1, reaction 2), a key enzyme in supplying precursors for pyrimidine biosynthesis, it will be interesting to determine the effect of 5-FU on neural tube closure. The potential protective effect of folic acid against 5-FU-induced defects remains to be tested.

Nitrous Oxide: Inhibitor of Methionine Synthase

Methionine synthase is a key enzyme that links the folate cycle with the methionine/homocysteine cycle, catalyzing the remethylation of homocysteine to methionine (Fig. 1, reaction 1). Inhibition of methionine synthase is predicted to be deleterious in several respects, including the accumulation of potentially teratogenic homocysteine and depletion of methionine thus reducing the supply of *s*-adenosyl methionine for trans-methylation reactions (Fig. 1, reaction 5). As described previously, elevated level of homocysteine is a risk factor for NTD *(82)*. Diminished methionine synthase activity is also predicted to lead to reduced production of THF which could, in turn, limit the availability of 5,10-methylene THF (Fig. 1, reaction 3), a requirement for dTMP and pyrimidine synthesis. Genetic studies in humans have so far failed to demonstrate a linkage between the methionine synthase gene and predisposition to NTD *(37,95)*.

Methione synthase can be inhibited in rat embryos by exposure of pregnant females, and, subsequently, embryos in culture, to nitrous oxide. This results in decreased synthesis of dTMP from the folate cycle and increased incorporation of labeled thymidine, via the "salvage" pathway (Fig. 1, reaction 7), from the culture medium *(96)*. In terms of embryonic development, nitrous oxide treatment in embryo culture causes generalized growth retardation and axial malformations but does not cause NTD *(97)*. The adverse effects of nitrous oxide appear to result from methionine depletion, as they can be prevented by concurrent supplementation with methionine, but not with folic acid *(98)*.

Maternal Heat Exposure

Maternal exposure to elevated temperatures early in pregnancy is another risk factor for NTD *(99)*. Exposure to brief periods of hyperthermia in humans can result from fever or recreational use of hot tubs or saunas and all have been linked to an increased risk of NTD in epidemiological studies. Laboratory animals, including mice, are also susceptible to hyperthermia-induced teratogenesis *(100)*. Exposure of pregnant mice to hyperthermia at E8.5, the stage at which the cranial neural tube is closing, causes a high

incidence of cranial NTD (exencephaly and anencephaly) *(101)*, which can be suppressed by maternal supplementation with folic acid during pregnancy. It is proposed that heat exposure may cause a transient reduction in cell proliferation that is overcome by folic acid *(101)*. Interestingly, in the same study, supplementation with myo-inositol also caused a reduction in the incidence of heat-induced NTD, although this was not statistically significant.

The susceptibility to heat-induced NTD is influenced by the genetic background in different mouse strains *(64)*. Owing to its strain-specific expression pattern, the growth arrest specific (*gas5*) gene has been suggested as a possible determinant of varying NTD susceptibility in the SWV/Fnn and LM/Bc/Fnn strains *(66)*. It seems likely, however, that the rostral position of the site of anterior neural tube closure (Closure 2) in the SWV strain is also a major predisposing factor compared with the more caudal site of Closure 2 in the less susceptible LM/Bc strain *(61,62)*. Whether expression of *gas5* influences the site of Closure 2 remains to be determined.

Valproate

In humans, it is clear that there is an increased risk of NTD, particularly spina bifida, following maternal treatment with the anticonvulsant valproic acid (VPA) *(102)*. In mice, maternal VPA treatment is embryotoxic, causing growth retardation and abnormalities that include skeletal defects and NTD, the precise phenotype depending on embryonic age at the time of treatment. VPA administration on E8 or E9 induces exencephaly and spina bifida, respectively *(103,104)*. These outcomes reflect the level of the body axis undergoing neural tube closure in the period immediately following VPA administration. At lower doses, the skeletal malformations appear to represent homeotic transformations of the vertebral column *(60)*. In cultures of rat or mouse embryos, VPA also causes cranial and caudal NTD as well as growth retardation and abnormalities of otic and optic vesicles and neuroepithelial ultrastructure *(105–108)*.

The relationship of VPA-induced teratogenicity to folate metabolism is controversial. In mice, maternal treatment with folinic acid (5-formyl tetrahydrofolate) has been reported to reduce the incidence of VPA-induced exencephaly *(109,110)*. In contrast, folinic acid was not protective in separate studies and neither was folic acid nor 5-methyl THF *(107)*. Despite the inconsistent results from folate treatment, the levels of several folate metabolites are altered in VPA-treated embryos, whereas a nonteratogenic analog of VPA does not have this effect *(110,111)*. Following VPA treatment, the levels of 5-formyl-THF (folinic acid) and 10-formyl-THF decrease, whereas

the level of THF increases, suggesting that the enzyme glutamate formyltransferase (Fig. 1, reaction 8) is inhibited by VPA *(110)*. Whether this effect is related to the induction of NTD is unclear.

Additional studies suggest that methionine may have a protective effect against VPA teratogenesis. Intraperitoneal injection of methionine into pregnant mice reduces the rate of spina bifida occulta induced by VPA treatment on E9 *(112)*. On the other hand, methionine treatment of rat embryos in culture does not reduce the incidence of VPA-induced abnormalities *(113,114)*, indicating that the action of methionine in vivo may be mediated through an effect on maternal metabolism, rather than correcting a methionine deficiency in the embryo *per se*. Indeed, maternal VPA treatment reduces plasma methionine levels and increases homocysteine and cysteine levels *(111)*, consistent with this hypothesis. Moreover, if the rats used as serum donors or embryo donors receive prior dietary supplementation with methionine, this does provide a significant protective effect against VPA-treatment in embryo culture *(113)*.

Several studies have addressed the possible mechanism for VPA-induced defects in terms of altered gene expression. For example, the induction of of homeotic transformations may be associated with altered expression of *Hox* genes *(60)*, whereas several transcription factors, including *Emx-1*, *Emx-2*, *c-fos*, *c-jun*, and *creb*, appear to be upregulated following VPA treatment *(115)*. However, is unclear whether these genes are significant in VPA-induced pathogenesis or merely part of a global misregulation of gene expression. Further analysis of gene expression following VPA treatment suggests that differences in the response of folate-related genes may be involved in the strain-specific sensitivity to VPA. Following VPA treatment, folate-binding protein-1 expression is reduced in SWV, a sensitive strain, whereas it is upregulated in the less sensitive LM/Bc strain. LM/Bc embryos also express MTHFR at a higher level *(116)*. These two differences may enable LM/Bc embryos to respond more effectively than SWV embryos to altered levels of folate metabolites following VPA treatment. In a much wider screen, RNA probes from VPA-treated and untreated tissues were used to screen a cDNA library derived from neural tube from the region of Closure 2. The gene-encoding ribonucleotide reductase subunit R1 (*rnr-r1*), a cell cycle regulatory protein, was found to be upregulated following VPA treatment, correlating with a decrease in cellular proliferation in the region of neural tube where Closure 2 occurs in normal untreated embryos *(117)*. Because growth retardation predisposes to the development of NTD in several other mouse models *(1)*, it is possible that upregulation of *rnr-r1* may be part of the pathogenetic mechanism leading from VPA treatment to cranial NTD.

SUMMARY AND CONCLUSIONS

Mouse models provide valuable experimental systems in which to study the role of folate during embryonic development. This chapter has reviewed the contribution of mouse models in one particular area: the pathogenesis and prevention of NTD.

Genetic models are providing both single-gene and multigene systems in which folate is implicated in normal neural tube closure and in the prevention of defective closure, leading to NTD. The genes identified, to date, can be broadly divided into those whose products either directly or indirectly affect folate metabolism. For example, genes encoding folate-binding proteins are directly involved, whereas more indirect effects are envisaged for transcription factors, such as *Pax3*, whose NTD phenotype includes altered folate metabolism. A third class of genes can be envisaged that have no relationship with the function of folate in normal development but whose mutant phenotype may be preventable by folate treatment. The *Cart1* model of NTD may be an example of this type of gene. All of these categories may be relevant to humans, in which the development of folate-preventable defects is likely to be influenced by a number of genes.

Mouse models are also providing valuable information about the role of folate in NTD induced by environmental factors such as hyperthermia and VPA. Perhaps most importantly, mouse models offer a route toward understanding the complex gene–gene and gene–environment interactions that are undoubtedly responsible for the majority of folate-responsive and folate-resistant NTD in humans.

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2

Genetic Variation in Folate Metabolism

Impact on Development

Rima Rozen

INTRODUCTION

Folate derivatives are utilized in single-carbon transfer reactions for many critical pathways, including methionine and DNA synthesis (*see* Fig. 1). For example, 5,10-methylene tetrahydrofolate (5,10-methylene THF) is required for conversion of dUMP to dTMP. It can also be converted to 10-formyltetrahydrofolate for the synthesis of the purine ring, or reduced to 5-methyltetrahydrofolate (5-methylTHF) for the remethylation of homocysteine to methionine. Methionine is the precursor for *S*-adenosymethionine (SAM), the methyl donor in numerous methylation reactions. The enzyme 5,10-methylene tetrahydrofolate reductase (MTHFR) converts 5,10-methylene THF to 5-methylTHF, thereby regulating a balance between folate required for DNA synthesis and folate required for methionine/SAM synthesis and methylation reactions. The importance of MTHFR to SAM supply is highlighted by the fact that SAM is a MTHFR inhibitor.

A common sequence variant in MTHFR has been implicated in several different complex conditions, including cardiovascular disease, neural tube defects, pregnancy complications, and cancer. This chapter will review our current knowledge on the biology of MTHFR and on the developmental problems associated with this common variant in the MTHFR gene. Other relevant enzymes in the homocysteine remethylation pathway and their sequence variants will be mentioned briefly, but additional studies are required to assess their impact on development.

BIOCHEMICAL AND MOLECULAR GENETIC ASPECTS OF MTHFR

5-Methylene tetrahydrofolate reductase is utilized to convert 5,10-methylene THF to 5-methylTHF in many different species. The bacterial and

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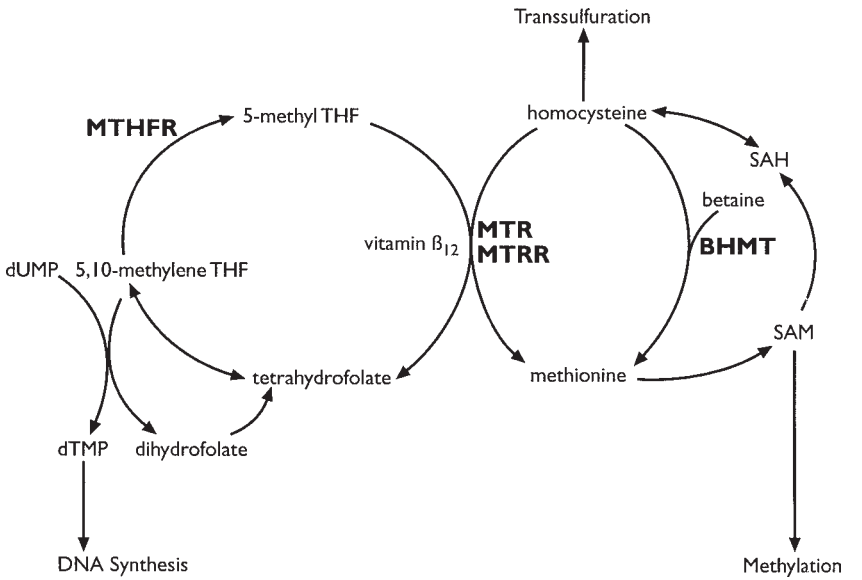

Fig. 1. Interconversion of folate derivatives by MTHFR and homocysteine remethylation pathway. MTR, gene for methionine synthase; MTRR, gene for methionine synthase reductase; BHMT, gene for betaine homocysteine methyltransferase.

porcine enzymes have been purified to homogeneity *(1,2)*. Although they both share a catalytic domain, the mammalian enzyme has an additional regulatory C-terminal domain that accounts for the larger size of the mammalian enzyme (77-kDa subunit in pig vs 33-kDa subunit in *Escherichia coli*). This C-terminal domain contains the binding site for SAM, an allosteric inhibitor of the enzyme. The bacterial enzyme is a tetramer, whereas the porcine enzyme is a dimer. The bacterial enzyme has recently been crystallized and its crystal structure determined; this work is critical for the understanding of how mutations in MTHFR impact on enzyme function *(1)*.

Using amino acid sequence information from the porcine enzyme, a partial cDNA for human MTHFR (1.3 kb) was isolated in 1994 by Goyette et al. *(3)*. This cDNA was used to isolate and express a cDNA of 2.2 kb *(4)*, which resulted in the synthesis of a 70-kDa protein that was catalytically active. Western blotting has suggested the presence of at least two protein isoforms of the human enzyme, with some degree of tissue specificity. A 77-kDa polypeptide was identified in most tissues, whereas the 70-kDa minor form was observed in human fetal liver *(4)*.

The human MTHFR gene has 11 exons encoding the 2.2-kb cDNA, with a size of approx 17 kb for this region of the gene that contains the coding sequence *(5)*. However, the presence of significant 5'UTRs and 3'UTRs, in addition to the 2.2-kb coding sequence, contributes to the large mRNAs of approx 8 kb and indicates that the final size of the gene will be greater than 17 kb *(5,6)*. The large size and complex pattern of splicing in the 5'UTR suggest that MTHFR may have some interesting regulatory properties.

The human gene maps to chromosome 1 (*1p36*) and the mouse gene maps to distal chromosome 4, a region that is homologous to the human *1p36* region *(3,7)*. The mouse gene structure is very similar to that of the human gene, with over 85% identity in the amino acid sequence *(5)*. There is 30% identity in amino acids between the *E. coli* enzyme, encoded by the *metF* gene, and the N-terminal catalytic domain of the human enzyme *(3,5)*.

SEVERE MTHFR DEFICIENCY

Severe MTHFR deficiency, with less than 20% of control enzyme activity, is the most common inborn error of folate metabolism *(8)*. These patients have dramatic elevations of plasma homocysteine with homocystinuria, and low or low–normal levels of plasma methionine. This relatively rare condition can have devastating consequences. Some patients die in the first year of life; others have variable clinical features, including developmental delay, motor and gait disturbances, seizures, and psychiatric manifestations. Pathological findings have included demyelination and vascular changes *(8)*. Over 20 different mutations in MTHFR have been reported in this group of patients *(3,9–12)*. Some of these mutations have been expressed in vitro and confirmed to impact enzyme function *(13)*.

The early-onset clinical symptoms in these homocystinuric patients emphasize the important role of MTHFR and folate metabolism in normal development, particularly in the development of the central nervous system. Other enzyme deficiencies, the most common being a deficiency of cystathionine-β-synthase (CBS), can cause homocystinuria through a disruption of homocysteine trans-sulfuration, but, in contrast to MTHFR-deficient patients, CBS deficiency is associated with high methionine levels in plasma *(14)*. This is the result of the fact that MTHFR deficiency results in a disruption of the homocysteine remethylation pathway, whereas CBS-deficient patients have normal remethylation, with a block in the first enzyme of the trans-sulfuration pathway. Although CBS-deficient patients also have some CNS problems, such as mental retardation, MTHFR deficiency appears to be associated with additional significant neurological features. Because

MTHFR-deficient patients are likely to be compromised with respect to SAM synthesis, it is possible that some of their distinct neurological problems are related to a decrease in methylation reactions in the CNS, such as decreased phospholipid synthesis or decreased neurotransmitter synthesis.

Common to both types of homocystinuria are vascular changes, presumably reflecting the increased levels of homocysteine in the circulation. With the recognition of hyperhomocysteinemia as a risk factor for cardiovascular disease, many studies have addressed the nature of the pathogenic effects of homocysteine on the vasculature. These include toxicity to the vascular endothelium, enhanced proliferation of smooth muscle cells, thrombogenic effects and an increase in oxidative stress *(15)*. However, because most studies have been performed in vitro, the physiologic relevance of these findings requires confirmation.

A mouse model for severe MTHFR deficiency has recently been generated *(16)*. Mice that are homozygous for a knockout of the MTHFR gene have severe hyperhomocysteinemia with reduced survival or delayed development and cerebellar pathology. Additional investigations of these mice should provide important information regarding the pathogenicity of hyperhomocysteinemia and the role of folate in normal development.

MILD MTHFR DEFICIENCY: 677C→T MUTATION

Based on the presence of thrombotic episodes in patients with severe hyperhomocysteinemia and homocystinuria, several investigators suggested that milder elevations in homocysteine could also be a risk factor for cardiovascular disease *(17)*. The observation that some patients with cardiovascular disease had a mild deficiency of MTHFR, with a thermolabile enzyme *(18)*, led to molecular genetic studies that identified a C to T mutation at bp 677 (an alanine to valine substitution) *(4)*. This mutation was expressed in vitro and shown to encode a thermolabile enzyme. Individuals who were homozygous mutant also had reduced enzymatic activity at 37°C (approx 35% of control values). These individuals are at risk for mild hyperhomocysteinemia, particularly when their plasma folate is low *(19)*. Folate supplementation has been demonstrated to lower homocysteine in these individuals *(20)*. These clinical observations are supported by biochemical studies that have demonstrated that the mutant human enzyme can be stabilized by both folate and its cofactor flavin adenine dinucleotide (FAD) *(1)*. Thus, supplemental folate might prevent hyperhomocysteinemia in mutant individuals by improving enzyme function. Studies with the mutagenized bacterial enzyme have yielded similar results to those of the human enzyme and have predicted, on the basis of crystal structure informa-

tion, that the mutant valine residue may indirectly affect FAD binding and/or increase the dissociation of the active bacterial tetramer into a dimer *(1)*.

The mutation has been shown to decrease total plasma folate *(21)*, as 5-methylTHF is the primary circulatory form of folate. It has also been shown to affect the distribution of folates in red blood cells *(22)*. Individuals with the mutation have decreased amounts of methylTHF and increased amounts of the formylated derivatives. Because 5,10-methylene THF conversion to 5-methylTHF is compromised by reduced MTHFR activity, there is an increased amount of 5,10-methylene THF available for conversion to formyltetrahydrofolate. The decreased 5-methylTHF accounts for hyperhomocysteinemia because of the reduced conversion of homocysteine to methionine by methionine synthase; this reduction could also affect methylation reactions. The increased amount of other folate derivatives might improve thymidine and purine availability for DNA synthesis (*see* Fig. 1). Although total folate in red blood cells was not different in mutant individuals in this study *(22)*, others have suggested decreased *(23)* or increased *(24)* total folate in erythrocytes. The discrepancy could be the result of the variable methodologies employed in different laboratories.

The 677C→T mutation is common in North American, European, and many Asian countries, with homozygosity frequencies ranging from 5% to 25% *(25)*. The highest frequencies of this variant have been reported in southern Mediterranean populations and Hispanic populations in North America. The mutation is relatively infrequent in African-Americans *(26)*.

The initial identification of this polymorphism was made on the basis of its role in the elevation of homocysteine, a risk factor for cardiovascular disease. However, because severe MTHFR deficiency is associated with a wide variety of developmental and neurologic problems, it is not too surprising that mild MTHFR deficiency might also have an impact on disorders involving the central nervous system (CNS).

DEVELOPMENTAL ABNORMALITIES ASSOCIATED WITH THE 677C→T VARIANT

Neural Tube Defects

Clinical studies have clearly demonstrated that folate supplementation reduces the occurrence and recurrence of neural tube defects (NTD) *(27,28)*. Mothers of children with NTD had been shown to have low folate levels and were suspected of having an altered folate metabolism *(29)*. The observation that mothers of NTD cases had mild hyperhomocysteinemia *(30,31)* led to the investigation of the MTHFR variant as a genetic risk factor for this

birth defect *(24)*. Several studies have reported that the 677C→T variant in the homozygous state in the child or in the mother can increase the risk for NTD. A recent review of the literature indicated a pooled odds ratio of 1.8 (95% confidence interval [CI] = 1.4 – 2.2) and 2.0 (95% CI = 1.5 – 2.8) for children and mothers, respectively, with the homozygous mutant genotype *(25)*; these values are quite similar to those reported in an earlier meta-analysis *(32)*. The combination of the mutant genotype in both the mother and child could have an even greater risk, according to one report *(33)*. As mentioned earlier for hyperhomocysteinemia, nutritional folate status is a critical determinant in the magnitude of the genetic risk conferred by this mutation. Low folate status combined with the homozygous mutant genotype may result in a higher risk than either variable alone *(33)*. The lack of an association between NTD and the MTHFR variant in some studies may reflect the nutritional status of the study group during the critical period of development of the neural tube.

The mechanism by which this mutation increases risk is not clear. Homocysteine has been shown to be teratogenic in studies of chicken embryos *(34)*. Alternatively, a decrease in methylation reactions or dysregulated DNA synthesis in critical cells could compromise neural tube closure.

Pregnancy Complications

Women with placental abruption *(35)* or recurrent pregnancy loss *(36)* have been reported to have mild hyperhomocysteinemia. These findings have culminated in several publications that have documented an increased frequency of the 677C→T variant in women with the aforementioned complications and with preeclampsia *(36–38)*. A recent review of the relevant literature has reported pooled odds ratios of 2.3 (95% CI = 1.1 – 4.9), 3.3 (95% CI = 1.2 – 9.2), and 2.6 (95% CI = 1.4 – 5.1) for placental abruption, recurrent pregnancy loss, and preeclampsia, respectively, in women who were homozygous mutant for the 677-bp variant *(38)*. The risk for these problems is also augmented in the presence of other thrombophilic risk factors, such as Factor V Leiden and the 20210-bp mutation in the prothrombin gene, either alone or in combination with the MTHFR mutation *(39–41)*. These various types of pregnancy complication could be caused by homocysteine-mediated effects on the placental vasculature.

A few reports in the literature have alluded to genetic selection, with possible intrauterine losses, based on MTHFR genotypes. One study has suggested that the frequency of the homozygous mutant genotype is increased in the younger population in Spain, compared to an older group, because folate supplementation during pregnancy in the past two decades has im-

proved the nutritional status of pregnant women and decreased the number of losses of mutants *in utero (42)*. Another study has suggested heterozygote advantage in families with NTD *(43)* and one publication has reported decreased amounts of control female newborns that are homozygous for this mutation *(44)*.

Other Emerging Developmental Problems

Oral Clefts

Maternal use of multivitamins with folic acid has been reported to reduce the risk of a cleft lip with or without cleft lip palate *(45)*. The first study of MTHFR in children with this congenital anomaly did not identify a statistically significant risk in a Hispanic California population, although there was an increased nonsignificant odds ratio for cleft lip (1.8, 95% CI = 0.3–7.9) in children of non-Hispanic white mothers who did not use vitamins *(46)*. The same group reported the absence of an effect on an isolated cleft palate *(47)*. In contrast, maternal hyperhomocysteinemia was observed in mothers of children with nonsyndromic orofacial clefts in the Netherlands *(48)*, and a recent small study in Ireland reported a significantly higher frequency of mutant MTHFR in subjects with an isolated cleft palate *(49)*.

Fetal Anticonvulsant Syndrome

One study has reported an increased frequency of this variant in women on anticonvulsant medication who had children with this syndrome *(50)*. Three common anticonvulsants (carbamazepine, phenytoin, and sodium valproate) have been shown to interfere with folate metabolism; consequently, these women may have a higher requirement for folate during pregnancy. In a related study, epileptic women on anticonvulsants were shown to have hyperhomocysteinemia and low folate, particularly when they were homozygous for this variant *(51)*.

Down Syndrome

Two studies *(52,53)* have reported an increase in the frequency of the MTHFR variant in mothers of children with Down syndrome. The postulated mechanism is an increase in DNA hypomethylation, which could promote nondisjunctional events by altering centromere methylation patterns or chromosome stability.

Schizophrenia

Abnormalities in methyl group metabolism have been observed in schizophrenics *(54)*, and patients with severe MTHFR deficiency can have

psychiatric disturbances *(8)*. Consequently, a few studies have examined the common MTHFR variant as a risk factor for this condition. Positive associations *(55,56)* have been observed in some but not all studies. This discrepancy could be the result of the fact that the association may only be present in subgroups of patients (e.g., those who are good responders to neuroleptic medication) as reported in a recent study *(56)*.

Congenital Heart Defects

Multivitamin supplementation has been shown to decrease the risk of congenital heart defects. Mothers of children with congenital heart defects have been reported to have higher homocysteine levels than control subjects *(57)*. Although the MTHFR variant was not examined in this study, the association between vitamin responsiveness and hyperhomocysteinemia suggests that the MTHFR variant could be a candidate risk factor, particularly because neural-crest cells contribute to the formation of the septum.

Cancer

The high frequency of a polymorphism that can affect genetic fitness in several different conditions raises the question of a possible selective advantage. One hypothesis relates to the fact that a mutation in MTHFR should increase DNA synthesis or repair through an elevation of 5,10-methylene THF levels for the synthesis of thymidine or purines. Several publications have reported a protective effect of the MTHFR variant in colorectal cancer *(58,59)*, possibly through the aforementioned mechanism; a recent study has demonstrated a similar protective effect in adult acute lymphocytic leukemia *(60)*. Although the age of onset of these disorders is in the adult period and therefore may have little impact on selection or early development, it is possible that enhanced DNA synthesis may be advantageous during early development or in the protection against early-onset childhood cancers.

OTHER COMMON VARIANTS IN FOLATE METABOLISM

MTHFR 1298A→C

A second common variant in MTHFR, at bp 1298, converts a glutamate to an alanine codon. This variant is present at a similar frequency (approx 9–10% homozygosity) to that of the 677-bp variant *(61,62)*. However, by itself, it may not sufficiently disrupt enzymatic function to alter homocysteine remethylation. The mutant enzyme has recently been expressed in vitro; the activity associated with this mutation (approx 68% of control activity) is intermediate between that of the wild-type enzyme and the enzyme

Table 1
Comparison of MTHFR Activities (at 37°C) Obtained by In Vitro Expression with Activities Reported in Lymphocyte Extracts

	Recombinant enzyme expression[a]	NTD patients and their parents and controls[b]	NTD mothers and controls	NTD children and controls[c]
AE	100%	100%	100%	100%
AA	68% (± 5.0)	61%	66%	57%
VE	45% (± 10.8)	25%	32%	31%
Residual activity (%)				
AE	31.1%	66%		
AA	48.9%	61%		
VE	11.9%	17.6%		

Note: The designations for the wild-type (AE) and mutant (AA, VE) enzymes are based on the single-letter amino acid code. AE (wild type) indicates the alanine codon at bp 677 and the glutamate codon at bp 1298. AA indicates the wild-type alanine codon at bp 677 and the mutant alanine codon at bp 1298. VE indicates the mutant valine codon at bp 677 and the wild-type glutamate codon at bp 1298. Residual activity refers to the activity after heating at 46°C for 5 min, an indicator of thermolability.

[a]The activities for the mutagenized enzyme in vitro were obtained from ref. *63*.

[b]The activities in lymphocyte extracts were obtained from ref. *61*.

[c]The activities in lymphocyte extracts were obtained from ref. *62*.

carrying a mutation at bp 677 (45% of control activity) *(63)*. These results are consistent with the enzyme activities determined in lymphocyte extracts in vivo (*see* Table 1). The mutation at bp 1298 does not affect thermostability of the enzyme, unlike the mutation at bp 677.

Hyperhomocysteinemia has not been observed in individuals who are mutant only for the 1298-bp mutation, reflecting the higher levels of enzyme activity associated with this mutation, as compared to the 677-bp change *(61,62)*. On the other hand, individuals who are heterozygotes for both the 1298 and 677 variants may be at risk for hyperhomocysteinemia *(61,63)* and, consequently, for the conditions associated with disruption of this pathway. One report has observed an increased odds ratio for neural tube defects in children who were double heterozygotes, but this risk was not statistically significant *(61)*.

Methionine Synthase (MTR) 2756A→G

This vitamin B_{12}-dependent enzyme utilizes 5-methylTHF for conversion of homocysteine to methionine. A polymorphism at bp 2756 has been

described, which converts an aspartate to a glycine residue *(64)*. This variant, however, is much less common than the MTHFR variants, with homozygosity frequencies of approx 4%. An increased risk for neural tube defects has not been observed *(33,65)*, but one report has suggested a protective effect in colon cancer *(66)*, as previously observed for the MTHFR variant.

Methionine Synthase Reductase (MTRR) 66A→G

This enzyme is required for the reductive activation of methionine synthase. A very common mutation has been identified that converts an isoleucine to a methionine codon *(67)*. Homozygosity frequencies of approx 25% have been observed in North Americans. This variant has been reported to increase the risk of neural tube defects when vitamin B_{12} levels are low or when the MTHFR 677-bp variant is present *(67)*. Of additional interest is the finding that this variant may also increase the risk of Down syndrome *(53)* or vascular disease *(68)*. However, all of the aforementioned disease associations have been the subject of single reports, which clearly require confirmation.

CONCLUSION

The important role of adequate dietary folate for normal development has been clearly established. The association of genetic variants in folate metabolism with increased risk for disease suggests that some individuals may have higher requirements for this nutrient.

The 677-bp variant in MTHFR has been investigated more thoroughly than other polymorphisms and appears to influence several developmental processes. The interaction between the genetic mutation and nutrient status is interesting and offers a reasonable approach (i.e. folate supplementation) to overcome the consequences of the mutation. In addition to nutritional influences, however, there may be other variants in the aforementioned enzymes or in other enzymes involved in remethylation, such as betaine homocysteine methyltransferase (BHMT), which can modify risk. The challenge will be to study interactions of multiple genetic and environmental factors in disorders that are clearly multifactorial in nature. Large numbers of individuals will have to be assessed, with various combinations of these risk factors, before a comprehensive understanding is achieved. The availability of mouse models with defects in folate metabolism *(16,69)* should complement clinical studies in elucidating the mechanisms of folate-dependent disease states.

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3

Folate Deficiency and the Molecular Determinants of Chromosome Instability

Possible Link to Meiotic Nondisjunction and Down Syndrome

S. Jill James and Charlotte A. Hobbs

BIOCHEMISTRY AND MOLECULAR GENETICS OF FOLATE AND DNA METABOLISM

Folate and One-Carbon Metabolism

Tetrahydrofolate (THF) is the metabolically active form of folate that is central to normal one-carbon metabolism. The interdependent and interconnecting pathways of folate and one-carbon metabolism are graphically presented in Fig. 1 with emphasis on the two major functions of folic acid: DNA synthesis and DNA methylation. Normal folate metabolism is essential for the synthesis and balance of deoxynucleotide triphosphate (dNTP) precursor pools required for error-free DNA synthesis and repair *(1)*. It is also essential for the establishment and maintenance of stable DNA methylation patterns required for tissue-specific gene expression and chromatin conformation *(2)*. Both of these functions in DNA metabolism are negatively affected by inadequate folate intake and/or by genetic polymorphisms in these pathways. Although the emphasis in this chapter is on folic acid, the interdependency and metabolic interaction of folate metabolites with methionine, vitamin B_{12}, and vitamin B_6 in maintaining normal one-carbon metabolism is apparent in the reactions diagrammed in Fig. 1. Folate and B_{12} are equally essential and inseparable for the methionine synthase reaction. Vitamin B_6 is a cofactor for the synthesis of 5,10-methylene THF and also for homocysteine catabolism to cystathionine and cysteine. Methionine transfers methyl groups derived from 5-methyl THF to *S*-adenosylmethionine (SAM), the major intracellular methyl donor.

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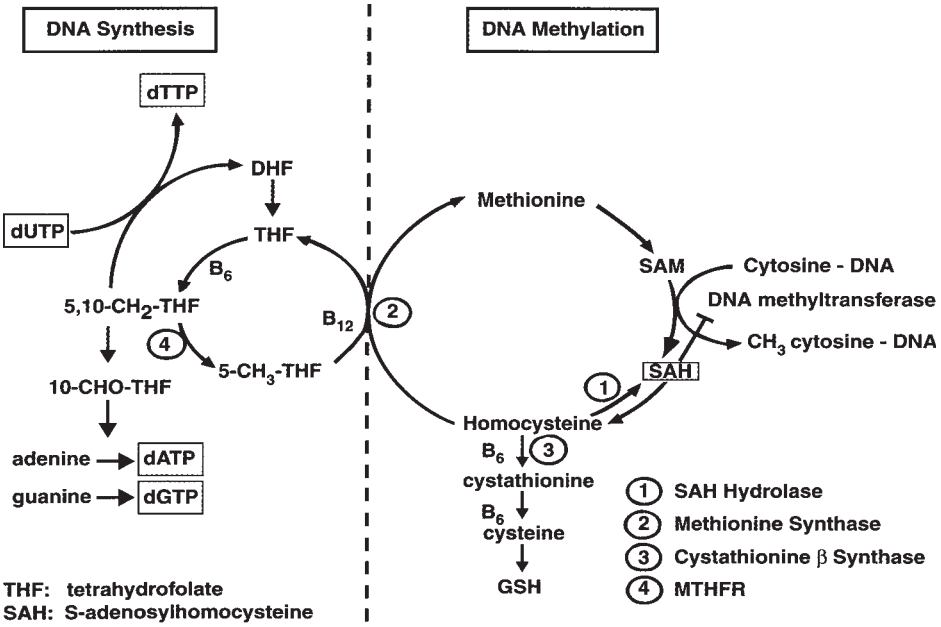

Fig. 1. Overview of the interactive and interdependent reactions involved in cellular one-carbon metabolism with emphasis on the two major functions of these pathways in DNA metabolism: normal DNA synthesis and normal DNA methylation. These two major functions intersect at the folate/vitamin B_{12}-dependent methionine synthase reaction that generates metabolically active tetrahydrofolate (THF) for DNA nucleotide precursor synthesis and, at the same time, regenerates methionine from homocysteine. Both DNA synthesis and DNA methylation are negatively affected by inadequate folate or vitamin B_{12} intake and/or by mutations in these pathways. Note that an elevation in homocysteine induces the reversal of the SAH hydrolase reaction and causes an elevation in SAH, a potent product inhibitor of the DNA methyltransferase reaction.

DNA Synthesis and DNA Methylation

The reactions on the left-hand side of the diagram describe the essentiality of folate for *de novo* dNTP synthesis. Because the dNTPs are the substrate for the DNA polymerases and have a half-life of seconds, the fidelity of DNA replication and repair synthesis is critically dependent on the maintenance of the correct balance of dNTP *(1)*. An imbalance in dNTP pools resulting from folate deficiency can delay replication fork progression, induce uracil misincorporation, and promote genomic instability *(3–6)*. Folate deficiency in vivo and in vitro increases the ratio dUTP/dTTP, resulting in the reiterative misincorporation of uracil into DNA *(5–7)*, fragile site expres-

sion *(8,9)*, DNA strand breaks *(10–13)*, and mutagenesis *(14–16)*. These DNA lesions induced by chronic folate deficiency can have significant negative consequences on DNA and chromosomal integrity.

The functions of folate in DNA synthesis and DNA methylation intersect at the central methionine synthase reaction (reaction 2). This reaction involves transfer of a methyl group from 5-methyltetrahydrofolate to B_{12} for the B_{12}-dependent remethylation of homocysteine to methionine. Methionine is the precursor for the synthesis of SAM, the major cellular methyl donor for DNA, RNA, protein, and phospholipid methylations. Methionine is converted to SAM, and subsequently to *S*-adenosylhomocysteine (SAH) by a variety of cellular methyltransferases present in all cells. This one-way reaction is subject to competitive product inhibition by SAH because of the higher affinity of SAH, relative to SAM, for the methyltransferase active site *(17)*. The pathologic accumulation of SAH results in a decrease in the SAM/SAH ratio and inhibition of most cellular methyltransferases *(18–20)*. SAH is subsequently degraded to homocysteine and adenosine by SAH hydrolase (reaction 1), a reversible reaction with thermodynamics that favor SAH synthesis rather than hydrolysis *(21,22)*. Thus, the direction of this reaction toward homocysteine is absolutely dependent on continuous product removal. It is important to note that metabolic conditions that interfere with the removal of homocysteine or adenosine will lead to SAH accumulation and inhibition of cellular methyltransferases *(17,23–26)*. The inhibition of DNA methyltransferase by SAH accumulation or SAM depletion is thought to contribute to DNA hypomethylation observed with folate deficiency.

Gene–Nutrient and Gene–Gene Interactions

Insufficient folate in the metabolic forms required to maintain normal DNA replication, DNA repair, and DNA methylation can result from either nutritional deficiency or from genetic polymorphisms in the folate pathway that affect folate-dependent reactions. Concomitant deficiencies in methionine, vitamin B_6, vitamin B_{12}, or choline may be expected to exacerbate a marginal folate deficiency and magnify the metabolic aberrations. Common polymorphisms in methylene tetrahydrofolate reductase (MTHFR), methionine synthase (MS), cystathionine-β-synthase (CBS), methionine synthase reductase (MTRR), and/or betaine–homocysteine methyltransferase (BHMT) would also be expected to interact with marginal folate status and associated micronutrient deficiencies. For example, although poor folate status alone is associated with increased risk of neural tube defects (NTDs), the combination of low folate status and the 677C→T polymorphism in the

MTHFR gene would be expected to confer a greater risk than either variable alone *(27)*. Similarly, the combination of low B_{12} status with a polymorphism in the MTRR gene magnified the risk of NTDs *(28)*. In addition to gene–nutrient interactions, gene–gene interactions in polymorphisms affecting folate metabolism have also been shown to elevate NTD risk. Increased risk can result from an interaction between the 677C→T polymorphism in the MTHFR gene and either a 68-bp insertion in the CBS gene or the common 2756A→G substitution in the MS gene *(29,30)*. These results suggest that the etiology of birth defects related to abnormal folate metabolism may extend to other interacting micronutrients, as well as to multiple interactive polymorphisms in the folate pathway.

FOLATE DEFICIENCY AND CHROMOSOME INSTABILITY

Chromosomal Aberrations and Micronuclei

Folate and/or B_{12} deficiency in humans has been long associated with chromosomal instability and multiple chromosomal aberrations. Early cytogenetic studies of folate- or B_{12}-deficient human lymphocytes or marrow cells revealed multiple chromosomal breaks and gaps, decondensed chromosomes, premature centromeric division, and centromeric spreading *(31,32)*. Chronic folate deficiency is manifested in a futile cycle of uracil misincorporation during DNA replication and repair *(5,6,12,33)*. Uracil misincorporation stems from a block in the folate-dependent methylation of dUTP to dTTP, resulting in the misinsertion of uracil in place of thymine *(5,34)*. The misincorporation of uracil for thymine *per se* is not a premutagenic lesion because the DNA polymerase will insert the correct adenine base opposite either thymine or uracil *(5,35)*. However, the presence of an active uracil glycosylase leads to site-specific abasic sites and single-strand breaks that represent significant premutagenic lesions *(12)*. These sites facilitate viral integration, recombination, and/or chromosomal breaks that result in the formation of micronuclei (small chromosome fragments). The increased frequency of micronuclei and DNA strand breaks in folate- or B_{12}-deficient human lymphocytes has been shown to be reversible with folate repletion, implying a causal role for folate deficiency in micronuclei formation *(13,36,37)*. In a recent study, folate deficiency in postmenopausal women was associated with an increased frequency of centromeric kinetochore-positive micronuclei *(13)*. In this metabolic study, folate repletion following the folate-depletion phase was associated with a significant decrease in kinetochore-positive, but not kinetochore-negative, micronuclei. Micronuclei containing centromeric kinetochores are surrogate markers for

abnormal chromosome segregation; thus, these findings implicate a role for folate deficiency as a risk factor for human aneuploidy.

Folate-Sensitive Fragile Sites

Chromosome fragile sites are nonrandom breaks and gaps that appear in metaphase chromosomes under cell culture conditions that induce folate depletion, dNTP imbalance, and/or block DNA polymerase progression *(38–40)*. The fragile X syndrome is the result of a rare folate-sensitive fragile site on the X chromosome and is a major genetic cause of mental retardation. Alteration in the higher-order structure of DNA, resulting from a CCG trinucleotide repeat expansion and abnormal methylation in the promoter region, leads to transcriptional inactivation of the *FMR1* gene and the phenotypic expression of the fragile X syndrome *(41)*. In addition to "rare" fragile sites, several "common" fragile sites are also induced under culture conditions that cause folate stress, dNTP starvation, or DNA hypomethylation *(42,43)*. Common fragile sites are inducible on all chromosomes and appear to be regions of unusual chromosome instability as evidenced by increased recombination, translocations, sister chromatid exchanges, and intrachromosomal gene amplification and deletions at these sites *(39,40,44,45)*. Pertinent to folate deficiency, breakage at sites of chromosomal fragility often occur in regions of decondensed and hypomethylated DNA that flank hypermethylated condensed regions. In addition, agents that induce DNA hypomethylation also induce fragile site expression *(42)*. It is tempting to speculate that dNTP imbalance, DNA hypomethylation, and stalling of the replication fork associated with nutritional folate deficiency in humans may promote induction of common fragile sites in vivo. The coincidence of several common fragile sites with specific deletions, chemically induced lesions, chromosomal translocations, and viral insertions further suggests that agents or conditions that predispose to fragile sites in vivo may also predispose to certain cancers *(47,48)*.

DNA METHYLATION, CHROMATIN CONFIGURATION, AND CHROMOSOME SEGREGATION

Origin and Stable Inheritance of DNA Methylation Patterns

In newly replicated DNA, cytosines occurring at CpG dinucleotides are enzymatically methylated by a DNA methyltransferase that catalyzes the postreplicative transfer of a methyl group from SAM to the fifth carbon of cytosine in DNA. The product of the methyl transfer reaction, SAH, has a higher affinity for the active site than the precursor, SAM *(18,19,21,22)*.

Under conditions of nutritional, drug-induced, or genetically-based folate deficiency, an accumulation of SAH secondary to an increase in homocysteine and reversal of the SAH hydrolase reaction, results in potent product inhibition of the DNA methyltransferase and global DNA hypomethylation *(48–50)*.

Following DNA replication, cytosines on the newly replicated strands are not yet methylated and, consequently, create a hemimethylated site opposite the methylated parental strand. This nascent hemimethylated site is the preferred target for the "maintenance" DNA methyltransferase at the replication fork. In this way, an unmethylated site on the daughter strand will be efficiently methylated by the maintenance methyltransferase, thereby preserving the parental methylation pattern. On the other hand, CpG sites that are unmethylated on the parental strand will remain unmethylated in the daughter strand. *It is important to note that postreplicative maintenance methylation represents an epigenetic self-perpetuating mechanism for the stable inheritance of methylation patterns and chromatin structural patterns between cell generations.* Under conditions of folate/methyl deficiency, the inheritance of methylation patterns becomes unstable, resulting in global hypomethylation, altered chromatin secondary structure, and inappropriate gene expression *(51–53)*.

Because the methyl group addition does not alter the primary base sequence of the DNA, this modification is considered an "epigenetic" phenomenon. Cytosine methylation within CpG dinucleotides induces transcriptional repression by directly blocking the binding of sequence-specific transcription factors or, indirectly, by recruitment of methyl-binding proteins that condense DNA into a repressive conformation that interferes with transcription factor accessibility. DNA methylation is essential for normal embryonic development, X chromosome inactivation, allelic imprinting, suppression of viral DNA, and normal chromosome structure and segregation *(59)*. Each of these functions of DNA methylation has been shown to be disrupted upon exposure to the potent demethylating agent, 5-azacytidine, or disrupted in transgenic mice lacking the *dnmt1* gene for the maintenance DNA methyltransferase *(57,59–63)*.

DNA Methylation and Pericentromeric Heterochromatin

Recent evidence linking DNA methylation to histone deacetylation and chromatin condensation supports the possibility that unstable DNA methylation patterns may be mechanistically linked to abnormal chromosome segregation *(62,64–66)*. MeCP2 is a methyl-binding protein that binds preferentially to cytosine methyl groups localized in repetitive pericentro-

meric heterochromatin. Recently, two groups demonstrated that MeCP2 binds in a complex with histone deacetylase, resulting in local histone deacetylation and chromatin condensation *(67,68)*. Chromatin must be condensed in order to resolve sites of strand recombination between homologous chromosomes into functional chiasmata, which are essential for homolog cohesion and chromosome alignment before entry into the metaphase of meiosis I *(69,70)*. In addition, epigenetic alterations in the secondary structure of centromeric heterochromatin are also required for kinetochore assembly and spindle attachment for normal segregation during anaphase of meiosis I and II *(71,72)*. Thus, a loss of methyl groups in the centromeric and pericentromeric regions would deplete binding sites for the MeCP2/deacetylase complex and promote chromatin *de*condensation, disrupt secondary and tertiary structure, and promote chromosome missegregation. In nonreplicating cells, such as oocytes, the loss of methyl groups at methylated CpG sites in DNA can occur during excision repair *(73)*, by spontaneous deamination of 5-methyl cytosine to thymine *(74)*, or during DNA strand exchange associated with recombination *(63)*. The failure of the DNA methyltransferase to remethylate the newly synthesized strands or failure to methylate the opposing strand during DNA strand crossover and recombination could result in permanent loss of the heritable patterns of methylation under conditions of intracellular folate/methyl deficiency.

The importance of stable pericentromeric DNA methylation and pericentromeric secondary and tertiary structures for normal chromosome segregation has been underscored by several recent discoveries. For example, lymphocytes from individuals with the rare autosomal disorder, ICF (immunodeficiency, centromeric instability, and facial anomalies) exhibit profound chromosomal abnormalities, including selective undermethylation of pericentromeric satellite DNA, pericentromeric decondensation, and complex multiradiate chromosomes *(75,76)*. The genetic origin of this disorder was recently discovered to be a mutation in the *de novo* DNA methyltransferase 3B (*DNMT3B*) and supports a causal association among DNA hypomethylation, chromosomal decondensation, and defective segregation *(77,78)*. Treatment of cultured cells with 5-azacytidine, a potent demethylating agent, similarly results in pericentromeric undercondensation and profound chromatid missegregation in mitotic anaphase *(62,79,80)*. Chromosomal deletions, translocations, and instability were recently reported in murine embryonic stem cells nullizygous for the maintenance DNA methyltransferase (*dnmt1*) gene *(81)*. Aneuploidy and chromosomal instability are present in virtually all human

cancers *(82)* and have been shown to be related to the extent of DNA hypomethylation *(83)*. Taken together, these data strongly suggest that stable heritable methylation patterns in pericentromeric DNA are an essential prerequisite for normal chromosome condensation, stabilization, and segregation.

MEIOTIC NONDISJUNCTION AND DOWN SYNDROME

Prevalence and Consequence of Down Syndrome

Down syndrome or trisomy 21 is a complex metabolic and genetic disorder that stems from the failure of chromosome 21 to segregate normally during meiosis *(84,85)*. It is the first clinically defined syndrome shown to be chromosomal in origin and, as a result, has been the prototype for intense clinical, cytogenetic, epidemiologic, and molecular investigation into mechanisms of human aneuploidy. The origin of the extra chromosome 21 has been shown to be maternal in almost 90% of cases, with approx 10% of cases resulting from paternal nondisjunction and a very small proportion of cases resulting from postzygotic mitotic nondisjunction (mosaics) *(86)*. Down syndrome is the most common genetic cause of human mental retardation and occurs with a prevalence of approx 1 in 600–1000 live births. In addition to mental deficiency, 40–50% of children with Down syndrome have congenital heart defects (primarily endocardial cushion defects), about 5% have gastrointestinal defects, and there is a 15 to 20-fold increased incidence of childhood leukemias *(85)*. The presence of three copies of the genes present on chromosome 21 results in overexpression of these genes and excessive synthesis of multiple-gene products that result in the phenotypic characteristics of the disease. The dysmorphic features and the neurologic, immunologic, endocrine, and biochemical abnormalities reflect a complex genetic and metabolic imbalance resulting from the presence of an extra copy of chromosome 21 *(85)*. Despite the prevalence and health consequences associated with Down syndrome, the biochemical and molecular bases for abnormal chromosome segregation and meiotic nondisjunction has not yet been elucidated.

More than 80% of trisomy 21 conceptions undergo spontaneous abortion, accounting for approx 1–2% of clinical miscarriages and thus represent a major source of human pregnancy loss *(86)*. Recent evidence suggests that the predominance of maternal nondisjunction is the result of the absence of a sensitive anaphase checkpoint in the oocyte that would detect and reject chromosomal errors *(87,88)*. In contrast, meiotic stages in sperm are subject to strict checkpoint controls such that most aneuploid sperm are effectively

eliminated *(89)*. The lack of oocyte checkpoint surveillance provides a plausible biological explanation for the predominance of maternal nondisjunction in aneuploid conceptions *(88,89)*. Trisomic conceptions are surprisingly common and account for approx 4% of all human conceptions *(86)*. Recent advances in the understanding of the process and stages of oocyte maturation and meiosis have contributed to the definition of molecular correlates of nondisjunction.

Stages of Oocyte Meiosis, Advanced Age, and Nondisjunction

Meiosis I (MI) involve reductional division, in which the duplicated sister chromatids stay attached and the paired homologs segregate (disjoin) to opposite poles. In meiosis II (MII), the sister chromatids undergo centromeric separation and segregate as in normal mitotic division. In the human female fetus, oocytes undergo DNA replication and genetic recombination and then remain arrested in meiotic prophase I (diplotene stage) for up to several decades until initiation of oocyte maturation and ovulation in the sexually mature adult female *(90)*. Nondisjunction can occur between homologs in anaphase of MI during oocyte maturation or can occur between sister chromatids in MII at the time of conception *(91,92)*. It is important to note that the actual nondisjunction event does not occur when the mother is a fetus, but occurs during oocyte maturation in the adult female or at the time of conception *(90,93)*. The risk of maternal nondisjunction increases exponentially after the age of 30 and reaches a frequency of 1 in 100 conceptions by the age of 40 *(86)*. Although advanced age remains the only well-established risk factor for nondisjunction, the biologic explanation for the age-related increase in risk is one of the major unanswered questions in human genetics. Interestingly, most children with Down syndrome are born to mothers under the age of 30, reflecting the higher birth rate despite lower risk in this age group. Whether meiotic nondisjunction in the younger mothers reflects an accelerated age-related phenomenon is not known. It has been speculated that the age effect may be initiated by genetic aberrations occurring in the fetal oocyte and subsequently promoted by biochemical or molecular perturbations occurring during the prolonged prophase I arrest, which can last up to four decades *(87,93,94)*.

Specific perturbations that have been proposed to enhance the risk of meiotic nondisjunction include disturbances in homolog pairing and genetic recombination before initiation of meiosis in the fetal oocyte, failure to maintain pairing throughout the prolonged suspension of prophase I, disturbances in chromatin condensation and centromeric instability before entry into metaphase, defects in chromosome alignment caused by abnormal kineto-

chore formation, and/or defects in spindle apparatus before entry into the anaphase *(88,92,94,95)*. Although the chromosomal basis for Down syndrome has been known for over 40 yr, the specific biochemical and molecular mechanisms that are responsible for meiotic nondisjunction remain undefined.

Altered Recombination in Meiosis I and II and Nondisjunction

It is now accepted that most human trisomies are associated with aberrant recombination patterns *(93,95,96)*. The sites of recombination (genetic crossing over between homologous chromosomes) resolve into "chiasmata," as the chromosomes condense before entry into metaphase. Chiasmata ensure normal chromosome segregation by establishing physical cohesion between homolog pairs that promotes proper alignment and capture of their kinetochores by opposite spindle poles *(91)*. Further, the chiasmata provide an essential counterbalancing tension to the kinetochore–microtubule pole forces that allows congregation at the spindle equator and initiates the signal for anaphase progression and segregation to opposite poles. In contrast, achiasmatic chromosomes fail to align at the spindle equator and move precociously and randomly to either pole, with smaller achiasmatic chromosomes migrating earlier than larger achiasmatic chromosomes *(91)*. The absence of tension between opposing kinetochores results in chromosome misalignment and missegregation. Because the mammalian female lacks an effective meiotic checkpoint control, the segregation failure and nondisjunction proceeds undetected.

Recent evidence based on gene-mapping methods and analysis of meiotic exchange configurations has confirmed that the number and location of chiasmata are important molecular correlates, but not the primary cause of nondisjunction *(93,95,97)*. The relationship between altered recombination and nondisjunction is complex and appears to involve at least three "susceptible" configurations that are more prone to nondisjunction *(92)*. In almost half of MI nondisjunctions, there is a total absence of detectable recombination on chromosome 21 that would be expected to result in disoriented chromosomes and random segregation of homolog pairs. A second type of recombination error associated with MI nondisjunction is a single distally placed exchange far from the centromere. Finally, an increase in proximal pericentromeric recombination is observed in MII errors *(92,95)*. It has been proposed that proximal chiasmata might predispose to chromosome entanglement and inability to separate properly *(94)*. An increase in proximal exchange could also interfere with sister chromatid centromeric cohesion, leading to premature chromatid separation in MI, and random single

chromatid migration that could be interpreted as an "MII" error. Taken together, these results challenge the notion that missegregation in MII is independent of MI and suggest that most maternal nondisjunction is initiated in MI and subsequently expressed at either MI or MII. This implies a "two-hit" process whereby the first hit requires a susceptible recombination profile (e.g., no exchange or misplaced exchange) that originates in the fetal ovary *(92,96)*. The second "hit" would involve a processing error during the protracted MI prophase arrest that would be enhanced with maternal age and/or specific environmental exposures.

Precocious Sister Chromatid Separation in Meiosis I: The Single Chromatid Hypothesis and Relationship to Folate Deficiency

The classic nondisjunction model for aneuploidy assumes that homologous chromosomes misalign at anaphase I such that both pairs of homologs migrate to one pole at MI or, alternatively, the sister chromatids fail to disjoin at MII. In either case, two chromosomes 21 would end up in a single oocyte. An alternative theory proposes that the extra chromosome derives from the inappropriate premature centromeric separation of sister chromatids in anaphase of MI resulting in the random migration of single chromatids to the same pole *(97)*. This would result in an apparent MI or MII nondisjunction by conventional centromeric mapping analysis when, in fact, the precocious chromatid separation occurred in MI. Supporting this theory, recent cytogenetic analysis of human MII metaphase oocytes obtained from in vitro fertilization patients revealed the unexpected presence of single chromatids at MII metaphase and no incidence of nondisjoined chromosome pairs *(93,97,98)*. The random migration of single chromatids at MI would increase the risk of two copies of chromosome 21 at MII and trisomy after fertilization. These results challenge the classic nondisjunction theory that would predict the presence of chromosome pairs in the abnormal MII metaphase oocytes. It is possible that an abnormal increase in proximal recombination and/or pericentromeric hypomethylation could destabilize centromeric cohesion holding the sister chromatids together and promote premature separation at MI. Supporting this possibility, increased pericentromeric recombination has been associated with missegregation of the X chromosome at both the first and second meiotic division *(99)*.

Of particular interest, nutritional folate deficiency has been shown to induce premature centromeric separation of sister chromatids that is reversible with supplementation. Metaphase spreads of bone marrow aspirates from patients with megaloblastic anemia resulting from folate and/or B_{12} deficiency revealed multiple chromosomal aberrations, including chromo-

some breakage, decondensation, and premature centromeric separation that were eliminated after vitamin replacement therapy *(31,32)*. Further, lymphocyte culture under conditions of folate and/or thymidine deficiency induced a sevenfold to ninefold increase in the incidence of premature centromere division that was reversible with repletion *(32,100)*. These results are consistent with the hypothesis that abnormal chromosome segregation associated with folate deficiency and pericentromeric hypomethylation may be secondary to premature sister chromatid separation at MI with subsequent random migration of two single chromatids to one pole, leading to trisomy 21 at conception.

GENE–NUTRIENT INTERACTIONS IN THE MATERNAL RISK OF DOWN SYNDROME: LINK TO ABNORMAL FOLATE METABOLISM

Multifactorial gene–environment interactions that compromise maternal folate status may promote chromosomal meiotic nondisjunction and risk of Down syndrome. The 677C→T polymorphism of the MTHFR gene causes an alanine to valine substitution that decreases enzyme activity by 35% with the heterozygous (C/T) genotype and 70% with the homozygous (T/T) genotype *(101)*. The reduction in enzyme activity associated with the MTHFR polymorphism raises the dietary requirement for folic acid to maintain normal remethylation of homocysteine to methionine *(102)*. Consequently, inadequate folate intake in individuals with the MTHFR polymorphism could lead to an increase in homocysteine and a decrease in methionine levels *(103)*. As shown in Fig. 1, chronic elevation in intracellular homocysteine can lead to a pathologic increase SAH that is associated with inhibition of the DNA methyltransferase and DNA hypomethylation *(19,104)*.

Based on this evidence, we proposed the possibility that gene–nutrient interactions associated with abnormal folate metabolism and DNA hypomethylation might increase the risk of maternal chromosome nondisjunction and Down syndrome. In a preliminary study, mothers of children with Down syndrome were found to have mildly elevated plasma homocysteine levels, reduced methionine levels, and a 2.6-fold increased frequency of the 677C→T polymorphism in the MTHFR gene compared to control mothers *(103)*. In a follow-up study that consisted of a larger population of mothers, we confirmed the initial finding that the MTHFR polymorphism associated with increased maternal risk of Down syndrome and further reported for the first time an association between a second polymorphism and Down syndrome risk *(105)*. Methionine synthase reductase (MTRR) is

a flavoprotein that maintains the methionine synthase enzyme in an active state for the remethylation of homocysteine to methionine and is also important for normal folate metabolism. Because the MTRR 66A→G polymorphism has been associated with increased risk for spina bifida *(28)*, we hypothesized that it might also be a maternal risk factor for Down syndrome. In this study, blood samples were obtained from three study sites within North America, from women who had a pregnancy affected by trisomy 21 (cases) and from women who did not have a pregnancy affected by Down syndrome (controls) *(103,106–108)*. Genomic DNA was analyzed for 677C→T MTHFR polymorphism and the 66A→G MTRR polymorphism and allele frequencies were calculated for each genotype and compared between cases and controls. Odds ratios for the mutant genotypes as compared to the wild-type were calculated as a measure of the association of the MTHFR and MTRR genotype with a Down syndrome-affected pregnancy, giving an estimate of relative risk. The interaction between the genotypes was evaluated by calculating the odds ratio for the exposure of one genotype while controlling for the other and by calculating the odds ratio for the presence of both genotypes compared to the absence of both.

Table 1 indicates the MTHFR and MTRR mutant allele frequencies for women with pregnancies affected by Down syndrome and for control women. The frequencies of the MTHFR 677T allele and the MTRR 66G allele were both significantly higher among cases than controls.

Table 2 shows that the presence of the MTHFR 677C→T substitution in one or both alleles was associated with an increase in the risk of having a child with Down syndrome. The odds ratio for the homozygous genotype was slightly higher than that of the heterozygous genotype.

In Table 3, the MTRR data are stratified by genotype and show that homozygosity for the 66A→G mutation was associated with an even greater increase in risk of having a Down syndrome-affected pregnancy compared to homozygous normal subjects. The heterozygous mutation, however, was not associated with a significant increase in risk.

Both MTHFR and MTRR polymorphisms were evaluated for the risk associated with each genotype independently and for the combined risk when both polymorphisms are present. As shown in Table 4, women who were heterozygous or homozygous for the MTHFR mutant genotype but did not have the MTRR mutant GG genotype were more likely to have a pregnancy affected by Down syndrome. Women who were homozygous for the GG MTRR polymorphism but were negative for MTHFR mutant allele were also at a greater increased risk. Although the presence of both polymor-

Table 1
Allele Frequencies of MTHFR 677C→T and MTRR 66A→G in Women with Down Syndrome-Affected Pregnancies and Controls

		Frequency					
		Cases		Controls			
Genotype	Allele	Alleles	%	Alleles	%	χ^2	*p*-Value
MTHFR	C	186	0.59	193	0.69		
	T	128	0.41	87	0.31	6.02	0.01
MTRR	A	116	0.40	146	0.53		
	G	174	0.60	132	0.47	8.95	0.003

phisms consistently conferred a greater risk of Down syndrome than either alone, the interaction does not appear to be synergistic.

Conducting studies of genetic polymorphisms such as MTHFR, with low frequency of the homozygous variant, can be challenging—the need for relatively large study populations to gain sufficient statistical power must be balanced against the need to minimize bias that may occur as a result of population admixture. In order to acquire sufficient statistical power for our study, cases were drawn from two birth defect registries and from a volunteer sample that included cases from 16 states and Canada. Control mothers were limited to women who were documented to have no children with a birth defect and were pooled from two previous studies. Thus, an inherent limitation in this study is the potential bias of population admixture introduced by selecting cases and controls from diverse geographical areas within North America. To minimize bias, cases and controls were restricted to North American Caucasians of European descent. The MTHFR 677T allele frequency in the control group was 0.31, which was within the range of frequencies reported by Botto and Yang in a recent meta-analysis *(109)*. The MTRR genotype distribution among our control mothers was compared to that among fathers of the cases, who might be more ethnically similar to the mothers than a convenience sample of controls. Among fathers of cases from Atlanta and California, the frequency of MTRR 66A→G homozygosity was 24.2%, whereas among the controls in the present study, it was 23.0%. This would suggest that the combined control samples were representative of both the MTHFR allele frequencies and the MTRR A66G homozygosity within North American Caucasians. Nonetheless, these findings need to be confirmed in a large prospectively designed population-based case-control study.

Table 2
Association Between Maternal MTHFR Genotype and Down Syndrome-Affected Pregnancies and Controls

	Cases (n=157)		Controls (n=140)				
Genotype	*N*	%	*N*	%	Odds ratio	95% Confidence interval	*p*-Value
CC	51	32	67	48	1.0		0
CT	84	54	59	42	1.87	1.14–3.06	0.02
TT	22	14	14	10	2.06	0.96–4.43	0.09
CT or TT	106	68	73	52	1.91	1.19–3.05	0.01

It is now well established that the metabolic imbalance and elevation of plasma homocysteine associated with reduced MTHFR activity in individuals with the T variant is largely preventable by supplemental folic acid *(108,110,111)*. Because MTHFR and MTRR enzymes require folate and B_{12}, respectively, to support the methionine synthase reaction, the metabolic impact of both polymorphisms is magnified by low folate or B_{12} levels *(28,102)*. Accordingly, risk estimates that stratify mutant genotypes by nutritional status result in more sensitive risk estimates than those based on genotype alone. For example, stratification of the MTHFR 677C→T genotype by folate status or the MTRR 66A→G genotype by vitamin B_{12} status has resulted in more precise risk estimates for neural tube defects *(27,28)*. The ability to analyze the MTHFR and MTRR genotypes in terms of specific metabolic biomarkers such as plasma homocysteine, folate, and/or vitamin B_{12} levels would increase the power to detect a significant impact on Down syndrome risk.

The association of DNA hypomethylation with chronic folate deficiency lends support to the possibility that the increased frequency of the MTHFR 677C→T and the MTRR 66A→G polymorphisms may be associated with chromosomal nondisjunction and Down syndrome. Interestingly, the presence of both polymorphisms conferred a greater risk than either polymorphism alone. Additional studies of other candidate genes in the folate pathway, as well as a systematic study of interactions with other micronutrients involved in folate/methyl metabolism in women with Down syndrome-affected pregnancies, may suggest opportunities to improve public health strategies for primary prevention of Down syndrome.

Table 3
Association Between Maternal MTRR Genotype and Down Syndrome-Affected Pregnancies and Controls

	Cases (n=145)		Controls (n=139)			95%	
Genotype	*N*	%	*N*	%	Odds ratio	Confidence interval	*p*-Value
AA	26	18	39	28	1.0		
AG	64	44	68	49	1.41	0.77–2.56	0.33
GG	55	38	32	23	2.57	1.33–4.99	0.01
AG or GG	119	82	100	72	1.78	1.02–3.13	0.06

FUTURE DIRECTIONS

In this chapter, we have explored the relationship between folate deficiency and the molecular determinants of chromosome instability. We reported data from preliminary studies that link genetic polymorphisms in two genes critical to folate metabolism, MTHFR and MTRR, to increased risk of Down syndrome. In this final section, we will review additional evidence that is consistent with our finding and recommend strategies for future studies to confirm or refute the association between folate metabolism and Down syndrome.

Our work is the first to suggest an association between abnormal folate metabolism and the occurrence of Down syndrome-affected pregnancies. Observations from previous studies may be considered supportive of our finding. In the Hungarian trial *(111,112)*, the only randomized controlled trial of the association between occurent neural tube defects and periconceptional use of multivitamins containing folic acid, there were five infants born with Down syndrome in the nonsupplemented group and two infants born with Down syndrome in the multivitamin group. The magnitude of the association is slightly less than that of the association between limb reduction defects and multivitamins. Five infants were born with limb reduction defects in the nonsupplemented group and only one infant with limb reduction defects was born in the supplemented group. Although these results were not statistically significant, several follow-up observational studies have been conducted that confirm the role of multivitamins in the occurrence of limb reduction defects *(113–115)*. No case-control studies have been conducted to determine the association between multivitamins containing folic acid and Down syndrome. Other evidence consistent with

Table 4
Interaction Between MTHFR and MTRR Genotype in Women with Down Syndrome-Affected Pregnancies and Controls

		Cases (n=157)		Controls (n=140)		Odds	95% Confidence	
MTHFR	*MTRR*	*N*	%	*N*	%	ratio	interval	*p*-Value
CC	AA or AG	27	19	52	39	1.0		
TT or CT	AA or AG	63	43	51	38	2.37	(1.31–4.31)	0.01
CC	GG	19	13	15	11	2.44	(1.07–5.54)	0.05
TT or CT	GG	36	25	17	12	4.08	(1.94–8.56)	0.001

an association between Down syndrome and maternal folic acid metabolism is the elevated rates of Down syndrome in women of Mexican and Central American origin compared to Caucasian women *(116)*. Hispanics have been shown to have an unusually high frequency of the MTHFR 677C→T polymorphism with an allelic frequency of 0.51 *(117)*. Further, the increased risk of Down syndrome in younger women with MII nondisjunction errors who smoke and use oral contraceptives was recently reported *(118)*. Interestingly, low folate status has been associated with each of these potential risk factors *(119,120)*.

Recommendations for Future Research Efforts

There are several possible explanations for the failure to conclusively identify risk factors for Down syndrome, other than advanced maternal age, in previous epidemiologic studies. It may be that relevant factors have not been included or, possibly, that the ability to identify such factors is limited by study design. We suggest that future epidemiologic studies of the association between Down syndrome and folic acid metabolism carefully consider several study design issues.

Case Ascertainment and Classification

In our preliminary study, case ascertainment was limited to mothers who had a live-born child with Down syndrome and could reflect a survival advantage. Multiple studies of early spontaneous abortions have documented the presence of trisomy 21 in about 1 in 100 abortuses, a frequency much higher than the 1 in 600–1000 among live-born infants with trisomy 21 *(121)*. In addition, elective terminations for congenital malformations are increasing *(122,123)*. Population-based ascertainment of Down syndrome

cases that have been spontaneously aborted or electively terminated requires substantial efforts and resources. However, without these efforts, our understanding of the genetic susceptibilities and environmental risks associated with Down syndrome will be incomplete.

Homogeneous classification of Down syndrome cases may result in inaccurate risk estimates. Hassold and Sherman *(96)* proposed that a major concern in most previous studies is that the etiology of nondisjunction has been considered to be homogeneous, however, there are different types of meiotic and mitotic errors, and the underlying mechanism may be quite different for each. In future studies, cases of Down syndrome should be stratified by type of meiotic and mitotic errors to determine the parental origin and timing of nondisjunction *(96)*. Improved classification of Down syndrome cases may result in improvements in risk estimation, as demonstrated by the results of the recent study of oral contraceptives and smoking *(118)*.

Control Selection

A major concern when reporting epidemiologic findings of an association between a disease and genetic polymorphism is the potential of reporting a spurious association that is unrelated to the underlying etiology of the disease. A major source of bias leading to a false association may be the result of population admixture such that the control group is ethnically different than the cases. In our study, we used a convenience control group drawn from the North American population, but from different geographical regions than the cases. By restricting cases and controls to those of Caucasian ethnicity, we attempted to minimize the bias that might be caused by population admixture. Nonetheless, the findings of our study need to be confirmed in a large prospectively designed population-based case-control study with controls stratified by ethnicity and chosen from the same geographical regions as the cases.

Gene–Nutrient Interactions

Khoury and Beaty *(124)* showed that in the presence of an interaction between genetic and environmental factors, failure to take both of these sets of factors into account leads to bias in the estimation of disease risk *(125–127)*. Because of the genetic complexity of folate metabolism, MTHFR and MTRR alleles may be expected to interact with other folate-related genes and with maternal folate status. Results from previous studies indicate that there is a clear interaction between the 677T allele of the MTHFR gene and folate intake. When folate intake is high, the homocysteinemia associated with the 677C-T mutation may be prevented *(128)*. The stratification of the

MTHFR 677C→T genotype by folate status or the MTRR 66A→G genotype by vitamin B_{12} status has resulted in more sensitive risk estimates for neural tube defects *(27,28)*. The ability to analyze the MTHFR and MTRR genotypes in terms of specific metabolic biomarkers such as plasma homocysteine, folate, and/or vitamin B_{12} levels would increase the power to detect a significant impact on Down syndrome risk. Investigations of gene–nutrient interactions will help to identify whether some women are more susceptible to having a Down syndrome-affected fetus and whether maternal intake of folic acid mitigates the adverse effects of genetic variants. Evaluating both genetic polymorphisms in genes involved in the folate pathway and maternal biomarkers of folate intake should better permit inferences about the mechanism underlying nondisjunction in women with Down syndrome-affected pregnancies.

Potential Confounders and Effect Modifiers

Causal relations and underlying mechanisms may emerge more clearly when etiologic research is focused on subgroups of women with increased vulnerability. Accordingly, future studies will need to include factors that may bias or modify the association among genetic polymorphisms, maternal folate intake, and Down syndrome. Folate antagonists such as smoking, oral contraceptives, and maternal aging may increase the need for folate and thereby increase genetic susceptibilities to some or all forms of nondisjunction.

The Importance of Elucidating the Biologic Mechanism of Nondisjunction

Through further discussion and collaboration between cellular and molecular biologists, biochemists, and genetic epidemiologists, we may be able to discover the underlying mechanism by which folate and other micronutrients are associated with congenital anomalies. Such efforts could increase the likelihood of detecting preventable causes and lead to the implementation of polices targeted to protect sensitive groups of women that are likely to bear disproportionate risk, thus reducing the risk of having a pregnancy affected by Down syndrome and perhaps other congenital anomalies. We are hopeful that further investigations will continue to provide new insights into the causation of human trisomies and that will help us to arrive at more effective strategies for primary prevention.

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4

Folic Acid, Vitamin B_{12}, and Genomic Stability of Human Cells*

Michael Fenech

INTRODUCTION

Folic acid and vitamin B_{12} play an important role in DNA metabolism *(2)* (Fig. 1). Folic acid is required for the synthesis of dTMP from dUMP and the maintenance of DNA methylation. Under conditions of folic acid deficiency, dUMP accumulates and, as a result, uracil is incorporated into DNA instead of thymine *(3)*. There is good evidence suggesting that excessive incorporation of uracil in DNA not only leads to point mutation but may also result in the generation of single- and double-stranded DNA breaks, chromosome breakage, and micronucleus formation *(4,5)*. The mutagenic effects of uracil are underscored by the observation that of eight known human glycosylases, four (UNG, TDG, hSMUG1, MBD4) are dedicated to the removal of uracil *(6)*. Folic acid and vitamin B_{12} are also required for the synthesis of methionine and *S*-adenosylmethionine, the common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation *(7)*. When the concentration of vitamin B_{12} and methionine is low, *S*-adenosylmethionine (SAM) synthesis is reduced, methylation of DNA is reduced, and inhibition by SAM of methylene tetrahydrofolate reductase (MTHFR) is minimized, resulting in the irreversible conversion of 5,10-methylene tetrahydrofolate to 5-methyltetrahydrofolate, thus favoring an increase in the dUMP pool and uracil incorporation into DNA. Deficiencies in folic acid and vitamin B_{12} therefore can lead to (1) an elevated DNA damage rate and altered methylation of DNA, both of which are important risk factors for cancer *(4–6)* and (2) an increased level in homocysteine status, an important risk factor for cardiovascular disease *(8)*. These same defects may also play an important role in developmental and neurological abnormalities *(4,5)*.

*Substantial parts of this chapter, including Fig. 1 and Table 1, were updated and/or reprinted from ref. *1*, with permission from Elsevier Science.

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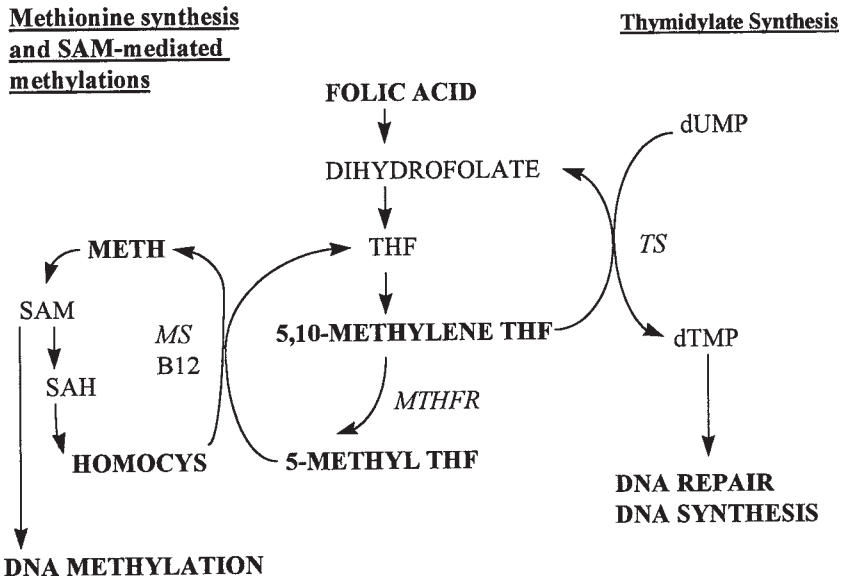

Fig. 1. The main metabolic pathways in folate and homocysteine (homocys) metabolism. Abbreviations: B12, vitamin B_{12}; meth, methionine; THF, tetrahydrofolate; TS, thymidylate synthase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosyl homocysteine. From ref. *1*.

The blood levels of folate and vitamin B_{12} required to prevent anemia and hyperhomocysteinemia are properly defined; however, it is still uncertain whether such accepted levels of sufficiency are in fact adequate to minimize chromosome damage rates and optimize DNA methylation status. In this review, evidence is provided from in vitro studies with human cells and in vivo cross-sectional and intervention studies in humans to identify the concentration or intake level at which potential genotoxic effects of low folate and vitamin B_{12} status may be prevented. In addition, the potential impact of genetic polymorphisms in key transport molecules and enzymes required for the metabolism and of folic acid and vitamin B_{12} are discussed as factors that should be considered when determining recommended dietary allowance (RDA) intake of these vitamins based on genomic stability *(9)*.

EVIDENCE FROM IN VITRO CULTURES WITH HUMAN CELLS

Fragile sites in chromosomes are expressed when human lymphocytes are cultured in the absence of folic acid and thymidine in culture medium

(10,11). Furthermore, under these conditions, chromosome breakage and micronucleus (MN) expression are increased simultaneously, suggesting a similar mechanism underlying the expression of fragile sites and chromosome breakage *(10–12)*. Reidy's experiments showed that lymphocytes cultured in folic-acid-deficient medium exhibit increased levels of excision repair during G_2 because the cytosine arabinoside-induced chromosome aberration level (which is indicative of excision repair activity) was more than doubled by this treatment and further enhanced by addition of deoxyuridine *(13,14)*. Treatment of human lymphoid cells in culture with methotrexate results in a large increase in the dUTP/dTTP ratio and a much increased incorporation rate of uracil in DNA *(15)*. The connection between uracil incorporation and the generation of DNA strand breaks was confirmed in more recent studies using the single-cell gel electrophoresis method; in this method, the extent of uracil incorporation was measured by treating the nuclei with uracil DNA glycosylase followed by measurement of resulting DNA breaks *(16,17)*.

To provide a more in-depth understanding of the impact of folic acid deficiency on chromosomal stability, we cultured lymphocytes for 9 d in culture medium with decreasing folic acid concentration within the physiological range (i.e, 10–120 nmol/L). We used a novel multiple end-point cytokinesis-block micronucleus assay measuring micronuclei (a marker of chromosome breakage and chromosome loss), nucleoplasmic bridges (a marker of chromosome rearrangement), and nuclear buds (a marker of gene amplification), necrosis, apoptosis, nuclear division index, as well as uracil in DNA. It was evident from the results of this study that chromosome damage rates (Fig. 2), uracil in DNA, and necrosis were minimized at a folic acid concentration of 120 nmol/L, and the nuclear division index was maximized at this concentration and there were no dose-related trends in apoptosis *(18,19)*.

It appears that no studies have yet been published showing a link between vitamin B_{12} deficiency in vitro and increased genomic instability in human cells. Conclusive experiments to answer this question are yet to be performed.

EVIDENCE FROM IN VIVO STUDIES WITH HUMANS

The early evidence of chromosome damage in human cells in vivo from folate and vitamin B_{12} deficiency was first obtained from studies linking the expression of Howell–Jolly bodies in erythrocytes with megaloblastic anemias *(20–22)*. Howell–Jolly bodies are whole chromosomes or chromosome fragments that lag behind at anaphase during production and maturation of

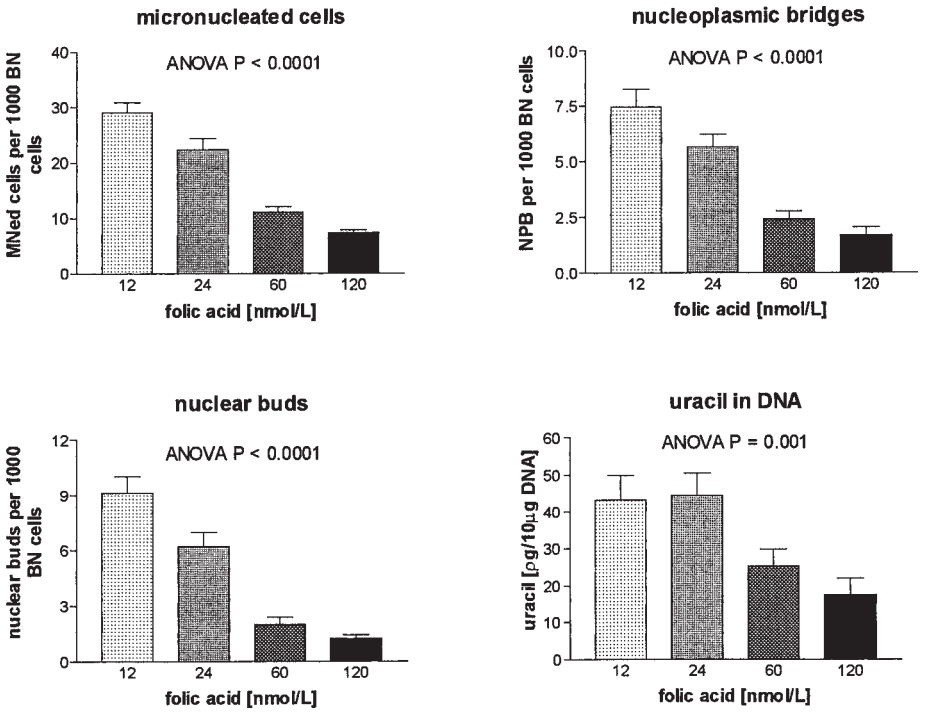

Fig. 2. Relationship between folic acid concentration and four different biomarkers of DNA damage: MNed = micronucleated cells, a biomarker for chromosome breakage and loss: NPB = nucleoplasmic bridges, a biomarker of chromosome rearrangement; nuclear buds = a biomarker for gene amplification. Results from long-term primary lymphocyte cultures of 20 different subjects (i.e., $N = 20$). (Data from ref. *18*.)

the red blood cell and, in fact, they are the same as micronuclei, the alternative and most commonly used term for this chromosome-damage biomarker. Micronucleated erythrocytes in humans are most readily observed in splenectomized subjects because the spleen actively filters micronucleated erythrocytes from the blood (*23,24*).

A case study of a 30-yr-old male with Crohn's disease with a very high level of micronuclei in erythrocytes (67/1000 cells) showed that this was associated with a low serum folate (1.9 ng/mL; normal range > 2.5 ng/mL) and a low red cell folate (70 ng/mL; normal range > 225 ng/mL). Micronucleus frequency was reduced to 12/1000 cells, serum folate increased to > 20 ng/mL, and red cell folate increased to 1089 ng/mL after 25 d with a daily oral dose of 25 mg folinic acid *(23)*. One of the main observations of this study was that minimum spontaneous MN frequencies were observed

only when serum folate levels exceeded 15–20 ng/mL, which was higher than the values accepted as normal by clinicians (i.e., 6–15 ng/mL).

A cross-sectional study of smokers (N = 30) and nonsmokers (N = 30) showed a significant inverse relationship between chromosome aberrations and blood folate status and that smoking and blood folate status are interrelated in their association with chromosome fragility. In this study of ex vivo expressed DNA damage, the cells were cultured in low folate medium and the results may therefore reflect the expression of fragile sites within chromosomes *(25)*.

Another small (N = 22) cross-sectional study on the influence of blood micronutrients on micronucleus frequency in erythrocytes of splenectomized subjects preselected because they had among the highest or lowest micronucleus frequencies from a larger population (N = 122) showed that the elevated micronucleus index was only strongly associated with low levels of serum folate (<4 ng/mL) and low levels of plasma vitamin B_{12} (<200 pg/mL). Vitamins C and E and beta-carotene did not show a strong inverse correlation with the micronucleus index *(26)*.

Blood samples from the same cohort of individuals analyzed in the studies of splenectomized individuals *(23,24)* were also analyzed for uracil content *(4,5)*. The results showed that uracil level in DNA was 70-fold higher in individuals with serum folate < 4 ng/mL relative to individuals with serum folate > 4 ng/mL. Uracil levels in DNA were rapidly reduced (within 3 d) after daily supplementation with 5 mg folic acid in both the deficient (<4 ng/mL serum folate) and nondeficient groups (>4 ng/mL serum folate). These changes were accompanied by corresponding reductions in erythrocyte micronucleus frequency but over a longer time frame *(4,5)*.

A folic acid depletion/repletion study (baseline: 195 µg/d; depletion: 5 wk 65 µg/d; repletion: 4 wk 111 µg/d followed by 20 d of >280 µg/d) of nine postmenopausal women in a metabolic unit showed a significant increase in micronucleus frequency in lymphocytes following depletion and a decrease following repletion; micronucleus frequency in buccal cells decreased after the repletion phase *(27)*. The depletion phase in this study also resulted in increased DNA hypomethylation, increased dUTP/dTTP ratio, and lowered NAD levels in lymphocytes *(28)*.

A cross-sectional study on buccal mucosal folate and vitamin B_{12} and its relation to micronucleus frequency in buccal cells revealed that buccal mucosal folate and vitamin B_{12} are significantly lower in current smokers than in noncurrent smokers *(29)*. Although current smokers in this study were three times more likely to have micronucleated buccal cells, this chromosome damage index was not associated with localized folate and vitamin

B_{12} deficiencies. However, an elevated salivary vitamin B_{12} was associated with a reduced micronucleus frequency in the buccal cells. This was the first study investigating the hypothesis that epithelial cancers such as those of the cervix, lung, bladder, and oropharyngeal region could be the result of localized deficiencies in folic acid and vitamin B_{12}, which was suggested from the observation that megaloblastic changes in such tissues can be corrected by folate/vitamin B_{12} supplementation *(30)*.

A small number of case studies link vitamin B_{12} deficiency with increased levels of chromosome aberrations *(31,32)*. Of 10 patients with pernicious anemia (which is a manifestation of vitamin B_{12} deficiency), 3 had elevated chromosome aberrations and 8 had increased levels of micronucleus frequency in bone marrow preparations *(33)*. A female infant with transcobalamin II (the transporter for vitamin B_{12} in plasma) deficiency showed elevated levels of aneuploidy (hypodiploidy in approx 30% of cells) and increased chromosomal breakage in the bone marrow with reduction in hypodiploidy to 10% of cells after 5 mo treatment with folate and vitamin B_{12} supplements *(34)*. Combined deficiency in folic acid and vitamin B_{12} was associated (1) with transient 7q– in one patient *(35)* and (2), in a series of patients, the production of a persistent abnormal deoxyuridine suppression test result (which is indicative of inadequate capacity to generate dTMP) and increased frequency of chromosomes showing despiralization and chromosomal breaks *(36)*. The latter studies showed that it took up to 84 d after supplementation with folic acid and vitamin B_{12} before the deoxyuridine suppression and the chromosomal morphology tests returned to normal. With regard to the question of chromosome despiralization, it may be important to note that the DNA methylation inibitor, 5-azacytidine, induces distinct undercondensation of the heterochromatin regions of chromosomes 1, 9, 15, 16, and Y and the specific loss of these chromosomes as micronuclei in human lymphocytes in vitro *(37)*. Similarly, the ICF (immunodeficiency, centromeric instability, and facial anomalies) immunodeficiency syndrome, which is caused by mutation in the DNA methyl transferase gene, is characterized by despiralization of heterochromatin of chromosomes 1, 9, and 16 and loss of this chromatin into micronuclei and nuclear blebs *(38)*.

A cross-sectional study in Japan involving 18 college students aged 19–23 yr and 15 laboratory workers aged 24–69 yr performed to investigate the age-adjusted micronucleus index in cytokinesis-blocked lymphocytes with serum vitamins found a protective effect of increased folic acid that was marginally significant (multiple regression beta value –4.00, $p = 0.06$) but no apparent protective effect associated with elevated vitamin B_{12} concentration; none of the subjects were vitamin B_{12} deficient (<200 pg/mL) or

folic acid deficient (<3.5 ng/mL) and mean values were 544 pg/mL and 7 ng/mL, respectively *(39)*. In contrast, low but not deficient levels of plasma vitamin B_{12} were associated with increased micronucleus frequency (r^2 = 0.11, p = 0.06) in nascent human erythrocytes of healthy blood donors *(40)*.

We have performed a series of studies to investigate the interrelationship between DNA damage in somatic cells and blood status for folate, vitamin B_{12}, and homocysteine. As a marker of chromosome damage, we have used the cytokinesis-block micronucleus method in lymphocytes, which has been shown in numerous studies to be a reliable and sensitive biomarker of chromosome breakage and chromosome loss that occurs spontaneously *(41)* or as a result of elevated exposure to genotoxins *(42)*.

Our preliminary studies comparing DNA damage rate and micronutrient status in vegetarians and nonvegetarians had indicated that there was a significant negative correlation between the micronucleus frequency in lymphocytes and plasma vitamin B_{12} status in young men *(43)*. Therefore, we investigated the prevalence of folate deficiency, vitamin B_{12} deficiency, and hyperhomocysteinemia in 64 healthy men aged between 50 and 70 yr and determined the relationship of these micronutrients with the micronucleus frequency in cytokinesis-blocked lymphocytes *(44)*. Twenty-three percent of the men had serum folate concentration less than 6.8 nmol/L, 16% had red blood cell folate concentration less than 317 nmol/L, 4.7% were vitamin B_{12} deficient (<150 pmol/L), and 37% had plasma homocysteine levels greater than 10 µmmol/L. In total, 56% of the apparently healthy men had nonoptimal values for folate, vitamin B_{12}, or homocysteine. The micronucleus index of these men (19.2 ± 1.1, N = 34) was significantly elevated (p = 0.02) when compared to that of men who had higher concentrations of folate and vitamin B_{12} and lower plasma homocysteine (16.3 ± 1.3, N = 30). Interestingly, the micronucleus index in men with normal concentrations of folate and vitamin B_{12} but homocysteine levels greater than 10 µmol/L (19.4 ± 1.7, N = 15) was also significantly higher (p = 0.05) when compared to those with normal folate, vitamin B_{12}, and homocysteine less than 10 µmol/L. The micronucleus index and plasma homocysteine were also significantly (p = 0.0086) and positively correlated (r = 0.415) in those subjects who were not deficient in folate or vitamin B_{12}. The micronucleus index was not significantly correlated with folate indices but there was a significant (p = 0.013) negative correlation with serum vitamin B_{12} (r = –0.315). It was apparent that elevated homocysteine status, in the absence of vitamin deficiency, and low, but not deficient, vitamin B_{12} status are important risk factors for increased chromosome damage in lymphocytes.

Subsequently, we performed a cross-sectional study (N = 49 males, 57 females) and a randomized double-blind placebo-controlled dietary inter-

vention study ($N = 31$ males, 32 females per group) to determine the effect of folate and vitamin B_{12} on DNA damage (micronucleus formation and DNA methylation) and plasma homocysteine (HC) in young Australian adults aged 18–32 yr *(45)*. None of the volunteers were folate deficient (i.e., red cell folate < 136 nmol/L) and only 4.4% (all females) were vitamin B_{12} deficient (i.e., serum vitamin B_{12} < 150 pmol/L). The cross-sectional study showed that (1) the frequency of micronucleated (MNed) cells was positively correlated with plasma HC in males ($r = 0.293$, $p < 0.05$) and (2) in females, MNed cell frequency was negatively correlated with serum B_{12} ($r = –0.359$, $p < 0.01$), but (3) there was no significant correlation between micronucleus index and red cell folate status. The results also showed that the level of unmethylated CpG (DNA) (measured using the Sss1 methylase method), was not significantly related to vitamin B_{12} or folate status. The dietary intervention involved supplementation with 700 µg folic acid and 7 µg vitamin B_{12} in wheat bran cereal for 3 mo followed by 2000 µg folic acid and 20 µg vitamin B_{12} via tablets for a further 3 mo. In the supplemented group, MNed cell frequency was significantly reduced during the intervention by 25.4% in those subjects with initial MNed cell frequency in the high 50th percentile, but there was no change in those subjects in the low 50th percentile for initial MNed cell frequency. The reduction in MNed cell frequency was significantly correlated with serum vitamins B_{12} ($r = –0.49$, $p < 0.0005$) and plasma HC ($r = 0.39$, $p < 0.006$), but it was not significantly related to red cell folate. DNA methylation status was not altered in the supplemented group. The greatest decrease in plasma HC (by 37%) during the intervention was observed in those subjects in the supplemented group with initial plasma HC in the high 50th percentile, and it correlated significantly with increases in red cell folate ($r = –0.64$, $p < 0.0001$) but not with serum vitamin B_{12}. The results from this study suggest that (1) MNed cell frequency is minimized when plasma HC is below 7.5 µmol/L and serum vitamin B_{12} is above 300 pmol/L and (2) dietary supplement intake of 700 µg folic acid and 7 µg vitamin B_{12} is sufficient to minimize MNed cell frequency and plasma homocysteine in young adults. Thus, it appears that elevated plasma HC, a risk factor for cardiovascular disease, may also be a risk factor for chromosome damage.

Other studies have shown that global DNA methylation in lymphocytes or colonic tissue is influenced by the extent of folate intake. The depletion–repletion study performed by Jacob et al. *(28)* with postmenopausal women in a metabolic unit showed more than a 100% increase in DNA hypomethylation after 9 wk on low-folate diet (56–111 µg/d) and a subse-

quent increase in DNA methylation after a further 3 wk on a high-folate diet (286–516 μg/d). Fowler et al. *(46)* and Cravo and colleagues *(47)* showed, using the Sss1 methylase assay, that cervical and gastric/colonic/rectal epithelium DNA methylation is significantly correlated to serum and tissue folate concentration. Furthermore, it was shown that intrinsic methylation of DNA was lower in the normal colorectal mucosa of adenoma and carcinoma patients; however, supplementation with 10 mg folic acid/d for 6 mo increased methylation 15-fold ($p < 0.0002$), and 3 mo after cessation of therapy, methylation decreased fourfold *(47)*.

Studies on breast cancer patients at the time of disease presentation and before chemotherapy showed that elevated mutant frequencies in the HPRT gene occurred in those individuals with serum folate in the deficient range and serum vitamin B_{12} levels were correlated negatively with sister-chromatid-exchange levels *(48)*. The extent of increase in HPRT mutant frequency in lymphocytes of breast cancer patients after chemotherapy tended to correlate negatively with serum folate level *(49)*. In this respect, it is important to note that murine cells deficient in DNA methyl transferase exhibit elevated point mutation rates mainly as a result of gene deletions caused by mitotic recombination or chromosome loss *(50)*.

Another important possibility for the prevention of genomic instability could be the prevention of integration of oncogenic virus DNA. Prevention of hypomethylation may enable a better surveillance of foreign DNA integration into human DNA because DNA methylation appears to have evolved partly for this purpose *(51)*. It is interesting to note in this regard that human papilloma virus (HPV) virus tends to integrate in fragile sites that may be folate dependent *(52)*, which raises the hypothesis that viral integration into DNA in vivo may be facilitated when folate status is low enough to cause fragile site expression. It is also important to note that transcription of retroviral or parasitic DNA sequences integrated into mammalian DNA is inhibited by cytosine methylation and, conversely, demethylation may activate transcription of endogenous retroviruses; the significance of these observations is underscored by the fact that the large majority of 5-methylcytosine in the genome actually lies within parasitic, retroviral, or transposon DNA *(53,54)*. Whether folate deficiency can activate transcription of retroviral DNA remains untested. Vitamin B_{12} may also play a direct role in the prevention of the integration of oncogenic viruses because it has been shown that cobalamin inhibits HIV integrase and the integration of HIV–DNA into nuclear DNA *(55)*. On the basis of these results, combination treatment with folic acid and vitamin B_{12} supplements has been used in the treatment of AIDS patients with apparent success (*56*).

ENVIRONMENTAL AND GENETIC FACTORS THAT DETERMINE THE BIOAVAILABILITY OF FOLIC ACID AND VITAMIN B_{12}

Alcoholism is associated with significantly reduced levels of tissue folate, vitamin B_{12}, and vitamin B_6 in humans; at intakes greater than 3.0 g/kg/d, there was a doubling in the level of DNA hypomethylation of lymphocytes *(57)*. The reduced folate level in alcoholics may be the result of reduced absorption or suboptimal dietary intake. However, if results in the rat model reflect the situation in humans, then there is a good probability that the microbial metabolism of alcohol can result in exceedingly high levels of acetaldehyde, which destroys folate in the intestine; this has been shown to be associated with localized folate deficiency in the colonic mucosa *(58)*.

Reduced absorption of protein-bound vitamin B_{12} resulting from atrophic gastritis caused by autoantibodies to gastric pareital cells and reduced absorption caused by autoantibodies to intrinsic factor are two of the main causes of vitamin B_{12} deficiency that may affect between 10% and 40% of the elderly (>60 yr) *(59)*. An increasingly important cause of cobalamin deficiency is exposure to nitrous oxide either because of abuse *(60)*, exposure during anesthesia *(61)*, or occupational exposure of hospital personnel during surgery procedures *(62)*. Nitrous oxide inactivates cobalamin, rendering exposed individuals effectively vitamin B_{12} deficient. It is interesting to note that hospital personnel exposed to nitrous oxide had four times the MN frequency of matched controls *(62)*, which could be explained in part by the genotoxic effect of functional vitamin B_{12} deficiency.

The conversion of dietary folate and vitamin B_{12} to an intracellular active coenzyme requires many physiological and biochemical processes, including stomach release of bound vitamin, intestinal uptake, blood transport proteins, cell uptake receptors, and enzymatic conversion to the active coenzyme *(63)*. In the case of vitamin B_{12}, at least five different peptides (R binder, intrinsic factor, ileal receptors, transcobalamin I, transcobalamin II) are required to deliver vitamin B_{12} from the gut to the tissues and a further four enzymes (cblF, cblC/D, microsomal reductase, cblE/G) are necessary to convert vitamin B_{12} to the appropriate reduced state to function as a coenzyme with methionine synthase. In the case of folate, a conjugase enzyme is required to deconjugate polyglutamated folate in the small intestine; receptors are required for active uptake into the intestinal brush-border epithelium, carried to the liver by the hepatoportal circulation where monoglutamated folate (i.e., folic acid) is reduced and methylated to form 5-methyltetrahydrofolate, which is exported into the blood; it is then taken

up by receptor/pinocytosis mechanism, where it is subsequently stored in cells in the polyglutamated form by the activity of folyl-γ-polyglutamate synthetase. The capacity of 5-methyltetrahydrofolate to donate its methyl group for the regeneration of methionine from homocysteine is dependent on the activity of methionine synthase. On the other hand, the activity of thymidylate synthase and methylene tetrahydrofolate reductase (MTHFR) determine the probability of 5,10-methylene tetrahydrofolate donating its methyl group for the conversion of dUMP to dTMP. All of the above indicates that genetic defects in one or more of the key enzymes and uptake proteins can limit the bioavailability of folate and vitamin B_{12}. Therefore, it may be necessary for above RDA intake of these vitamins to overcome defects relating to the uptake or reduced activity of enzymes, as, in fact, has been shown in subjects defective in intrinsic factor or cobalamin reductase in the case of vitamin B_{12} and subjects defective in MTHFR in the case of folate *(63–65)*. It is also interesting to note that in the case of MTHFR, polymorphisms reducing its activity such as the C677T mutation may, on the one hand, protect against cancer and on the other hand increase the risk for developmental defects such as Down syndrome and neural tube defects *(66–71)*. The most plausible explanation is that the MTHFR mutation minimizes incorporation of uracil into DNA and therefore chromosome breakage and rearrangement, whereas it has only a relatively minor impact on DNA methylation—this implies that chromosome breakage/rearrangement may be more critical than hypomethylation for carcinogenesis, although this emphasis may change depending on folate intake and the extent to which hypomethylation of DNA causes aneuploidy, a potential carcinogenic event *(66,67,72)*. However, the impact of the MTHFR mutation on DNA methylation status may be important enough during the finely tuned development process when the concerted and timely expression of genes is critical and possibly more susceptible to appropriate DNA methylation status. Current in vivo evidence suggests that MTHFR C677T homozygotes have lower global DNA methylation than wild types in lymphocytes *(73)*. In vitro studies suggest that under conditions of adequate riboflavin, C677T homozygotes are equally susceptible as wild types to uracil incorporation into DNA and chromosome damage induced by folate deficiency *(18,19)*. Either way, the observations with the C677T and A1298C MTHFR polymorphism with respect to cancer risk and developmental defects *(69,70)* suggest the potential importance of folate supplements to overcome metabolic limitations and the expectation is that the same may apply to vitamin B_{12} for defects of other key enzymes such as methionine synthase and methionine synthase reductase *(65,74)*.

Table 1
Concentration and Dietary Intake of Folic Acid that Minimizes Genomic Instability in Human Tissue

Genomic instability biomarker	Concentration in culture medium—in vitro (ng/mL)	Concentration in plasma—in vivo (ng/mL)	Concentration in RBCs—in vivo (ng/mL)	Daily dietary intake (μg/d)
SSB/DSB—comet assay	100 (*16*)			
Micronuclei	80 (*9*)[a]	15 (*22*)	600 (*22*)	5000 (*22*)
	53 (*18*)	7 (*26*)	313 (*44*)	228 (*26*)
				700 (*44*)[b]
Nucleoplasmic bridges	53 (*18*)			
Nuclear buds	53 (*18*)			
Uracil in DNA	53 (*17*)	53 (*3,4*)	480 (*3,4*)	5000 (*3,4*)
CpG hypomethylation		24 (*46*)		10,000 (*46*)
		7 (*27*)		516 (*27*)

[a]In the presence of thymidine (4.0 mg/L).
[b]Together with 7 µg/d vitamin B_{12}.
Note: 1 ng/mL of folic acid = 2.26 nmol/L. From ref. *1*.

RECOMMENDED DIETARY ALLOWANCES FOR FOLATE AND VITAMIN B_{12} BASED ON GENOMIC STABILITY

There is now increasing interest to redefine recommended dietary allowances (RDAs) of minerals and vitamins not only to prevent diseases of extreme deficiency but also to prevent developmental abnormalities and degenerative diseases of old age as well as optimizing cognition *(75)*. Prevention of chromosome breakage and aneuploidy is an important parameter for the definition of new RDAs for micronutrients *(9)* such as folic acid and vitamin B_{12} because increased rates of DNA damage have been shown to be associated with increased cancer risk *(76–78)* and accelerated aging *(79)*. Table 1 summarizes the information from in vitro and in vivo controlled experiments in human cells and human subjects with a view to defining, based on current knowledge, the optimal concentration and dietary intake of folic acid for minimizing genomic instability. The results from a variety of DNA damage biomarkers suggest that above RDA levels of folic acid intake are required to minimize DNA damage; furthermore, the current sufficiency levels of folate in plasma (i.e., 2.2 ng/mL or 4.9 nmol/L) and red blood cells

(i.e., 132 ng/mL or 298 nmol/L), which are based on prevention of anemia *(80)*, are much lower than the averaged concentration levels at which DNA damage is minimized (i.e., 21 ng/mL [range: 7.3–53.0 ng/mL] for plasma and 464 ng/mL [range: 313–600 ng/mL] for red blood cells) (results from Table 1). In this regard, it is interesting to note that the red cell folate concentration that corresponds to minimization of risk of neural tube defects in the unborn child is approx 400 ng/mL *(81,82)*, which is of a similar magnitude as that which appears to be required for minimizing genomic instability. The data from our intervention studies *(44,45)* also suggest that the current sufficiency level of vitamin B_{12} in plasma for the prevention of anemia (i.e., 150 pmol/L or 203 pg/mL) is almost half of the level at which the micronucleus index in lymphocytes is observed to be minimized (i.e., 300 pmol/L or 406 pg/mL); intakes of three and a half times the current Australian RDA were required to achieve this level in plasma. Only with careful choice and sufficient intake of folate-rich foods such as aleurone flour, certain fruits and vegetables, and vitamin B_{12}-rich foods such as liver is it possible to achieve the required above RDA intake of folate and vitamin B_{12} *(83,84)*. The use of fortified foods or tablet supplements may be a more practical proposition for achieving these levels of intake *(85)*. Combining folic acid with vitamin B_{12} in supplements or in a fortification program not only has the benefit of maximizing the impact of these vitamins because vitamin B_{12} makes folate more bioavailable both for synthesis of dTMP and methionine, but it also enhances the homocysteine-lowering capacity of folate *(44)* and prevents the possibility of masking neuropathies (associated with pernicious anemia) when folate supplements are taken on their own in individuals who have an underlying vitamin B_{12} deficiency *(85)*.

CONCLUSION

The accumulated evidence to date suggests that folate and vitamin B_{12} play an important role in genomic stability. Above RDA intakes of these vitamins may be required for a large proportion of humans because of the increasing evidence for common single-nucleotide polymorphisms that alter significantly the activity of proteins required for the absorption, transport, and metabolism of these vitamins to their active forms. Current evidence from prospective studies suggest a reduced risk of cancer in those with lower chromosomal damage rates regardless of exposure to man-made carcinogens. It is therefore anticipated that increased genomic stability resulting from adequate intake of folate and vitamin B_{12} may result in reduced cancer risk, and some epidemiological evidence for this view is already emerging *(66,67,86,87)*.

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5

Molecular Genetics of Folate Metabolism

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INTRODUCTION

Folate is a critical nutrient in maintaining normal cell growth and division and is thus important for human development. Food (fresh green vegetables, liver, yeast, and some fruits) largely contains folate polyglutamates that are hydrolyzed by folylpolyglutamate conjugase (enzyme commission [EC] number 3.4.19.9) to monoglutamates before absorption in the jejunum (reviewed in detail in refs. *1* and *2*). After uptake in the enterocytes, intracellular polyglutamate synthesis occurs, followed by reconversion into monoglutamates that are reduced to biologically active tetrahydrofolate. After methylation, the folates enter the portal circulation as 5-methyltetrahydrofolate. The majority of this folate is taken up by the liver, which plays a central role in folate homeostasis. The cellular uptake is mediated via specific folate receptors. Plasma 5-methyltetrahydrofolate (30–40%) is bound to albumin, α2 macroglobulin, transferrin, and folate-binding protein.

In the cell, 5-methyltetrahydrofolate serves as a methyl donor and as a source of tetrahydrofolate. Tetrahydrofolate acts as an acceptor of one-carbon units, producing a variety of other folates, which, in turn, are specific coenzymes in intracellular reactions. One of these is 5,10-methylenetetrahydrofolate that is reduced by the enzyme 5,10-methylenetetrahydrofolate reductase to 5-methyltetrahydrofolate. 5-Methyltetrahydrofolate carries the methyl group required for conversion of homocysteine to methionine (remethylation pathway of homocysteine). This methyl group is initially transferred to cob(I)alamin, revealing methylcobalamin that is demethylated by homocysteine to form methionine by the cobalamin-dependent enzyme

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methionine synthase. Occasionally, cob(I)alamin may be oxidized to cob(II)alamin, resulting in the inhibition of methionine synthase. To maintain the enzyme activity, a reductive methylation by the enzyme methionine synthase reductase is required.

Because cobalamin serves as an acceptor of the methyl group from 5-methyltetrahydrofolate, cobalamin deficiency can be associated with "folate trapping," where folates are metabolically dead because they cannot be recycled as tetrahydrofolate back into the folate pool. The resulting failure to regenerate methionine causes depletion of methionine and ultimately leads to hyperhomocysteinemia.

Characterization of inherited disorders of folate transport and metabolism revealed that folate status is under genetic control. The recent cloning of genes encoding proteins required for intestinal absorption of folates, delivery of folates to the cells, as well as the folate cycle provided the basis to identify rare mutations associated with severe enzyme deficiencies and genetic polymorphisms affecting folate and homocysteine status. These include the gene coding for 5,10-methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), methionine synthase reductase (*MTRR*), folate receptor 1 (*FOLR1*), reduced folate carrier 1 (*SLC19A1*), folate hydrolase (*FOLH1*), and the serine hydroxymethyltransferase genes (*SHMT1* and *SHMT2*).

THE 5,10-METHYLENETETRAHYDROFOLATE REDUCTASE GENE

The human 5,10-methylenetetrahydrofolate reductase gene (*MTHFR*) is located on chromosome 1p36.3 *(3)*. The *MTHFR* gene (GenBank accession number AH007464) consists of 11 exons, with a length ranging from 102 to 432 bp, and introns ranging from 250 to 1.5 kb, except for one intron of 4.2 kb *(4)*. The entire coding region encompasses 1.980 bp (GenBank accession number NM_005957). So far, two mRNAs of 7.5 kb and 8.5 kb have been identified in tissues. Several alternatively spliced 5' noncoding regions have been reported, suggesting complexity of the *MTHFR* gene expression.

The MTHFR Gene Product MTHFR

The *MTHFR* gene product consists of 656 amino acid residues with a predicted molecular mass of 74.6 kDa *(3–6)* and is termed 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20). The amino acid sequence is highly conserved, showing 90% homology with the mouse polypeptide *(4)*. MTHFR consists of two identical subunits of approx 70 kDa *(7)* and represents a key enzyme in the folate cycle. It reduces 5,10-

methylenetetrahydrofolate to 5-methyltetrahydrofolate, thus catalyzing the only reaction in the cell that ultimately generates 5-methyltetrahydrofolate, the biologically active folate derivative. Interestingly, knockout mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition *(8)*.

Common Polymorphisms in the MTHFR *Gene*

Three polymorphisms exist in *MTHFR* that are located at nucleotide position 677 (*MTHFR* 677C→T) *(5)*, position 1298 (*MTHFR* 1298A→C) *(9–11)*, and position 1317 (*MTHFR* 1317T→C) *(10)*. *MTHFR* 677C→T occurs in exon 4 at the folate-binding site of 5,10-methylenetetrahydrofolate reductase and changes an alanine into a valine residue (A222V). *MTHFR* 1298A→C is located in exon 7 within the presumptive regulatory domain changing a glutamic acid into an alanine residue (E429A). The *MTHFR* 1317T→C is a silent mutation that is also located in exon 7. *MTHFR* 677C→T, *MTHFR* 1298A→C, as well as compound heterozygosity for 1298A→C and 677C→T are associated with a reduced enzyme activity of 45%, 68%, and 42%, respectively *(12)*.

Allele Frequencies of MTHFR *Polymorphisms*

The *MTHFR* 677C→T polymorphism has a relatively high frequency throughout the world. The 677TT genotype is present in about 12% of the general population and shows a heterogeneous distribution among ethnic groups with an allele frequency ranging from 0.045 in Sri Lanka to 0.3 in Caucasians and Americans *(13)*. *MTHFR* 1298A→C shows an allele frequency of 0.3 in Canadians, Austrians, and Dutch individuals *(10,11,14)*. The allele frequency of *MTHFR* 1317T→C is 0.05 in Canadian individuals *(10)* and 0.059 in a Turkish population *(15)* and was quite common in a small population of Africans (the allele frequency among nine healthy Africans was 0.39) *(10)*. In the study of Meisel et al., none of 1000 healthy Caucasians and only 1 of 1000 coronary artery disease patients tested positive for *MTHFR* 1317T→C *(16)*.

Metabolic Effects of MTHFR *Polymorphisms*

The *MTHFR* 677C→T mutation is associated with decreased formation of 5-methyltetrahydrofolate and an accumulation of formylated tetrahydrofolate polyglutamates in erythrocytes *(17)*. Furthermore, *MTHFR* 677TT is frequently associated with significantly higher total homocysteine plasma levels as compared to heterozygotes or people without mutation. This effect is observed in all countries and regions of Europe, indicating a steady impact of the mutation on total homocysteine plasma levels *(18)*.

The *MTHFR* 1298A→C mutation alone does not influence folate status or total homocysteine concentrations. By contrast, compound heterozygosity for the 677T and the 1298C alleles can be associated with decreased folate plasma concentrations *(11,14)* and higher total homocysteine concentrations *(11)*.

Disease Associations of MTHFR *Polymorphisms*

Some of the clinical implications of *MTHFR* 677C→T are summarized in Chapter 2. The implications of *MTHFR* 677C→T and *MTHFR* 1298A→C in cardiovascular disease, cerebrovascular disease, venous thrombosis, longevity, neural tube defects, pregnancy, congenital abnormalities, preclampsia, diabetes, cancer, psychiatry, and renal failure are reviewed in refs. *19* and *20*.

More recently, Dekou et al. reported that the frequency of the *MTHFR* 677T allele of the coronary heart disease high-risk town of Dewsbury was significantly higher than in the coronary heart disease low-risk town of Maidstone and was associated with increased total homocysteine plasma concentrations *(21)*. This effect was seen in men but not in women and was not observed for *MTHFR* 1298A→C. Furthermore, a higher susceptibility for malignant lymphoma has been observed in individuals with the combined *MTHFR* 677CC/1298AA genotype, as well as those with the *MTR* 2756GG genotype *(22)*. Moreover, the *MTHFR* 677C→T mutation, smoking, and folate status were strong interactive determinants of high-risk adenomas of the colorectum *(23)*. In this study, the risk was particularly high in smokers with low folate and the *MTHFR* 677CT or the *MTHFR* 677TT genotype, and in smokers with high folate and the *MTHFR* 677CC genotype. This risk pattern was also observed for colorectal hyperplastic polyps. Together, these data demonstrate a strong gene–nutrition interaction involving the *MTHFR* 677C→T polymorphism.

Most recently, Meisel et al. observed no association of the *MTHFR* 677/1298 genotypes with coronary artery disease or total homocysteine plasma levels *(16)*. In another study, the *MTHFR* 1298C allele was associated with early-onset coronary artery disease, even when total homocysteine levels were not elevated *(24)*.

The *MTHFR* 677TT and the *MTHFR* 677CT/1298AC genotype can modulate the efficacy of oral or intravenous folic or folinic acid therapy *(25–27)*. Furthermore, the effect of drugs that interfere with the folate status is suggested to be influenced by the *MTHFR* 677C→T polymorphism *(20)*.

The *MTHFR* 1317T→C mutation was not associated with either deep vein thrombosis *(15)* or with coronary artery disease *(16)*.

Rare Mutations in the MTHFR *Gene*

Rare mutations exist in *MTHFR* that are associated with severe enzyme deficiency (enzyme activity less than 20% in fibroblast cultures; an overview is provided in ref. *28*). The first mutations in severely MTHFR-deficient patients were identified in 1993 by Goyette and coworkers *(29)*. During the following years, evidence accumulated that severe MTHFR deficiency results from heterogeneous mutations associated either with extremely low enzyme activity and early onset of symptoms, or with moderately reduced enzyme activity and later onset of symptoms *(30–32)*. To date, approx 25 different mutations have been reported that may, at least partly, explain the heterogeneous clinical phenotypes *(30–34)*.

THE METHIONINE SYNTHASE GENE (*MTR*)

The human methionine synthase gene (*MTR*) is located on chromosome 1q43 *(35,36)*. The entire coding region (GenBank accession number U71285) has a length of 3.795 bp, with a predicted molecular mass of 140 kDa *(37,38)*. Two mRNAs of 7.5 kb and 10 kb are present in human tissues *(38)*. Furthermore, a minor mRNA of 4.4 kb as well as other partially spliced larger mRNAs have been detected *(35)*.

The MTR *Gene Product Methionine Synthase*

The *MTR* gene product consists of 1.265 amino acid residues *(37,38)* and is termed methionine synthase (MS, EC 2.1.1.13, alternative titles: 5-methyltetrahydrofolate–homocysteine *S*-methyltransferase, tetrahydropteroylglutamate methyltransferase). The amino acid sequence shows about 55% and 65% homology with the polypeptide of *Escherichia coli* and *Caenorhabditis elegans*, respectively *(37,38)*.

Methionine synthase is a cytoplasmic enzyme that requires methylcobalamin for activity and catalyzes the remethylation of homocysteine to methionine. In this reaction, the methyl group of 5-methyltetrahydrofolate is transferred to the enzyme bond cob(I)alamin to generate methylcobalamin followed by the transfer of the methyl group to homocysteine to reform methionine. The enzyme consists of four separate regions referred to as the homocysteine-binding region (residues 2 – 353), the methyltetrahydrofolate-binding region (residues 354 – 649), a region responsible for binding the cobalamin prosthetic group (residues 650 – 896), and an *S*-adenosylmethionine-binding domain (residues 897 – 1227). Importantly, functional integrity of the latter region is required for reductive activation of methionine synthase by the enzyme methionine synthase reductase (EC 2.1.1.135) *(36)*.

Common Polymorphisms in the MTR *Gene*

In *MTR*, a polymorphism exists that is located at nucleotide position 2756 (*MTR* 2756A→G) *(35,37,38)*. *MTR* 2756A→G occurs at the C-terminal end of the α/β domain of the enzyme and changes an aspartic acid into a glycine residue (D919G). Because *MTR* 2756A→G is located in a helix between the cobalamin domain and the *S*-adenosylmethionine-binding domain, the glycine substitution could affect the secondary structure of the enzyme. However, the functional significance of this polymorphism will have to be examined in expression experiments to characterize its impact on the protein.

Allele Frequency of MTR *Polymorphism*

The *MTR* 2756A→G has an allele frequency of 0.15 *(37)* and 0.16 *(39)*, showing differences among study populations (allele frequencies ranging between 0.18 and 0.38) *(22,35,40,41)*.

Metabolic Effects of MTR *Polymorphism*

The mutation alters formyltetrahydropteroylglutamic acid (H4PteGlu) disposition of erythrocytes *(42)* in that the *MTR* 2756AG genotype is associated with more formyl-H4PteGlu, relative to 5-methyl-H4PteGlu, as compared to individuals with wild-type alleles. This relationship is not present in red blood cells of individuals with a neural tube defect *(42)*.

The influence of *MTR* 2756A→G on total homocysteine plasma levels is a matter of debate. Harmon et al. reported an association with increased total homocysteine levels *(43)*, which has not been confirmed by others *(39,40,42,44–46)*. Furthermore, an association of *MTR* 2756A→G with low plasma total homocysteine levels has been observed *(47,48)*.

Disease Associations of MTR *Polymorphism*

An association of *MTR* 2756A→G with severity of coronary artery disease and dose of lifetime smoking has been demonstrated *(49)*. Furthermore, the *MTR* 2756AG genotype was associated with a longer event-free survival in coronary artery disease patients as compared to AA genotype patients *(50)*. Other investigators found no association of *MTR* 2756A→G with birth defects *(39,42,46)*, cerebrovascular and cardiovascular disease *(44,51)*, and early-onset vascular thrombosis *(45)*. A decrease of the odds ratio for neural tube defects has been described for patients with the GG genotype *(41)*. Furthermore, a higher susceptibility for malignant lymphoma has been observed for individuals with the *MTR* 2756GG genotype *(22)*.

Effect of Combined MTHFR *and* MTR *Genotypes*

Only few studies addressed the effect of the combined *MTR*/*MTHFR* genotypes on homocysteine-related disorders. A lack of association of the

combined *MTR* 2756A→G and *MTHFR* 677C→T genotypes with neural tube defects *(42)* and hyperhomocysteinemia of patients with early-onset vascular thrombosis *(45)* has been described. The association of the combined genotypes with vascular disease was not clear in the study of Morita et al. *(52)*. No combined effect of *MTHFR* 677C→T, *MTR* 2756A→G and of the cystathionine β-synthase (*CBS*) polymorphism *CBS* 844ins68 on fasting or post-methionine-loading total homocysteine levels has been observed among vascular disease patients *(48)*. These findings are in line with the results of the study of Harmon et al. *(43)*, who did not detect an interaction of *MTR* 2756A→G and *MTHFR* 677C→T genotypes with respect to total homocysteine levels in healthy subjects.

The *MTR* 2756A→G polymorphism was equally distributed among healthy individuals as well as subjects with renal insufficiency with extremely high or with extremely low total homocysteine plasma concentrations *(53)*. By contrast, the combination of *MTR* 2756AG and 2756GG with the *MTHFR* 677TT/1298AA and the *MTHFR* 677CT/1298AC genotypes was associated with extremely high total homocysteine plasma concentrations *(53)*.

Rare Mutations in the MTR *Gene*

Two point mutations, three insertions, and two deletions have been identified among six patients with severe methionine synthase deficiency associated with a defective synthesis of methylcobalamin (referred to as the complementation G type of cobalamin deficiency, cblG type) *(37,54–56)*. Two of these mutations were located in the vincinity of the cobalamin-binding site *(37,55)*. Another mutation is embedded in a sequence that makes direct contact with bond adenosylmethionine, probably disrupting activation of the enzyme *(55)*.

The clinical symptoms of patients with severe methionine synthase deficiency include developmental delay, neurological deteriorations, mental retardation, and megaloblastic anemia, which often leads to diagnosis. Typical laboratory characteristics include hyperhomocysteinemia, homocystinuria, hypomethioninemia, and low methylcobalamin levels without methylmalonic aciduria.

THE METHIONINE SYNTHASE REDUCTASE GENE

The human methionine synthase reductase gene (*MTRR*) is located on chromosome 5p15.2 – p15.3 *(36)*. The *MTRR* gene (GenBank accession numbers AF121202 – AF121213) encompasses approx 34 kb and comprises 15 exons, varying in size from 43 to 1.213 bp, and 14 introns, with sizes

ranging from 108 bp to 5 kb *(57)*. The entire coding region has a length of 2.094 bp (GenBank accession numbers AF025794 and AF121214), encoding a protein with a predicted molecular mass of 77.7 kDa. A predominant mRNA of 3.6 kb is present in human tissues *(36)*. An alternative splicing of exon 1 suggests that translation can be initiated at two different ATG codons *(57)*.

The MTRR *Gene Product Methionine Synthase Reductase*

The *MTRR* gene product consists of 698 amino acid residues and is termed methionine synthase reductase (MSR, EC 2.1.1.135, alternative title: 5-methyltetrahydrofolate–homocysteine–methyltransferase reductase). The amino acid sequence shares 38% identity with human cytochrome P–450 reductase and 43% identity with the polypetide of *C. elegans (36)*. Methionine synthase reductase is a member of electron transferases termed the "ferredoxin-NADP(+) reductase (FNR) family." It is involved in the remethylation of homocysteine by reductive methylation of cob(II)alamin, which, in its unmethylated state, inactivates methionine synthase. Thus, the central role of methionine synthase reductase is maintenance of the function of methionine synthase by restoration of methionine synthase activity *(36)*.

Common Polymorphisms in the MTRR *Gene*

In *MTRR*, a common polymorphism exists that is located at nucleotide position 66 (*MTRR* 66A→G) (*58*). *MTRR* 66A→G is located at a site that is within the flavin mononucleotide-binding domain of the enzyme and changes an isoleucine into a methionine residue (I122M). Because the frequency of both alleles was nearly identical in the study by Wilson et al., the designation of a wild-type allele on the basis of the allele frequency was not possible *(58)*. However, because of a significant homology with related flavin mononucleotide-binding proteins of other species, it has been suggested that the ancestral human *MTRR* sequence contains the isoleucine codon and that the G allele represents the mutation *(58)*. The consequence of *MTRR* 66A→G on the function of the protein is currently unknown.

Allele Frequency of MTRR *Polymorphism*

The allele frequency of *MTRR* 66A→G is 0.51 among white Caucasians of French, British, and mixed European ancestry *(58)* and 0.47 among North American whites *(59)*.

Metabolic Effects of MTRR *Polymorphism*

The *MTRR* 66A→G mutation influences neither total homocysteine concentrations *(58,60)* nor serum folate and vitamin B_{12} levels of children with

neural tube defects and their mothers *(58)*, as well as of Caucasian individuals *(60)*.

Disease Associations of MTRR *Polymorphism*

The frequency of *MTRR* 66A→G was significantly higher in mothers of children with Down syndrome as compared to control mothers *(59)*. Furthermore, the *MTRR* 66GG genotype was independently associated with Down syndrome with a 2.57-fold increase in estimated risk. The combined presence of the *MTHFR* 677C→T and the *MTRR* 66A→G polymorphism was associated with a greater risk of Down syndrome than was the presence of either alone *(59)*. Furthermore, *MTRR* 66A→G showed a trend toward an increased prevalence among case mothers and cases of spina bifida *(58)* and was associated with an increased risk of neural tube defects when combined with a low serum cobalamin level *(58)*. A trend in risk has also been reported for premature coronary artery disease in individuals with the *MTRR* 66GG genotype *(60)*. Additional studies are required to confirm that *MTRR* 66A→G confers an increased risk for Down syndrome, neural tube defects, and coronary artery disease.

Rare Mutations in the MTRR *Gene*

An infrequent mutation has been described at nucleotide position 997 (*MTRR* 997C→G) that changes a leucine into a valine residue (L333V) *(61)*. *MTRR* 997C→G is located between the putative flavin mononucleotide- and flavin adenine dinucleotide-binding sites of methionine synthase reductase. This mutation was present in 1 of 50 Canadian control alleles *(61)* but has not been found in more than 1500 alleles of Austrian renal transplant patients and children with metabolic stroke (unpublished observation), suggesting that the *MTRR* 997C→G mutation represents a rare event. Thirteen mutations have been identified among 11 patients with methionine synthase reductase deficiency *(36,61)*. These mutations include large insertions or deletions, smaller deletions, as well as point mutations and are associated with a defective reductive activation of methionine synthase (cblE complementation type of cobalamin deficiency, defective synthesis of methylcobalamin, defective reducing system). The mutations are located in the *MTRR* coding region, possibly not inhibiting the production of a complete protein *(36,61)*.

Patients with methionine synthase reductase deficiency exhibit a range of clinical symptoms similar to those observed in patients with methionine synthase deficiency. These include severe developmental delay, ataxia, cerbral atrophy, seizures, and blindness. The laboratory findings are identical with

methionine synthase deficiency and include hyperhomocysteinemia, homocystinuria, hypomethioninemia, megaloblastic anemia, and low methylcobalamin levels without methylmalonic aciduria.

THE REDUCED FOLATE CARRIER 1 GENE

The human reduced folate carrier 1 gene (*SLC19A1*, solute carrier family 19; alternative titles and symbols: *RFC1*; folate transporter, *FOLT*; intestinal folate carrier 1, *IFC1*) is located on chromosome 21q22.3 *(62)*. The *SLC19A1* gene (GenBank accession numbers AL163302, AP001757, BA000005 of the human chromosome 21 segment HS21C102; this segment contains the sequence of the *SLC19A1* gene) consists of five exons (exon 2 to exon 6) *(63)*. Exon 2 is spliced from at least four 5´ exons (preferentially from exon 1) and contains the translational start site. The entire coding region encompasses 1.776 bp (GenBank accession numbers U19720 and U15939) *(64,65)*, corresponding to a mRNA of 2.7 kb detectable in human tissues *(65)*.

The SLC19A1 *Gene Product Human Folate Transporter*

The *SLC19A1* gene product consists of 591 amino acid residues with a predicted molecular mass of 65 kDa and is termed human folate transporter (FOLT; alternative titles and symbols: placental folate transporter 1; reduced folate carrier protein, RFC; intestinal folate carrier, IFC–1) and belongs to the SLC19A family of transporters. The protein shows 65% homology with the mouse and the hamster polypeptide *(65)*. The human folate transporter is an integral membrane protein. Transfection experiments with human *SLC19A1* cDNAs revealed an increased uptake of 5-methyltetrahydrofolate *(65)* and 5-methyltetrahydrofolic acid *(66)*, suggesting involvement of the *SLC19A1* gene product in internalization of folates into cells. In mice, a 58-kDa protein has been detected in the brush-border membrane of the intestine, which mediates intestinal folate transport *(67)*. Furthermore, the folate transporter is possibly important for the development of embryos, providing the folate transport across the placenta *(68)*.

Common Polymorphisms in the SLC19A1 *Gene*

In *SLC19A1*, a polymorphism exists that is located at nucleotide position 80 (*SLC19A1* 80G→A) *(69)*. This polymorphism occurs in exon 2 and changes an arginine into a histidine residue (R27H).

Allele Frequency of SLC19A1 *Polymorphism*

Among French healthy adults, homozygosity for the mutant A allele has been observed in 21.9% of individuals (heterozygotes: 50.9%, homozygotes for the G allele: 27.2%) *(69)*.

Metabolic Effects of SLC19A1 *Polymorphism*

The *SLC19A1* 80G→A mutation did not affect plasma folate or red blood cell folate concentrations of French unselected healthy individuals but showed a trend toward higher total homocysteine levels in subjects with the *SLC19A1* 80GG genotype *(69)*. In some individuals showing the combined *SLC19A1* 80GG/*MTHFR* 677TT genotype, significantly higher total homocysteine levels have been observed as compared to individuals with the *SLC19A1* 80GG/*MTHFR* 677CC or the *SLC19A1* 80GG/*MTHFR* 677CT genotype *(69)*.

Disease Associations of SLC19A1 *Polymorphism*

Currently, no data are available about the association of the *SLC19A1* 80G→A polymorphism with human diseases.

Rare Mutations in the SLC19A1 *Gene*

Mutations in the *SLC19A1* gene that are associated with a severe deficiency of the folate transporter in humans have not been reported yet.

THE FOLATE HYDROLASE GENE

The human folate hydrolase gene (*FOLH1*, identical with human prostate-specific membrane antigen, gene symbols: *PSM*, *PSMA*; alternative title and symbol: glutamate carboxypeptidase II, *GCP2*) is located on chromosome 11p11 – p12 *(70)*. The *FOLH1* gene encompasses approximately 60 kb and consists of 19 exons (GenBank accession number AF007544) *(70,71)*. The entire coding region has a length of 2.653 bp (GenBank accession number M99487) *(72)*, with a predicted molecular mass of 84 kDa excluding carbohydrate. Two mRNAs of 2.8 kb and 1.5 kb have been detected in human tissues *(73)*. In addition to the full-length transcript, a shorter mRNA lacking exon 18 has been observed, suggesting the presence of splice variants *(73)*.

The FOLH1 *Gene Product Folylpoly-γ-Glutamate Carboxypetidase*

The *FOLH1* gene product consists of 750 amino acid residues with an apparent molecular weight of 100 kDa and is termed folylpoly-γ-glutamate carboxypetidase (FGCP; alternative titles and symbols: prostate-specific

membrane antigen, PSM; N-acetylated α-linked acidic dipeptidase, NAALADase; folate hydrolase 1, FOLH) *(72)*. The amino acid sequence shows 92% identity with the pig polypetide *(74)*. Folylpoly-γ-glutamate carboxypetidase is an exopeptidase that is anchored to the apical brush-border membrane and shows folate hydrolase and N-acetylated α-linked acidic dipeptidase activity. Folylpoly-γ-glutamate carboxypetidase hydrolyzes the terminal glutamate residues of dietary folylpoly-γ-glutamates before absorption. Thereafter, the monoglutamyl folate derivatives are transported through the membrane via the folate transporter. Therefore, folylpoly-γ-glutamate carboxypetidase possibly regulates the availability of dietary folates (*75*).

Common Polymorphisms in the FOLH1 *Gene*

In *FOLH1*, a polymorphism exists that is located at nucleotide position 1561 (*FOLH1* 1561C→T) and changes a histidine into a tyrosine residue (H475Y) *(73)*. This polymorphism is located in exon 13 at the putative catalytic domain of the enzyme and is associated with a 53% reduction of enzyme acitivity *(73)*.

Allele Frequency of FOLH1 *Polymorphism*

Among 75 healthy Caucasians, 6 heterozygotes and no homozygous individual have been identified, corresponding to an allele frequency of 0.04 and a genotype frequency of 8% for heterozygotes and 92% for the homozygote wild-type genotype *(73)*.

Metabolic Effects of FOLH1 *Polymorphism*

The *FOLH1* 1561C→T mutation is associated with lower serum folate levels and higher serum total homocysteine concentrations, whereas no relation has been found with red blood cell folate or vitamin B_{12} levels *(73)*. Furthermore, no relationship was observed between the presence of the *MTHFR* 677C→T mutation and *FOLH1* 1561C→T with respect to folate status and homocysteine concentrations *(73)*.

Disease Associations of FOLH1 *Polymorphism*

At the time of writing, the association of the *FOLH1* 1561C→T with human diseases had not been investigated.

Rare Mutations in the FOLH1 *Gene*

So far, no mutations in the *FOLH1* gene that could be related to severe deficiency of folyl-γ-glutamate carboxypeptidase in humans have been reported.

THE FOLATE RECEPTOR 1 GENE

The human folate receptor 1 gene (*FOLR1*; alternative titles and symbols: folate-binding protein, *FBP*; folate receptor, *FOLR*; folate receptor-α, *hFR*; ovarian-cancer-associated antigen Mov18, CaMov18) is located at chromosome 11q13.3 – q13.5 *(76,77)*. The *FOLR1* gene (GenBank accession number U20391) encompasses 6.8 kb and consists of 7 exons *(78)*. The entire coding region is 771 bp long (GenBank accession number J05013) *(79)* and is encoded by exons 4–7 *(78)*. Two complementary DNA isoforms exist showing 5´ termini that are encoded by exons 1–4. A single mRNA species of 1.1 kb is expressed in human tissues *(79)*.

The FOLR1 *Gene Product Folate-Binding Protein*

The *FOLR1* gene product consists of 257 amino acid residues with a calculated molecular mass of 29.8 kDa and is termed folate-binding protein (FBP; alternative titles and symbols: folate receptor 1, FOLR; human folate receptor-α, hFR; ovarian-cancer-associated antigen, CaMOv18) *(79)*. The protein shows 80% homology to the bovine soluble folate-binding protein and more than 99% homology to the human soluble folate-binding protein *(79)*.

The folate-binding protein is a membrane receptor that is mainly expressed in the kidney and human placenta, but also in thymus epithelium, the brain, and several cell lines. It is required for the uptake and the delivery of 5-methyltetrahydrofolate to the cytoplasma of the cells *(80)* and plays a critical role in the folate homeostasis during development *(81)*. Furthermore, soluble forms have been found in human serum and milk. These two forms of folate-binding protein have similar binding characteristics for folates, are immunologically crossreactive, and are nearly identical in amino acid sequence. The function of the soluble form is only partially understood *(80)*.

Common Polymorphisms in the FOLR1 *Gene*

Three polymorphisms that are located in the promotor region of the folate receptor-α gene (identical with *FOLR1*) have been reported *(82)*. These are located at nucleotide position 762 (*FOLR1* 762G→A), position 610 (*FOLR1* 610A→G), and position 631 (*FOLR1* 631T→C). Two of these polymorphisms were always present in tandem (*FOLR1* 610A→G and *FOLR1* 631T→C) *(82)*.

A further three polymorphisms have been identified in the gene coding for ovarian-cancer-associated antigen (identical with *FOLR1*) using the probe cHTMOv18 *(83)*. Two of these were detected by the restriction enzyme *Pst*I, including a four-allele polymorphism showing bands of 7.5 kb,

6.8 kb, 6.3 kb, and 5.9 kb, respectively. The other polymorphism included three alleles of 4.2 kb, 3.9 kb, and 3.8 kb, respectively. The third polymorphism was detected by the restriction enzyme *Msp*I, showing two allelels of 4.5 kb and 3.2 kb *(83)*.

Allele Frequencies of FOLR1 *Polymorphisms*

Among unrelated individuals, the frequencies of the *FOLR1* four-allele polymorphism detectable by the enzyme *Pst*I ranged between 0.04 and 0.5 *(83)*. The frequencies for the three-allele polymorphism were 0.02, 0.27, and 0.61, respectively. The frequencies of the two alleles detectable by the enzyme *Msp*I were 0.07 and 0.93, respectively *(83)*. The prevalence of *FOLR1* 762G→A and the *FOLR1* 610A→G/*FOLR1* 631T→C polymorphisms was 8.33% and 4.2%, respectively, among children with spina bifida and 6.25% for all three polymorphisms among healthy control individuals *(82)*.

Metabolic Effects of FOLR1 *Polymorphisms*

The influence of the polymorphisms detectable by *Pst*I and the *Msp*I in the *FOLR1* gene, as well the influence of *FOLR1* 762G→A and *FOLR1* 610A→G/*FOLR1* 631T→C on total homocysteine plasma levels and folate status has not been investigated yet.

Disease Associations of FOLR1 *Polymorphisms*

There seems to be no association of the *FOLR1* 762G→A and the *FOLR1* 610A→G/*FOLR1* 631T→C polymorphisms with the phenotype of spina bifida *(82)*. Furthermore, no polymorphisms were identified in exons 3–6 of *FOLR1* in individuals with neural tube defects *(84)*. No polymorphisms were present in another study of individuals with spina bifida, where the intron–exon boundaries as well as the signal sequences in addition to the *FOLR1* coding region had been screened *(85)*.

Rare Mutations in the FOLR1 *Gene*

Four *de novo* insertions within exon 7 and the 3´ untranslated region of *FOLR1* have been found in patients with sporadic neural tube defect *(86)*. These mutations affected the carboxy-terminal tail or the glycosylphosphatidylinositol anchor region of the folate-binding protein. One of these insertions has also been found in 1 of 150 control individuals *(86)*.

THE SERINE HYDROXYMETHYLTRANSFERASE GENES

The cytosolic serine hydroxymethyltransferase gene (*SHMT1*) and the mitochondrial serine hydroxymethyltransferase gene (*SHMT2*; alternative

titles and symbols: glycine auxotroph A, Gly A+; human complement for hamster) are located at chromosomes 17p11.2 and 12q13.2 *(87)*. The *SHMT1* gene (GenBank accession numbers Y14489 – Y14492) spans approx 40 kb *(88)*. It consists of 13 exons and 12 introns and is alternatively spliced *(89)*. The entire coding region of *SHMT1* encompasses 1.449 bp (GenBank accession number L11931). The *SHMT2* gene (GenBank accession number U23143) spans 4.5 kb and consists of 11 exons and 10 introns *(90)*. The entire coding region includes 1.422 bp (GenBank accession number L11932) *(87)*. Furthermore, a pseudogene exists (GenBank accession number X85980) that has been mapped to chromosome 1p32.3 – p33 and shows 90% sequence identity with *SHMT1 (91)*.

The SHMT1 *Gene Product Cytosolic Serine Hydroxymethyltransferase*

The *SHMT1* gene product is 483 amino acid residues long and encodes the cytosolic serine hydroxymethyltransferase (cSHMT), with a predicted molecular mass of 53 kDa. The amino acid sequence shares 65% identity with the mitochondrial serine hydroxymethyltransferase polypeptide *(87)*. Cytosolic serine hydroxymethyltransferase represents one of the two isoforms of human serine hydroxymethyltransferase (SHMT, EC 2.1.2.1). It is a pyridoxal phosphate-containing enzyme that catalyzes the interconversion of 5,10-methylenetetrahydrofolate and glycine to tetrahydrofolate and serine in both directions *(92)*.

The SHMT2 *Gene Product Mitochondrial Serine Hydroxymethyltransferase*

The *SHMT2* gene product is 474 amino acid residues long and encodes the mitochondrial serine hydroxymethyltransferase (mSHMT), with a predicted molecular mass of 52.4 kDa. Mitochondrial serine hydroxymethyltransferase is the other isoform of human serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) and shares 65% sequence identity with the cytosolic polypetide *(87)*. Because of the high degree of sequence identity between the cytosolic and the mitochondrial serine hydroxymethyltransferases, the genes most likely arose from a duplication event. Mitochondrial serine hydroxymethyltransferase is a pyridoxal phosphate-dependent enzyme that is suggested to catalyze glycine synthesis from serine in the mitochondria as well as the reversible conversion of tetrahydrofolate into 5,10-methylene tetrahydrofolate.

Common Polymorphisms of SHMT1 *and* SHMT2

In *SHMT1*, a polymorphism that is located at nucleotide position 1420 (*SHMT1* 1420C→T) has been identified *(93)*. *SHMT1* 1420C→T is located in exon 13, which represents the last exon and leads to an amino acid substitution of leucine to phenylalanine (L474F).

In *SHMT2*, a polymorphism exists that is located at position 1721 (*SHMT2* delTCTT1721 – 1724) *(93)*. *SHMT2* delTCTT1721 – 1724 is located in the 3´ untranslated region and represents a deletion of 4 bp.

Allele Frequencies of SHMT *Polymorphisms*

The *SHMT1* 1420C→T polymorphism showed an allele frequency of 0.319 among Dutch healthy individuals *(93)*. In this study population the allele frequency of *SHMT2* delTCTT1721 – 1724 was 0.027 *(93)*.

Metabolic Effects of SHMT *Polymorphisms*

The *SHMT1* 1420C→T polymorphism did not influence total homocysteine plasma concentrations of healthy individuals *(93)*. By contrast, the *SHMT1* 1420CC genotype was associated with increased total homocysteine plasma levels in mothers of children with neural tube defects. Furthermore, the *SHMT1* 1420TT genotype was related to significantly higher folate levels in the plasma and in red blood cells of healthy individuals, which was not the case for patients with neural tube defects and the case mothers. The *SHMT2* delTCTT1721 – 1724 mutation did not alter either total homocysteine or folate levels of healthy individuals *(93)*.

Disease Associations of SHMT *Polymorphisms*

Neither *SHMT1* 1420C→T nor *SHMT2* delTCTT1721 – 1724 led to an increased risk for neural tube defects *(93)*.

Rare Mutations in the SHMT1 *and* SHMT2 *Gene*

In *SHMT1*, a mutation that is located at nucleotide position 1181 (*SHMT1* 1181G→A) has been identified in a patient with a neural tube defect *(93)*. *SHMT1* 1181G→A is located in exon 12 and changes a serine into an asparagine residue (S394N). This mutation was not present in 400 control alleles.

In *SHMT2*, a mutation that is located at position 850 (*SHMT2* 850C→T) has been found in a patient with a neural tube defect *(93)*. *SHMT2* 850C→T results in the replacement of an arginine by a tryptophan residue at codon 284 (R284W). This mutation was not present in 400 control alleles. Furthermore, a nonsense mutation (*SHMT2* 906T→G) has been observed in one case of neural tube defect *(93)*.

CONCLUSIONS

Today, there is no doubt that the folate status of an individual is influenced by genetic factors. Many polymorphisms have been identified in genes related to conversion of dietary folates, intestinal absorption, receptor- and carrier-mediated transport, as well as the metabolism of folate. Several of these have already been found to exert biochemical effects. In contrast, only a limited number of studies have investigated the association of genetic polymorphisms with human diseases. In particular, studies are scarcely focusing on the polygenetic background of folate status in specific disease entities. Because of the existence of many folate-dependent pathways required for the generation of methionine and for nucleotide biosynthesis in humans, prevention of folate deficiency is clinically relevant. In this context, it is worth mentioning that a substantial number of individuals worldwide are prone to development of folate deficiency as a result of the presence of a common genetic polymorphism (*MTHFR* 677C→T). The manifestation of the phenotype (suboptimal folate status, folate deficiency) resulting from *MTHFR* 677C→T shows a strong dependence on gene–nutrient interactions. Furthermore, this polymorphism as well as another allelic variant of *MTHFR* (*MTHFR* 1298A→C) have been shown to modulate response to folic acid therapy, suggesting that therapy with drugs interfering with folate metabolism is influenced by genetic factors. Much is yet to be learned about the impact of folate-related genetic variants on human diseases, the gene–nutrient interactions, as well as the pharmacogenetic consequences. Large-scale studies are of utmost importance to precisely characterize the multiple genetic polymorphisms underlying folate-related disorders in humans.

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6

Folate Receptor-α

An Update

Barton A. Kamen

Folate is a water-soluble vitamin essential for a number of critical metabolic pathways involving the synthesis of amino acids and nucleic acids *(1)*. It was isolated in 1941 and synthetically prepared in 1945. The importance of folate for cell growth was so recognizable that antifolates were used as antineoplastic agents in 1947, and by 1950, a Lederle monograph *(2)* already had 367 citations. There are over 25,000 citations in "PUBMED" for methotrexate, the most commonly used antifolate, antineoplastic agent between 1966 and 1999 (reviewed in ref. *3*). Knowledge of folate-mediated reactions (e.g., thymidylate synthetase and *de novo* purine synthesis and transmethylation of homocysteine to methionine) accumulated more rapidly than studies of folate conservation. This is likely the result of the lability of reduced folates and the presence of large amounts of folic acid in standard tissue culture medium (1–5 μ*M*) compared to plasma concentrations of reduced folates (e.g., human plasma folate is 0.01–0.03 μ*M*).

This chapter will review progress in work to more completely understand the function and regulation of the folate receptor, a high affinity, lipid-anchored protein with a limited tissue distribution. Based on the presence of the folate receptor-α (FR-α) in placenta, choroid plexus, and proximal tubules of the kidney, it is our assumption that the primary roles of the receptor are to concentrate and/or conserve folate in selected compartments, such as the fetus and the central nervous system.

INTRODUCTION

Why conserve folate?—because mammalian organisms lack the capacity to synthesize this "vital amine" (i.e., a vitamin). Lack of folate results in an excess of some toxic amino acids and the lack of synthesis of nucleic acids.

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The clinical manifestations of a folate deficiency are significant and include birth defects, vascular disease, anemia, and perhaps even cancer. Before initiating a review of the folate receptor, a few "bullet points" from the view of a pediatric hematologist–oncologist to put into perspective (or at least justify the time and effort in studying the receptor) the importance of folate and antifolate homeostasis seem appropriate.

HOMOCYSTEINE, EXCITOTOXIC AMINO ACIDS, AND VASCULAR DISEASE

Although the underlying mechanisms are not completely defined, there is a very strong association between increased plasma homocysteine and cerebrovascular, coronary artery, and peripheral vascular disease. Recently, in our ongoing studies of the neurotoxicity of antifolates such as methotrexate, we documented marked increases in the cerebrospinal fluid (CSF) content of adenosine and homocysteine and its metabolic products, the so-called excitotoxic amino acids *(4)*. Excitotoxic amino acids activate the NMDA receptor, which may result in a cascade of events that are associated with neuronal/glial cell death *(5)*. These recent findings add to the large body of literature showing that a folate deficiency leads to homocystinemia *(6,7)*. Most recently, decreases in CSF *S*-adenosylmethione (SAM) and increases in *S*-adenosylhomocysteine (SAH), resulting in a very abnormal SAM/SAH ratio, has been found in two children who had developed methotrexate leukoencephalopathy *(8)*.

FOLATE DEFICIENCY AND CANCER

The biochemical manifestations of a folate deficiency can be seen in a decreased supply of *S*-adenosylmethionine and then in loss of methylation of CpG islands as well as a decrease in dTMP and a rise in the dUMP pool, which has been shown to result in misincorporation of deoxyuridine (du) into DNA. Recently, Ames and colleagues showed the significant extent of misincorporation of dU into DNA in folate-deficient patients *(9)* and, like others *(10)*, speculated on the consequences. The protective effects of adequate folate, and of genetic polymorphisms altering the composition of the intracellular folate pool, with regard to the risk of colon cancer and perhaps other carcinomas has also been recently reviewed *(11,12)*. Recent work by James and her colleagues have also shown that cells transformed in vitro are more tumorgenic in vivo *(13)*.

FOLATE AND BIRTH DEFECTS

It is well established that an inadequate amount of folate results in fetal wastage and/or neural tube defects (NTD) *(14)*. Moreover, periconceptional supplementation with folic acid has been shown to reduce the occurrence of NTD, orofacial clefts, and conotruncal heart defects (reviewed in ref. *15*). This is of such significance that as of January 1998, bread and cereal grains were supplemented with folate. In this regard, our folate receptor (FR) knockout animals (null phenotype for folate receptor, specifically FR-α) are nonviable and have neural tube defects (detailed in Chapter 8).

ANTIFOLATES

Despite (or because of) the metabolic significance of the above-noted reactions and the potential problems associated with a folate deficiency, the acute disruption (inhibition) of folate-mediated reactions have proven important in medicine. Antifolates are antineoplastic and antimicrobial agents as well as drugs used for the treatment of patients with psoriasis, arthritis, and asthma *(16)*. In particular, even after more than 40 yr of use, methotrexate remains a mainstay in the treatment of children with leukemia *(3,17)*.

OVERVIEW OF FOLATE ECONOMY AND FOLATE ACCUMULATION

Both eucaryotic and procaryotic organisms must have effective barriers to separate the environment from the intracellular milieu. The extracellular environment contains molecules that must be excluded as well as critically essential molecules that must be concentrated. Vitamins represent a class of these latter compounds. For example, cobalamin is concentrated 100,000-fold from seawater by certain bacteria. In higher organisms, having plasma, vitamins must be concentrated in order to have effective intracellular concentrations. Folates represent such an example. Although many folate-mediated reactions and the reductions of folic acid and dihydrofolic acid to tetrahydrofolate are well studied [reviewed in several multivolume texts in the past decade *(1,18)*] and some of the enzymes have been sequenced, cloned, and/or analyzed by X-ray crystallography, it is only since the late 1980s that proteins important in folate transport have been purified (reviewed in ref. *19*) and the process of receptor-coupled transport, which we termed potocytosis, have been characterized *(20)*. It was not until the mid-1990s that human folate polyglutamate synthetase was cloned *(21)* and several groups obtained cDNA for the reduced folate carrier(s) as detailed next *(22,23)*.

REDUCED FOLATE CARRIER

Carrier-Mediated Folate (and Antifolate) Transport

A model for folate (antifolate) transport has evolved through the extensive work of several laboratories (reviewed in refs. *1*,*18*, and *19*). The essential components are a reduced folate carrier (RFC) for influx (facilitated transport) and a separate energy-dependent efflux system. Transport is inhibited by selected anions such as bromosulfophthalein and phthalate, further suggesting the importance of an anion pump in folate transport *(24)*. It also appears that there may be multiple routes of entry. For example, methotrexate (MTX) will block 5-methyl[^{3}H]tetrahydrofolate transport in isolated hepatocytes, but the converse experiment shows only partial inhibition of [^{3}H]MTX uptake by 5-methyltetrahydrofolic acid. Studies of murine leukemia cells have also recently provided evidence for a MTX-specific pathway *(25)*.

Molecular Biology/Biochemistry of RFC

Using NHS-esters of radiolabeled methotrexate or folate and/or photoaffinity labeling, candidate RFC molecules have been identified *(26–28)*. The molecular mass range is from 35 to >100 kDa and the protein is glycosylated. Difficulties in purification may be related to small quantities of carrier, that the carrier may not be a single protein, or perhaps that it is unstable. Currently, a number of investigators are tackling the problem of isolating RFC. Using complementation cloning, it appears that at least two groups have identified a gene whose product restores MTX sensitivity to "transport-resistant" cells *(22,23)* and another has antibodies directed against the putative RFC *(28)*. The cDNA predicts that the transport protein is 55–60 kDa and the staining characteristics suggest that it is glycosylated. In the last few years, there has been an explosion in the basic knowledge regarding the reduced folate carrier(s). Cowan and Moscow and others have isolated the cDNA for a human RFC. The gene mapped to the long arm of chromosome 21 *(29,30)*. This location is of importance because hyperploidy of chromosome 21 is a common finding in acute lymphoblastic leukemia and is the karyotypic finding of Down syndrome. We have found a strong correlation between MTX accumulation and chromosome 21 ploidy *(31)* and it is well known that children with trisomy 21 are extremely sensitive to MTX.

In addition to these studies, others studying normal tissue (e.g., intestinal cells, hepatocytes, and kidney proximal tubules) have also isolated candidate RFC proteins as well as a family of molecules that may be important in basolateral and/or apical transport of folate and, even more specifically,

antifolates *(32,33)*. The intestinal RFC is identical to the murine species. At least part of the renal and hepatic system seems to be in the family of organic anion transporters (OATs appear to be related to MOATs [multiorganic acid transporters] in hepatocytes). The consensus sequence for the RFC suggests that it may be regulated by protein kinase C (PKC). However, Said and colleagues found that a general protein tyrosine kinase (PTK), but not PKC inhibitors, interfered with intestinal cell RFC function *(34)*. An anion transporter may be working in tandem with the folate receptor.

FOLATE RECEPTOR

Receptor-Mediated Folate Transport: An Example of Potocytosis

Studying cells grown in low levels of folate (<1 n*M*), several laboratories identified a folate receptor (FR) on the membrane of KB (human nasopharyngeal carcinoma) cells, $CACO_2$ (colon carcinoma) cells, IGROV–1 (ovarian carcinoma), MA104 (monkey kidney epithelial) cells, and, more recently, in both murine and human leukemia cells (*35–39*: reviewed in refs. *19* and *40*). A soluble form of the protein was initially found in milk 30 yr ago and in some chronic myelogenous leukemia cell extracts more than 25 yr ago (in early history is reviewed in ref. *40*).

We proposed that receptor-coupled folate transport occurs in discrete areas of the cell membrane called caveolae *(20)*. Although the anatomic clustering is the subject of much debate *(41–43)*, the kinetics of 5-methyl[^{3}H] tetrahydrofolate in MA104 cells are suggestive of a coupled or channeled process (Fig. 1). First, the ligand binds to the surface via an "externalized" FR. This folate is removable with an acid/saline wash; second, the receptor/ligand complex is sequestered into an acid-resistant but still membrane-bound compartment; third, the compartment is acidified and folate dissociates from the receptor and is transferred to the cytoplasm; fourth, the receptor returns to the cell surface (acid labile fraction) to begin another round of folate binding and internalization.

Molecular Biology/Biochemistry of FR

Several laboratories have reported the isolation of cDNA clones for FR *(44–47)*. The cDNA from $CACO_2$, KB, and human placenta are identical and there is only one difference near the amino terminus in the ovarian carcinoma cDNA. The predicted amino acid sequence of the membrane form is >99% identical to the actual analysis of the soluble receptor from milk. This correlation at the nucleic acid level and the relationship of the membrane and soluble form assessed by [^{35}S]-labeling KB cells in vitro *(48)* supports

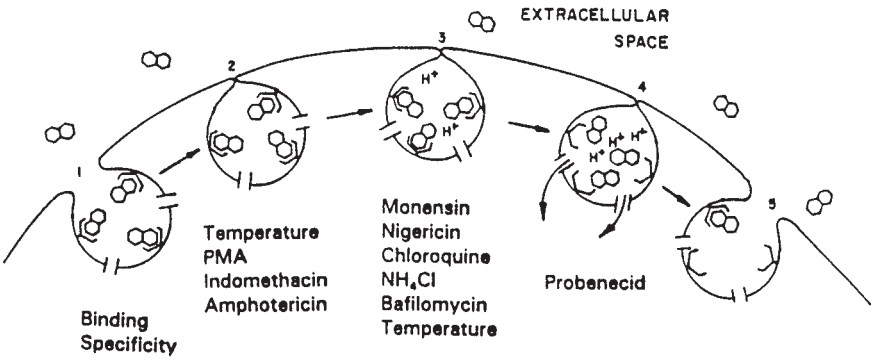

Fig. 1. Receptor-coupled transport of folate (potocytosis).

the hypothesis that the soluble form of the receptor is a processed product of the membrane form, specifically that the source of the soluble folate-binding protein is FR that was released from the cell membrane. In this regard, we showed that the cDNA sequence correctly predicted that the receptor would be coupled to the membrane via a glycosyl–phosphatidylinositol linkage (GPI) by releasing the receptor with phospholipase C specific for a GPI linkage *(45)*. Others have confirmed this through direct analysis and enzyme release in both KB cells and fresh tissue samples *(49,50)*. There is also some evidence that the receptor may be released through the action of a Mg^{2+}-dependent protease. In addition, Ratnam and colleagues found a second clone in a human placental library *(51)* and a third species predominating in marrow elements *(52)*. The first species, initially referred to as MFP2, is now termed FR-α; the second, also from placenta and originally named MFP1, is FR-β; the third, recently described by molecular techniques, is FR-γ. FR-β is about 70% identical to FR-α and maintains a GPI anchor. FR-γ is also about 70% identical to FR-α and is GPI anchored; however FR-γ' is truncated at the carboxy terminus and is a secreted protein. The function of FR-β and FR-γ are not known. Preliminary studies in receptor-negative CHO cells transfected with FR-β allowed us to show that membrane binding had no influence on the accumulation of 5-methyl[^{3}H]tetrahydrofolate. Moreover, despite the molecular detection of FR-β using polymerase chain reaction (PCR) technology in a number of different tissues, the total amount of message is very small compared to FR-α and there are no known cell lines that express FR-β naturally.

Recently, it has been suggested that the nomenclature for the whole family may be somewhat misleading. Da Costa and Rothenberg, noting that the molecules are so similar, suggested that tissue identification may be more useful *(53)*. Based on studies of soluble as well as membrane-bound protein in selected overexpressing cell lines as well as transfected cells, we believe that there is some merit to this classification. Moreover, it is our opinion, especially because plasma folate across species lines vary by more than 10-fold (e.g., man is 5–15 n*M* and mouse is 150–250 n*M*), that even the function of the receptors, which have differing affinities for 5-methyl-tetrahydrofolate, may be different. Regardless, the role and regulatory pathways for the receptor(s) need to be determined. For convenience, we will continue to use the currently accepted terminology as outlined in recent reviews *(19,40)* and, moreover, unless otherwise noted, the studies presented here are derived exclusively from studies of FR-α unless otherwise indicated. In addition, there are very little data on the fate/function of the soluble protein and our early studies showed that, at least in vitro, it did not deliver bound folate to some cells in a specific manner; therefore, it will not be reviewed here.

STUDIES OF FOLATE RECEPTOR-A-MEDIATED FOLATE UPTAKE

The model of potocytosis (Fig. 1) was developed using a near-confluent culture of MA104 cells, a monkey kidney cell line growing in physiological folate (i.e., folic acid free medium containing 2–10 n*M* reduced folate). Drugs or conditions that affect the process are listed under each step in Fig. 1. In addition, *N*-ethylmaleimide stops receptor cycling and the nonsteroidal anti-inflammatory agent indomethacin also alters folate receptor distribution and interferes with accumulation of folic acid. This latter effect appears significant only at a concentration of 400–500 m*M*, which is about 50–100 times greater than the clinically achieved plasma concentration. Although slowing down or inhibiting receptor-coupled uptake could be done with folate analogs, temperature, monensin, nigericin, and probenecid, until we used phorbol myristic acid (PMA), we had not found a way to increase folate uptake, alter folate receptor distribution, and charge the rate of cycling *(54)*. The mechanism by which PMA works (i.e., PKC activation), however, is not yet explained. Potent inhibitors of PKC such as staurosporine and bryostatin, although inhibiting the PMA-mediated increase in receptor cycling, did not inhibit the increased delivery of the 5-methyltetrahydrofolate, whereas trifluoperazine and chlorpromazine did. Most recently, we have shown that F-actin disrupting agents such as cytochalasin D reversibly externalized the folate receptor and increased 5-methyltetrahydrofolate deliv-

ery *(55)*. The cellular "analog" for this cytochalasin D effect has not yet been identified.

It is clear from the above brief description and published work that MA104 cells have served us well in describing the kinetics of a process that has led to further studies and the sudden increase in "spelunkers," as investigators concerned with caveolae have been called *(56–59)*. Knowing that FR-α was found in kidney proximal tubule, choroid plexus, and placenta led to the hypothesis that its function in a normal tissue or organ is to prevent loss of folate (i.e., urine) and to package and move folate into specific body compartments (e.g., spinal fluid folate is three to four times greater than plasma and fetal plasma is two to three times a matched maternal sample). We and others continue to study receptor function in a number of different cell types and under different conditions (log phase, near-confluent, and stationary phase of growth) in vitro. Thus far, we have found at least four variations of the original model for potocytosis, which may be, at least in part, cell-line dependent. These variations are discussed next. Moreover, because the function of FR-α may be to conserve and concentrate folate in specific compartments in vivo, as suggested earlier, it should be realized also that the best model may be a cell line that grows with tight junctions and polarizes in vitro!

SPECIFICS OF POTOCYTOSIS AS DESCRIBED IN MA104 CELLS

1. Was developed in near-confluent cells growing in physiological (1–10 n*M* reduced folate) rather than 2–5 μ*M* folic acid found in most standard tissue culture medium.
2. The receptor was in a 1:1 distribution between the external (acid labile [AL]) and internal, but membrane bound (acid resistant [AR]) pools and the pools exchanged about once an hour (Fig. 1).
3. Receptor-mediated uptake accounted for >90% of total cytoplasmic 5-methyl[^{3}H]tetrahydrofolate when assessed under physiological concentrations (1–10 n*M*).

Variation on a Theme I

In MA104 cells, we found that the accumulation of 5-methyl[^{3}H] tetrahydrofolate is markedly dependent on the rate of cell growth. The rate of folate accumulation in d 3 exponentially growing cells was three to four times greater than in the very confluent d 8–9 cells. Over this period, thymidine labeling also decreased by >80%. Interestingly, FR antigen, mRNA, and [^{3}H]-folic acid binding increased threefold to fivefold as the cells became confluent and we also noted that the external/internal ratio (i.e., AL/

AR), which was 2:1 on d 3, 1:1 on d 5, was 1:2 on d 8–9. Thus, there is an inverse relationship between cell replication and the amount of folate receptor on the membrane, and the receptor pool shifted from a predominantly external to a predominantly internal pool as the culture aged *(60)*. This report also showed that the activity of the enzyme folylpolyglutamate synthetase (FPGS), which adds the glutamate moiety in the unique γ-carboxy linkage, is coupled to cell replication, a result also found for HL-60 *(61)* cells during drug-induced differentiation and our studies of mitogen-stimulated peripheral blood lymphocytes *(62)*.

Variations on a Theme II

Not all receptors cycle between the external and internal compartment and, as a corollary, when folate accumulation was assessed under limiting extracellular folate (1–10 n*M*), the accumulation is related more to the cycling fraction than the apparently fixed external pool. To show these phenomena, we measured the uptake of 5-methyl[^{3}H]tetrahydrofolic acid in several malignant cell lines that had three to five times more receptor than MA104 cells. For example, IGROV–1 (ovarian carcinoma) cells bind 12 pmol [^{3}H]-folic acid/10^6 cells. However using an experimental design similar to that used to study MA104 cells, we concluded that all of the receptor does not participate in potocytosis. Of the 12 pmol of receptor, it appears that only 4 pmol cycle between the external and internal compartment. Uptake of 5-methyl[^{3}H]tetrahydrofolic acid was more directly proportional to the cycling fraction. Although the initial rate of ligand uptake is nearly 2 pmol/h/12 pmol binding, this is a lower efficiency of uptake/unit binding compared to MA104 cells (1 pmol/h/1pmol binding). Additional studies with other receptor-positive cell lines are in progress. In particular, Huennekens shared his L1210-JF cell line, which has upregulated [^{3}H]-folic acid binding *(63)*. Although there are 6 pmol of [^{3}H]-folic acid bound/10^6 cells, we found little acid-resistant [^{3}H]-folic acid and little cytoplasmic 5-methyl[^{3}H] tetrahydrofolic acid even after a 24-h incubation at physiological concentrations (1–20 n*M*) of folate. This was recently confirmed by others *(64)*. Moreover, these authors showed that the protein sequence was identical to another L1210 line selected in low-folate medium that also upregulated FR-α; however, this line (L1210-A) accumulated folate. Glycosylation did not affect function, but, interestingly, there was a difference in the GPI anchor as assessed by sensitivity of the protein to be released by PI-PLC and nitrous acid. Thus, heterogeneity in the anchor, most likely posttranscriptionally, appears to alter FR function. In this regard, we found that FR-negative CHO cells transfected with human cDNA for FR-α bound 25 pmol of folic acid/

10^6 cells, but uptake of folate was less than 1 pmol and we are in the process of analyzing the GPI anchor.

Variations on a Theme III

Even when present and cycling, FR was not required for cells to accumulate 5-methyl[^{3}H]tetrahydrofolate. We noted that blocking the receptor with folic acid (which has such a large affinity compared to 5-methyltetrahydrofolate that it is not released from FR or delivered to the cytoplasm) had little effect on the accumulation of the 5-methyl[^{3}H]tetrahydrofolate in exponentially growing cells. We also found that folic acid could block 85–90% of the binding of 5-methyl[^{3}H]tetrahydrofolate in a choriocarcinoma cell line (JAR), yet it only inhibited 10–15% of the cytoplasmic accumulation. Thus, FR is not needed for folate uptake in growing cells. It still remains a more likely possibility that FR function in normal cells is to move and/or package folate (i.e., FR may be a marker [hallmark] of differentiation) *(65)*.

In order to study the phyisological function of FR-α in vitro, it may be critical to study folate accumulation and transcellular movement in cells such as kidney proximal tubule cells that will polarize in vitro. It was not surprising that we found in our initial experiments that confluent OKP (opossum kidney proximal tubule cells) bound and accumulated folate (Fig. 2), but only exponentially growing, low-density cells synthesized polyglutamates (Fig. 3). These results are consistent with change in folate uptake in the MA104 cells noted earlier and support the notion that FR-α activity needs to be studied in confluent, differentiated, even polarized cells.

Variations on a Theme IV

The effects of modulators such as PMA, nonsteroidal anti-inflammatory agents, steroids, and retinoid are both cell lineage and cell density dependent. The effects of PMA on growing cells were different from the effects seen in confluent cells. Although the major observation (i.e., the shift of receptor to a primarily AL state) was even more dramatic in more confluent cells (d 5 shifts from AL/AR of 1:1 to about 2:1, whereas the d 8–9 cells shift from 0.5:1 to 2–2.2:1), we also noted that the receptor moved faster and the accumulation of 5-methyl[^{3}H]tetrahydrofolate was increased the same-fold (1.8 times) *(54)*. Although 4α-PMA and 4α-PDBu (analogs that do not activate PKC) were without effect and the PDBu effect was reversible with time, we are entertaining the possibility that the effect of PMA is not PKC mediated because a number of inhibitors have no effect *(54)*. Recent publications suggest that PMA has non-PKC-binding sites *(66)* and show that phospholipase D is activated by the regulatory domain of PKC but is

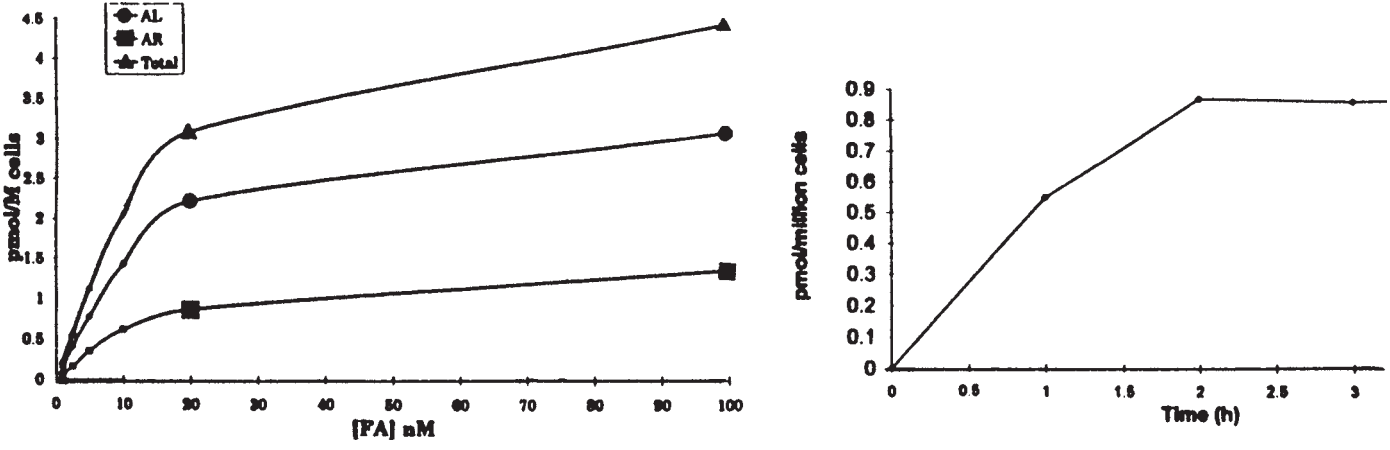

Fig. 2. [^{3}H]-Folic acid binding and 5-methyl[^{3}H]tetrahydrofolate uptake by confluent OKP cells.

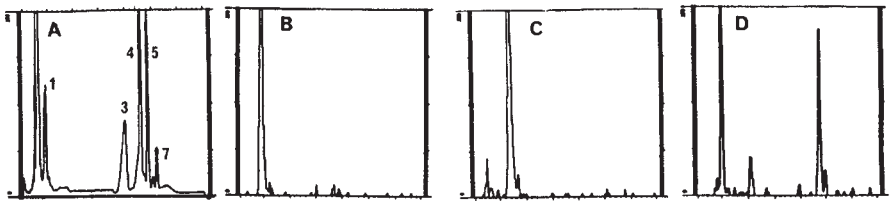

Fig. 3. Folylpolyglutamate profile. Panel **A** is a tracing of a high-performance liquid chromatogram of folate polyglutamate standards. Numbers are flutamate residues (e.g., 1 is mono, 3 is tri glutamate derivative). Panels **B** and **C** are ring-labeled and methyl-labeled 5-methyltetrahydrofolate accumulated in the cytoplasm of confluent OKP cells. Panel **D** is 5-methyl[^{3}H] tetrahydrofolate accumulated in only a 1-h pulse of d 1 (exponentially growing OKP cells). Note the marked amount of pentaglutamate synthesized compared to the absence of polyglutamates in panels B and C.

independent of the kinase activity *(67)*. Experiments to identify possible mechanisms for and to more completely determine the specificity of this PMA effect have been recently presented and it appears that although the receptor is modulated by PMA, it may not be PKC mediated *(54)*.

With respect to differences in observed effects of the agents noted earlier between cell lines and between cells in log phase vs stationary phase growth, it should also be noted that the FR gene has been mapped to chromosome region 11q13.3 near cyclin D (PRAD1) and FGF3, FGF4, and the MEN loci *(68)*. This region is often amplified in carcinoma of the breast and the head and neck region *(69)*. In collaboration with Dr. Tom Carey (University of Michigan), Dr. Rebecca Orr, a former graduate student in the laboratory noted that SCC38, a cell-line hexaploid at the 11q13.3 locus, synthesizes less FR than MA104 cells as assessed by immunoblotting. Moreover, the uptake of 5-methyl[^{3}H]tetrahydrofolic acid by these cells in vitro did not occur via potocytosis and a membrane preparation from these cells did not bind [^{3}H]-folic acid. The effects of steroids and retinoids and the sequencing of three FR-α mutations, one which had a dominant negative effect on normal FR-α, have been published *(70,71)*. Because the polyploidy is partly based on an 11p–11q inversion, it is possible that some of the effects are the result of gene rearrangement and/or loss of heterozygosity associated with 11q.

IN VIVO AND CLINICAL CORRELATES ("ONCOLOGICAL TANGENTS")

During the tenure of and even prior to the basic experiments of folate receptor function, we have studied the effects of antifolate therapy on folate

stores in mice, rats, monkeys, and humans. This data have been reported over the past decade *(72–74)*. In brief, repetitive, low-dose methotrexate results in a folate deficiency in the liver, red cells, and, of interest for this symposium, the brain. In particular, 1 yr of a "typical" methotrexate dose and schedule for treatment of children with acute lymphoblastic leukemia resulted in a 90% loss of folate in the brain of subhuman primates *(73)*. Rats also showed a decrease in brain folate after only weeks of antifolate therapy, whether or not cranial radiation was given *(72)*. Humans developed folate deficiency in liver and red cells as determined by biopsy and routine blood sampling *(74)*. Concomitantly with our studies, the work of others *(75)* has revealed that therapy with methotrexate causes at least a transient increase in plasma homocysteine and we have recently found a striking increase (threefold to fivefold) in the amount of homocysteine in the CSF of children taking methotrexate, even with leucovorin (5-formyltetrahydrofolic acid) rescue as part of a planned treatment for acute lymphoblastic leukemia *(4)*.

Because it is hypothesized that the folate receptor serves to maintain the CSF folate three to five times that of plasma, abnormalities in its synthesis or function may further compromise folate in the central nervous system (CNS), especially when antifolates are being taken. There is already a paradigm for abnormal synthesis: paroxysmal nocturnal hemoglobinuria (PNH) is an X-linked disease of the bone marrow in which GPI-anchored proteins are not synthesized. Consider the effects of blocking synthesis of or inhibiting the function of FR-α on the choroid plexus. Recently, a 60-yr-old woman presented to a neurologist with somnolence, ataxia, and signs of dementia. Routine studies revealed no diagnosis. She was found to have a normal plasma folate and homocysteine, but a markedly decreased CSF folate and elevated homocysteine. Treatment with folate and dextromethorphan, the latter a noncompetitive antagonist of the NMDA receptor triggered by homocysteine resulted in a neurological recovery. We postulated a failure to concentrate folate in the CSF as the basis for her physical findings. The cause remains unknown, but this case serves as an example that compartmentation of folate is important. Studies of the homozygous and heterozygous FR-α transgenic mice should yield a better understanding of folate homeostasis in the CSF *(76)*.

CONCLUSION

Like many observations in science, from the identification of a molecule, to deciphering its regulation, function of and impact of malfunctioning or absent often takes many years. The folate-binding protein in milk was iden-

tified in the late 1960s; a cellular form was identified in the early 1970s, finally placing it on the membrane in the late 1970s, and the "molecular" explosion with sequences, cloning, and expression in the late 1980s. The 1990s saw studies of genetically altered cells (transfectants) and studies of the distribution of the receptor in normal and malignant tissues. The availability of genetically engineered animals (1999) may herald a new phase of experiments that will result in the elucidation of function, regulation, and medical impact of an aberrant, absent, or overexpressed folate receptor, one of the few GPI-anchored proteins. We suggest that abnormal FR-α will be at least associated with CNS disease and birth defects.

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7

Folate, Homocysteine, and Neural Tube Defects

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NEURAL TUBE DEFECTS: DISABLING MALFORMATIONS

Neural tube defects (NTD) are one of the most disabling congenital defects in the human. NTD applies to malformation of the brain and/or spinal cord. The process of the formation of the nervous system is called neurulation. During neurulation, the flat neural plate transforms into a cylindrical neural tube with canalization and rostral and caudal closure. When the closure does not occur at the rostral end, the brain does not develop. This defect is clinically recognized as anencephaly.

When caudal closure does not occur, spina bifida will be the end result. Spina bifida can result in paralysis of the legs, the bladder, the rectum, and hydrocephalus.

This closure process of the neural tube occurs very early in human pregnancy: from d 21 until d 28 after conception or 1 wk after the expected menstruation. This illustrates the fact that any measure of prevention has to start before conception.

NTD AS A RESULT OF GENETIC AND ENVIRONMENTAL FACTORS

The birth prevalence of NTD depends on the country and socioeconomic and ethnic groups. The numbers range from 1:2500 in Finland, 1:300 in Mexico, to 1:80 in South Wales.

Neural tube formation as well as NTD are guided or caused by a multifactorial process, involving both genetic and environmental factors. Recognized genetic factors are sex differences, ethnic differences, consanguinity, increased rate of concordance in monozygotic twins, increased prevalence in siblings and in children of affected patients, single-gene mutation and chromosomal abnormalities. Suggested environmental influences are nutri-

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tion, diabetes, hyperthermia, teratogens like aminopterin, thalidomide, valproic acid, and other antiepileptics, alcohol, or profession.

NTD AND NUTRITION

Numerous reports have suggested that nutritional deficiencies in general would cause adverse birth outcomes. As an example, a Dutch midwife found an increase in NTD in 1722 and 1732, 2 yr that were linked with poor crops. She also noted that the children with NTD came from the poorest homes in urban areas *(1)*. A similar observation was made in the children who were exposed *in utero* to severe food shortage during the Second World War in Holland. In addition to a significant decrease in birth weight, there was also a significant increase in the rate of NTD *(2)*.

NTD AND FOLATES

The possibility that folate was specifically linked to NTD in humans was first reported by Hibbard *(3)*. Using the FIGLU test, he observed that women who had pregnancies associated with fetal malformations had a higher incidence of aberrant folate metabolism. Hibbard and Smithells *(4)* subsequently repeated this finding. Also, a key role of folate in preventing NTD was suggested.

Folate status is now assessed by the determination of folate in serum/plasma and red blood cells. Folate can be determined with a microbiological assay with an interassay and intraassay variation of less than 5% (O'Brien and Keller 1992). In more recent reports, the radioassay technique is used with a variation coefficient of less than 10%. The concentration of folates in red blood cells is considered to represent the body storage over the last 7 wk, especially in the liver (Herbert 1990). In most studies, the method of determination of folate and the intraassay and interassay variation coefficient is not mentioned.

It is striking that in all studies in pregnant women who received no vitamin supplementation, a strong decrease of maternal folate serum or plasma levels during the course of pregnancy were reported. Therefore, studies on folate status in women in relation to NTD have to take into account the existence of pregnancy or not.

Studies on blood folate and red cell folate levels in nonpregnant women with NTD offspring did not show significant differences with controls *(5–8)*. This implies that folate values are not predictive for NTD.

Maternal folic acid levels studies in the first trimester of pregnancy in mothers with NTD offspring tended to be lower than in controls (Tables 1

Table 1
Maternal Blood Folate Levels in Mothers with NTD Offspring and Controls in the Pregnant and Nonpregnant State

Population	Folate conc.	Method	Results	Remarks	Ref.
		Nonpregnant Population			
44 NTD	16.2 nmol/L	Radioassay	NS	Nonpregnant	*(5)*
18 Controls	13.5 nmol/L	v.c.:<5–10%		mean values	
41 NTD	14 nmol/L	Radioassay	NS	Nonpregnant	*(6)*
50 Controls	12 nmol/L	v.c:<5–10%		mean values	
20 NTD	3.8 ng/L?	Radioactive	?	Nonpregnant	*(7)*
46 Controls	4.9	binding assay		median values	
47 NTD pat.	10.0 nmol/L	Radioassay	0.00001	Nonpregnant	*(9)*
60 NTD	12.5 nmol/L	v.c:<5–10%	NS	median values	
51 Fathers	12.0 nmol/L		0.01		
94 Controls	14.0 nmol/L		—		
12 NTD	11 nmol/L	Immunoassay	NS	Nonpregnant	*(8)*
15 Controls	12 nmol/L	v.c.:<6–10%		median values	
		Pregnant Population			
5 NTD	4.9 ng/mL	Microbiological	NS	First-trimester	*(10)*
953 Controls	6.3 ng/mL	assay		mean values	
11 NTD	6.3 ng/mL	Not mentioned	NS	First-trimester	*(11)*
>1000 Controls	6.7 ng/mL			mean values	
32 NTD	3.4 ng/mL	Microbiological	NS	First-trimester	*(12)*
384 Controls	3.4 ng/L	assay		mean values	
89 NTD	4.1 ng/mL	Microbiological	NS	First-trimester	*(13)*
172 Controls	4.2, 4.3 ng/mL	assay		mean values	
81 NTD	3.5 µg/L?	Microbiological	0.002	First-trimester	*(14)*
247 Controls	4.6 µg/L	assay		median values	
81 NTD	3.5 µg/L?	Microbiological	0.004	Pregnancy mean	*(15)*
323 Controls	4.5 µg/L	assay		values	
31 NTD	6.1 ng/L?	Radioimmuno-	NS	First-trimester	*(16)*
63 Controls	7.1	assay		median values	
16 NTD	4.3 ng/mL	Radioactive	NS	First-trimester	*(7)*
36 Controls	4.4, 5.7 ng/mL	binding assay		median values	

Note: Results are expressed in *p*-values.

and 2). Statistical significancy, however, was only reached in two out of eight studies .

Two studies (Table 1) did show significant lower serum folate values in women with NTD offspring *(14,15)*. These studies were performed in an Irish population known to have a relatively high prevalence of NTD.

Table 2
Red Cell Folate Levels in Mothers with NTD Offspring and Controls in the Pregnant and Nonpregnant State

Population	Folate conc.	Method	Results	Remarks	Ref.
		Nonpregnant Population			
44 NTD	519 nmol/L	Radioassay	NS	Nonpregnant	*(5)*
18 Controls	823 nmol/L	v.c.:<5–10%		mean values	
41 NTD	558 nmol/L	Radioassay	NS	Nonpregnant	*(6)*
50 Controls	515 nmol/L	v.c:<5–10%		mean values	
17 NTD	163 ng/mL	Radioactive	NS	Nonpregnant	*(7)*
35 Controls	159 ng/mL	binding assay		median values	
47 NTD pat.	470 nmol/L	Radioassay	NS	Nonpregnant	*(9)*
57 Mothers	540 nmol/L	v.c:<5–10%	NS		
49 Fathers	520 nmol/L		NS		
72 Controls	520 nmol/L				
		Pregnant Population			
6 NTD	141 ng/mL	Microbiological	0.001	First-trimester	*(10)*
959 Controls	228 ng/mL	assay		mean values	
178 NTD	178 ng/mL	Compet.	0.005	First-trimester	*(11)*
286 Controls	268 ng/mL	Protein-binding assay		mean values	
81 NTD	269 ng/mL	Microbiological	0.001	First-trimester	*(14)*
247 Controls	338 ng/mL	assay		mean values	
84 NTD		Microbiological		Dose-response	*(18)*
266 Controls		assay		relationship	
14 NTD	156 ng/mL	Radioactive	NS	First-trimester	*(7)*
26 Controls	162 ng/mL	binding assay			
31 NTD	801 ng/mL	Radioimmuno-	NS	First-trimester	*(16)*
63 Controls	921 ng/mL	assay v.c.: 10.6–12.8%		median values	

Note: Results expressed in *p*-values.

Using red cell folate levels (Table 2), three studies showed lower values in the first trimester of pregnancy than controls. Daly et al.. *(18)* found a dose-response relationship with NTD. No significant differences were found in the nonpregnant state

Studies performed in the postpartum period did not showing differences between groups *(17)*. Wald *(20)* analyzed the folate status of women within the MRC study *(19)*. Whole blood and red cell folate were found to be

highly correlated ($r = 0.99$). Pregnancies with NTD tended to have low blood folate levels, particularly in the first trimester of pregnancy. This is consistent with the knowledge that the lack of dietary folate is one of the causes of NTD.

There was a significant trend in the proportions of women who had two or even three previous NTD pregnancies according to the quartile of whole blood folate: 5.6%, 2.5%, 3.4%, and 1.8%, respectively ($p = 0.03$). This was not significant for serum folic acid.

It has to be noted that the range of folate values in a population is relatively narrow. This has been described as the "narrow window" effect *(20)*. Much of the variation in risk reflects the background genetic predisposition rather than folic acid differences.

Observational and Intervention Studies with Folic Acid/ Folate on NTD Risk

The observational studies on the effect of periconceptional nutritional folate and/or folic acid supplementation on the risk of NTD are summarized in Table 3. Three observational studies demonstrated a relationship between the intake of folate and a reduction of NTD *(23,26,27)*. Five studies using periconceptional multivitamins containing folic acid reduced the risk of NTD. Only the study of Mills et al. *(24)* reported no reduction of NTD. It has to be emphasized, however, that this study was performed in a low-NTD-prevalence area.

The results of the observational studies can be judged as level II evidence in regard to clinical efficacy. Two population-based case-control studies (Atlanta 1968–1980 and California 1989–1991) demonstrated a substantial risk reduction associated with periconceptional multivitamin use and neural tube defects with multiple other birth defects *(28)* with an odds ratio (OR) of 0.36 and a 95% confidence interval (CI) of 0.18–0.72.

Six intervention studies on the effect of periconceptional supplemental folic acid intake on the risk of NTD are reported (Table 4).

Credit has to be given to the first attempt to perform a randomized trial by Laurence et al. *(29)* and the pioneering work of Smithells et al. *(30)* in Leeds. The protocol of the Leeds group containing a placebo double-blind controlled study was rejected by three hospital research ethics committees. This led to a less satisfactory design *(33)*.

The best study organized by the Medical Research Council *(19)* was a multicenter, double-blind, randomized trial. This trial fulfilled all the criteria for a randomized investigation. Daily supplementation with folic acid

Table 3
Observational Studies on the Effect of Periconceptional Nutrional Folate and/or Folic Acid Supplementation on the Risk of NTD (Level of Evidence II)

Population	Exposure	Methods	Results	Remarks	Ref.
764 NTD 764 Controls (UK)	Multivitamins folic acid dose??	Through prescription	OR<1	Records complete?	*(21)*
347 NTD 2829 Controls (USA–Atlanta)	Multivitamins with folic acid up to 800 µg/d	Interview	OR: 0.40 95% CI: 0.26–0.66	Recall up to 16 yr	*(22)*
75 NTD 150 Controls (Australia)	Diet+vitamins; diet: total (20–1787 µg/d) and free folate (8–1678 µg/d)	Questionnaire diet recall	OR: 0.7 95% CI: 0.27–182	Recall up to 25 mo	*(23)*
571 NTD 573 Controls (USA)	Vitamins and/or diet folic acid up to 800 µg/d	Interview	NS	Low-prevalence area	*(24)*
22,776 (Women)	Vitamins: users vs nonusers folic acid: 100–1000 µg/d	Prenatal diagnosis and and interviews	OR: 0.29 95% CI: 0.15–0.55	Recall up to 15 mo	*(25)*
456 NTD	Vitamins with dietary folate	Interview	OR: 0.4 95% CI: 0.2–0.6	Recall up to 20 mo	*(26)*
2615 Controls Hospital-based study	31–2195 µg/d	Food frequency questionnaire	OR: 0.6 95% CI: 0.4–1.1	Exclusion when folic acid was known	
549 NTD 540 Controls	Vitamins and folic acid 0.4–0.8 mg/d; dietary folate 10–1660 µg/d	Interview Folate intake estimation	OR: 0.39 95% CI: 0.20–0.70	Recall up to 18 mo	*(27)*

Results are expressed as odds ratio (OR) with confidence interval (CI).

Table 4
Intervention Studies on the Effect of Periconceptional Folic Acid Intake on the Risk of Neural Tube Defects (Level I Evidence, P-Values or Relative Risk)

Population	Exposure	Methods	Results	Remarks	Ref.
11 NTD (Wales, UK)	Folic acid 4000 µg/d and placebo	Randomization double blind	0.05	Small study; statistical transfer of noncompliers	*(29)*
973 NTD (UK)	Multivitamin with folic acid 360 µg/d	Non-randomized	RR = 0.14 0.05	Group differences	*(30)*
195 NTD (Cuba)	Multivitamin with folic acid 5000 µg/d	Non-randomized	0.05	NTD not defined	*(31)*
1195 NTD (Multicentered)	Folic acid 4000 µg/d	Randomized double blind	RR = 0.12–0.716	Well designed	*(19)*
261 NTD (Ireland)	Multivitamins with folic acid 4000 µg/d	Randomized double blind	NS	Numbers too small	*(14)*
4156 Women (Hungary)	Supplements with folic acid 800 µg/d	Randomized double blind	0.05	Occurrence study	*(32)*

(4 mg/d), folic acid (4 mg/d) with other vitamins, other vitamins without folic acid, and placebo, led to a strong reduction of the NTD recurrence rate in the folic acid groups. The rationale for the choice of such a high dose of folic acid was to avoid the risk of an ineffective low dose and the impossibility of repeating such a long-lasting and costly study. The trial was closed prematurely because of the reached significance before the calculated numbers were to be reached.

The Hungarian randomized trial *(32)* also provided evidence that the occurrence of NTD could be prevented. Subjects took a daily multivitamin containing 800 µg folic acid per day. The control group used a placebo containing trace elements. It could not be disclosed if the significant result was the result of folic acid alone or to folic acid embedded within the multivitamin preparation.

One has to realize that the MRC and the Hungarian study were performed in a rather high-NTD-prevalence area. The results of the intervention stud-

ies can be judged as level I evidence in regard to the need for clinical application.

The rather high dose of folic acid used in the MRC study was probably chosen to avoid a negative answer in a large-scale and costly study. Almost all studies were performed with folic acid doses below 1.0 mg. A randomized study from Denmark also showed that there was no difference in outcome between 1.0 and 2.5 mg of folic acid per day *(34)*.

A large-scale intervention study on the prevention of neural tube defects was carried out in China *(35)*. In the northern region of China, having high rates of neural tube defects, and in the southern region, having low rates, women were asked to take 0.4 mg of folic acid per day in the periconceptional period. When folic acid was taken, the rates of neural tube defects decreased to 1.0 per 1000 in the northern region and 0.6 per 1000 in the Southern region. The intake of folic acid reduced the risk of neural tube defects in areas with high rates of these defects as well as in areas of low rates.

To understand the role of folic acid, we have to discuss homocysteine metabolism.

HOMOCYSTEINE AND NTD

Homocysteine is a sulfur-containing amino acid, formed at demethylation of the essential amino acid methionine. Three B vitamins are involved in homocysteine metabolism: the biological active form of vitamin B_6 as a cofactor for the enzyme cystathionine β synthase, vitamin B_{12} as a cofactor for methionine synthase, and 5-methyltetrahydrofolate (methyl-THF) as a substrate. The formation of methyltetrahydrofolate is catalyzed by methylene tetrahydrofolate reductase (MTHFR), a vitamin B_2-dependent enzyme *(36)*. Homocysteine metabolism (and therefore also folates) plays an important role in the so-called "one-carbon metabolism" providing methyl groups to all kinds of substance, including the synthesis and regulation of DNA and mRNA, which are essential also for embryonic cells.

Derangement of Homocysteine Metabolism Is the Possible Basis for NTD

Steegers-Theunissen et al. *(37)* were the first to report on a possible derangement of folate-dependent homocysteine metabolism in women who had NTD offspring (OR = 6.8; 95% CI = 1.2 – 48.7). This finding was extended and confirmed *(6,9,15)*. Van der Put et al. *(9)* demonstrated that the risk of spina bifida was increased at the 75th percentile of homocysteine

values and at the 5th and 25th percentile of vitamin B_{12} in mothers with spina-bifida affected offspring.

Mills et al. *(38)* found that mothers of children with a neural tube defect had higher homocysteine values than did vitamin B_{12} controls. The difference was highly significant ($p = 0.004$) in the lower half of the vitamin B_{12} distribution after adjusting for plasma folate. No methylmalonate levels above the normal range were observed, indicating that the vitamin B_{12}-dependent methylmalonyl–coA mutase functioned normally in both groups. Because an elevated plasma methylmalonate concentration indicates vitamin B_{12} deficiency, the authors concluded that the vitamin B_{12} status of both groups was normal. This indicates that although the vitamin B_{12} status of the cases was normal, they, nevertheless, had more difficulty in metabolizing homocysteine compared with control subjects with comparable vitamin B_{12} levels. Therefore, the authors suggested adding vitamin B_{12} to fortified food, thereby reducing the dose of folic acid required.

When folate intake is inadequate, as demonstrated in controlled studies, plasma homocysteine concentrations will rise. Plasma homocysteine concentrations are inversely correlated with red cell and serum folate concentrations *(39)* and is a sensitive indicator of the folate status.

Folic acids supplements are clearly capable of lowering plasma homocysteine concentrations, as demonstrated in meta-analysis of randomized controlled trials *(40,41)* and extensively reviewed *(42)*. These studies were performed in cardiovascular patients, volunteers, and elderly people.

It is interesting to note that the use of folate or folic acid in healthy volunteers is also followed by a decrease in plasma homocysteine concentrations *(8,43–45)*.

Dietary intake patterns also relate to plasma folate and homocysteine concentrations, as demonstrated in the Framingham study *(46)*.

The Derangement of Homocysteine Metabolism in Mothers with NTD Offspring Is Based on Gene Mutations of the MTHFR Enzyme

When it became clear that a derangement of homocysteine metabolism was one of the possibilities for the pathogenesis of NTD, research efforts focused on possible enzyme deficiencies and underlying polymorphisms in the metabolic methionine–homocysteine chain in which folic acid and vitamin B_{12} play an essential role. Kang et al. *(47)* had already found that thermolability of the MTHFR enzyme could be a risk factor for coronary heart disease.

One common polymorphism (C677T) was reported *(48)*. A change of alanine to valine at the 225th amino acid of MTHFR decreased the activity

of this enzyme by 35% *(49)*. The mutation predisposes to mild hyperhomocysteinemia in the presence of a low folate status *(50,51)*. The distribution of the MTHFR genotypes among the population is in Hardy-Weinberg equilibrium.

A Shortage of MTHFR in Families with NTD Resulting from a Genetic Mutation

Van der Put et al. *(52)* found a shortage of MTHFR as a result of the C677T mutation located on chromosome 1 in Dutch families with NTD. The gene mutation was found in 16% of mothers, 10% of fathers, 13% of children with spina bifida, and in 5% of controls. This finding was confirmed in the mothers of Irish patients with NTD *(53,54)*, Irish families *(55)*, and Canadian families *(56)*.

The study of Van der Put et al. *(57)* pulled three pieces of the etiologic jigsaw together: first, the proven protective effect of periconceptional folic acid supplementation; second, the familial clustering; third, the increased risk of neural tube defects in the presence of elevated maternal homocysteine levels.

The B vitamin status of these women was not deficient, although folate levels were in the lower range of the control values *(9,14,52,57)*. The altered folate and vitamin B_{12} metabolism in families with spina bifida offspring *(9)* is due to decreased MTHFR activity caused by the C677T mutation *(57)*.

Meta-analysis of studies on the MTHFR gene mutation resulted in an odds ratio of 1.7 (95% CI: 1.2–2.6) for spina bifida patients, 1.8 (95% CI: 1.1–3.1%) for mothers, and 1.9 (95% CI: 1.3–2.8%) for fathers. International controls were used for this calculation *(58)*.

The risk of NTD was mostly associated with sporadic rather than inherited spina bifida offspring *(57)*. The C677T mutation was associated with a sevenfold increased risk if both the mother and the child were homozygous for this mutation. This finding suggested that a defective homocysteine metabolism might also be present in the NTD patients themselves. Studies from France *(59)*, China *(60)*, Germany *(61)*, North America *(62)*, South Africa *(63)*, and Turkey *(64)*, failed to demonstrate an association between the TT mutation and an increased risk of NTD. This "controversy" stresses the need for the study of variation of dietary sources of folate, which modulate the C677T mutation.

The Prevalence of the Genetic Mutation C677T Varies in Different Ethnic Groups

The prevalence of the C677T mutation varies in different ethnic groups *(58,65–67)* and varies between 2.3% and 16.6%: in Italians, 16.3%; in

French Canadians, 12.0%; in British controls, 12.0%; in white Australians, 10.7%; in Utah Mormons, 9.0%; in Dutch controls, 8.4%; in Irish, 6.1%; in Japanese, 2.0%. This finding might explain the geographical and racial differences in the risk for NTD. A meta-analysis *(67)* concluded that the corresponding attributable fraction of the C677T mutation was 10.8%. Thus, other genes involved in folate–homocysteine metabolism and their interaction should be investigated. The C677T mutation is associated with an elevated homocysteine plasma level and a low folate status.

More Mutations Found and They Interact!

Recently, another mutation in the MTHFR gene was found: A1298C *(68,69)*. This mutation reduces the MTHFR activity to a lesser extent than the C677T mutation. The pathology risk for neural tube defects for the C677T and A1298C mutations together had an odds ratio of 2.4 (95% CI: 1.1 – 5.5). The combined heterozygosity for either the C677T or the A1298C mutation or the combined heterozygosity was associated with reduced MTHFR specific activity *(57)*, higher plasma homocysteine levels, and decreased plasma folate levels. This combined heterozygosity was observed in 28% of 86 NTD patients and in 20% of 403 controls. Both mutations can explain 35–50% of the clinical effect of folic acid *(70)*. Folate status and folic acid supplementation do modulate plasma homocysteine concentrations also when the C677T mutation is present *(71)*.

Homozygotes have elevated homocysteine and have an enhanced response to the homocysteine lowering effect of folates *(72)*. Research on other mutations or enzymes are so far negative, at least for methionine synthase *(73)* and cystathionine β-synthase *(74)*.

It can be concluded that the common MTHFR C-T mutation occurs in about 10% of the white populations, conferring an enhanced risk of NTD *(75)*. In a search for other candidate genes, Morrison et al. *(76)* found that a Gly919MS variant occurred more frequently in combination with the MTHFR thermolabile variant in mothers of NTD offspring.

A Substantial Minority of People May Have Increased Folate Needs

Five to 15% of general populations are homozygous for the thermolabile variant of the thermolabile variant of 5,10-MTHFR (C677T), which causes hyperhomocysteinemia. The C677T mutation causes an increased red cell folate and low plasma folate, mainly present as 5-methyltetrahydrofolate (THF), in these homozygous individuals *(70)*. Only 5-methyl-THF can be transported across the plasma membranes of cells; all other forms of folate

remain in the cell. This explains the raised folate concentrations in red cells. Because of a shortage of 5-methyl-THF, homocysteine cannot be remethylated to methionine, explaining the raised homocysteine values. These results suggest that a substantial minority of people may have increased folate needs *(77)*.

There is a close relationship among the common C677T mutation, low folate status, and hyperhomocysteinemia. When folate status is normalized, plasma homocysteine concentrations decrease and the adverse effects of the mutation are hidden *(9,71,72,78)*.

The C677T Mutation and Hyperhomocysteinemia Are Possibly Also Involved in Some Birth Defects Other than NTD

There is startling evidence that folic acid, homocysteine, and the MTHFR mutation are involved in the pathogenesis and/or prevention of congenital heart disease *(79)* and schisis *(80)*. In cases of early pregnancy loss, next to hyperhomocysteinemia *(81–83)* the missense mutation C677T increases the risk threefold *(71,84)*. An interesting observation is an abnormal folate metabolism and a mutation in the MTHFR gene in mothers of children with Down syndrome *(85,86)*.

Homocysteine and the Embryo

To understand the role of homocysteine and folic acid in the pathogenesis of NTD, almost all evidence comes from animal studies. The method of whole-embryo culture has been extensively studied in analyzing the mechanisms of neural tube closure *(87)*. A complex panorama of cellular and molecular mechanisms is now unfolding.

Methionine can prevent cranial NTD in rat embryos cultured in cow or human serum *(88)*. Homocysteine can disturb embryonic development or prevent defects, depending on the milligram or microgram doses. It seems likely that homocysteine reduces the *S*-adenosylmethionine (SAM)/*S*-adenosylhomocysteine (SAH) ratio thereby inhibiting the methyl-group donation necessary for the methyl-group reaction *(89)*.

Methylation of contractile proteins in the cells of the neural epithelium is an important step in the closing process of the neural tube *(90)*. Because the enzyme cystathionine-β-synthase only appears when the fetal liver is present, the homocysteine moiety is conserved in the embryonic homocysteine–methionine cycle. Methionine synthase and *S*-adenosylhomocysteine hydrolase are present in all embryonic tissues throughout the neurulating period *(91)*.

The studies of Rosenquist et al. *(92)* in avian embryos in vitro support the direct role of homocysteine and folic acid in the closure mechanism of the neural tube. Homocysteine acts as an agonist at the *N*-methyl-D-aspartate receptor and as a coagonist at the glycine site.

The studies of Zhao et al. *(93)* suggest that the mutant Cart1 mouse, which causes abnormal apoptosis in the forebrain and subsequent absence of forebrain mesenchyme cells, is a homolog of one of the human genes involved in the development of NTD. It is noteworthy that prenatal treatment with folic acid of these knockout mouse embryos suppresses the development of NTD.

Other studies report evidence that many folate-resistant NTD can be prevented by the supplementation of the complex B vitamin myoinositol, at least in the curly tail mouse *(94)*. Previously, these authors showed that the neural tube defect in curly tail mutants resulted from a cell proliferation defect in the hindgut endoderm that is causally related to downregulation of retinoic acid receptor-β expression.

Antifolate drugs like methotrexate, trimethoprim, and aminopterin act as potent inhibitors of dihydrofolate reductase. Among the anticonvulsants valproic acid causes a 5- to 20-fold increase of NTD. In a murine model, valproic acid decreases the embryonic levels of formyltetrahydrofolate *(95)* and also induces methionine deficiency *(96)*.

Homeobox genes and paired box (Pax) genes dictate the development of the central nervous system. Several intrinsic and extrinsic factors that can disturb essential cellular events have to be elucidated to discover and finally prevent potential teratogens.

Homocysteine and the Trophoblast

Habibzadeh et al. *(97)* showed that the methylgroup of 5-methyltetrahydrofolate is more slowly incorporated into the trophoblast DNA of NTD-affected fetuses than from controls. This indicates a disturbance in the uptake or utilization of 5-methyltetrahydrofolate and therefore in the pathway of folate metabolism.

Using umbilical cord blood samples and full thickness biopsies of the placenta, Daly et al. *(98)* demonstrated that there was a reduced enzyme activity in placental tissue associated with the C677T variant of MTHFR. This indicates that an important step in folic acid metabolism is also deficient in the human placenta and might explain why this variant is associated with NTD.

HOMOCYSTEINE AND THE FETUS

Amniotic fluid is the mirror image of the composition of the early embryo because of the transparent and totally permeable skin. When pregnancy advances, the amniotic pool reflects the fetus' own composition derived from the mother and the placental membranes.

Steegers-Theunissen et al. *(99)* found elevated homocysteine levels in amniotic fluid of NTD fetuses (n = 27) compared to controls (n = 31). The gestational age of both groups differed: 22 versus 16 wk median. The total homocysteine concentration in amniotic fluid of both groups were significantly lower in the amniotic fluid than in plasma (a factor of 3–4), as was demonstrated previously by Kang et al. *(100)* in normal pregnancies. The mean concentration of folate, vitamin B_{12}, and vitamin B_6 were not significantly different between groups. Serum vitamin B_{12} concentrations were comparable in the NTD group and control women. In both groups, vitamin concentrations in amniotic fluid were significantly higher (factor of 2) than those in serum. This suggests an active transplacental transfer of this vitamin against a concentration gradient.

Lower vitamin B_{12} levels in the amniotic fluid of NTD fetuses *(101,102)* or normal pregnancies in women who had previously had an infant with a NTD have been determined in relation to increased transcobalamin levels *(103,104)*.

Dawson et al. *(105)* found a 60% decrease of amniotic fluid vitamin B_{12} and folate levels between 15 and 20 wk gestation associated with an increase in amniotic fluid volume. The vitamin B_{12} and folate levels of amniotic fluid of NTD fetuses were below the range of unaffected pregnancies. Wenstrom et al. *(106)* demonstrated that high amniotic fluid homocysteine levels, heterozygosity or homozygosity of 5,10-MTHFR mutations were associated with defects of the cervical lumbar spine and occipital encephalocele. Anencephaly, exencephaly, and sacral defects did not seem to be related to altered homocysteine metabolism.

HOMOCYSTEINE AND THE CHILD

Disturbance of the Folate-Dependent Homocysteine Metabolism in Children with NTD Is the Cause of the Disease

In children with spina bifida, folate and homocysteine metabolism is disturbed *(70)*. A strong correlation was found between plasma homocysteine and B vitamins with age in 70 spina bifida children and 185 controls. Adjustment for age was performed. Homocysteine levels above the 95th percentile showed an odds ratio of 6.4 (95% CI: 2.7–15.2) and for the 5th

percentile of plasma folate, an odds ration of 6.0 (95% CI: 2.5–14.5), demonstrating a highly increased risk for NTD. Adjustment for plasma folate resulted in a folate-independent odds ratio of 4.1 (95% CI: 1.6–10.5) on having NTD. This indicates that elevated homocysteine and decreased plasma folate are partly independent risk factors for NTD. Vitamin B_6 levels were decreased in NTD patients with elevated homocysteine levels. This might indicate an impaired cystathionine β-synthase activity. In addition, plasma folate levels separate of homocysteine decreased in NTD patients when compared to controls. This might imply that folate metabolism not related to homocysteine is impaired in these patients.

The determined etiologic fractions of homocysteine above the 95th percentile and plasma folate levels below the 5th percentile are 30.7% and 25.7, respectively. The etiologic fraction of elevated homocysteine levels that are not associated with plasma folate was 9.6%.

No indication was found for a major role of methionine synthase or cystathionine β-synthase *(73,74)* or MTHFD in the etiology of NTD *(107)*.

This major role for homocysteine and folate in the etiology of NTD was not found by Graf et al. *(108)* and Bjørke-Monsen et al. *(109)*. These studies were small, however, and were not adjusted for age, a crucial factor in judging homocysteine plasma values. No polymorphism was found for folate receptors in patients with spina bifida *(110)*.

Altered Distribution of Folates in Red Blood Cells in Individuals Homozygous for the C677T Mutation

Red cell folate is an indicator for the stored folate derivates in the cell. Some studies have reported decreased red cell folate levels in mothers with NTD offspring, indicating a possible nutritional folate deficiency in these individuals *(15,111)*.

Van der Put et al. *(52)* found an increased red cell folate concentration and decreased plasma folate in NTD patients and their parents homozygous for the C677T mutation in comparison with the heterozygotes and the wild-type individuals. Kirke et al. *(14)* observed decreased red cell folate, plasma folate, and vitamin B_{12} levels in mothers with NTD offspring. Because red cell folate levels of these mothers showed a correlation with vitamin B_{12}, they concluded that methionine synthase function is directly or indirectly impaired in these mothers. Theoretically, however, a methionine synthase or vitamin B_{12} deficiency should lead to increased plasma folate and plasma homocysteine and possibly to decreased red cell folate levels. This combination was not observed by Van der Put et al. *(9)* in a study that comprised 63 mothers, 56 fathers, and 55 children with spina bifida. Therefore, the

involvement of methionine synthase in NTD etiology is unlikely. The distribution of red cell folate levels in NTD families resembled that in controls. Mutations in genes encoding for methylene tetrahydrofolate dehydrogenase and serine hydroxylmethyl transferase are good candidates to explain lower vitamin B_{12} levels as observed in a small subset of NTD family members.

Bagley and Selhub *(112)* found an accumulation of formylated tetrahydrofolates in red blood cells of individuals homozygous for the C677T mutation. In individuals with the wild-type genotype, only 5-methyltetrahydrofolate polyglutamates were found. This points toward the possibility that there is an in vivo impairment in the activity of the thermolabile variant of MTHFR and an altered distribution of folates in red blood cells. Excessive accumulation of 5-methyltetrahydrofolate could be detrimental for the *de novo* synthesis of nucleic acids that use non-methylated folates as substrates. A shift in folate distribution could favor DNA synthesis and repair, explaining the beneficial side of a mutation: protection for colon cancer *(113,114)*.

Mitchell et al. *(115)* reported that the 46% of the variance in red blood cell folate levels, as studied in 440 pairs of monozygotic twins and 331 dizygotic twins, could be attributed to additive genetic effects. Measured phenotypic covariates counted for 16% and 38% were the result of random environmental effects.

Birth Defects Other than NTD Can Also Be Prevented with Folic Acid

Various epidemiological studies focused on the possible effect of the periconceptional use of multivitamins including folic acid or folic acid alone on a possible reduction of birth defects other than NTD *(116)*. Also, the intervention studies, although not designed to test birth defects other than NTD, yielded additional information. Overall, one might conclude that specific birth defects like those of the urinary tract, the cardiovascular system, and schisis have been reduced in women using folic acid containing multivitamins. For the use of folic acid alone, there is also observational evidence for the same conclusion. The MRC intervention study *(19)* did not show a reduction of birth defects in the folic-acid-only arm, but this study was not designed for that purpose.

In regard to congenital heart defects, Czeizel *(117)* reported that in a randomized trial with periconceptional multivitamins containing 0.8 mg folic acid, a reduction of congenital anomalies, neural tube defects and congenital heart anomalies, in particular.

Congenital Heart Disease

In a epidemiologic study on maternal risk factors for placental abruption, Raymond and Mills *(118)* reported a five times increased rate (OR: 4.63). It is quite possible that mothers who experienced placental abruption and had hyperhomocysteinemia are the subgroup that also induced heart defects in their offspring.

Kapusta et al. *(79)* found that in a case-control study of 27 mothers with infants born with congenital heart disease and 56 controls, a significantly higher homocysteine concentration in the study group than in the controls (OR: 4.9; 95%, CI: 1.0–25.3), without differences in folate status in both groups.

Schisis

Wong et al. *(80)* studied 35 women who had given birth to a child with orofacial clefts and 56 controls. Both fasting and after load, plasma homocysteine values were significantly higher in the study group than in the control group (OR: 5.3.8; 95%, CI: 1.1 – 24.2). The median concentrations of serum and red cell folate were significantly higher in mothers of cases compared to the mothers of controls.

For children with a cleft lip/palate, a large-scale population-based case-control study of Shaw et al. *(27)* showed a 50% reduction in the occurrence risk by periconceptional multivitamin supplementation. This was not confirmed by the case-control study of Hayes et al. *(119)*.

The nonrandomized intervention study by Tolarova and Harris *(120)* demonstrated a 65% reduction in the recurrence risk of mothers who were treated with a daily multivitamin supplement including 10 mg folic acid in the periconceptional period.

In 512 Argentine families with cleft palate and palatum anomalies and 218 control families, Tolarova et al. *(121)* found a three times more frequent C677T homozygoty of the MTHFR gene in cases than controls. The frequency was higher in cases of bilateral clefts (22.2%) compared to unilateral clefts (17.2%). Red blood cell and plasma folates showed significantly lower levels in Argentineans than in Dutch, possibly reflecting differences in diet.

UNEXPLAINED PREGNANCY LOSS

With the start of the homocysteine research in relation to NTD and the genetic mutations found, it was logical to also explore this fascinating field in early embryonic development. Hibbard *(3)* was the first to suggest a possible relationship between miscarriage and folate deficiency. An increased

FIGLU excretion was found after histidine loading in 32% of women with an isolated unexplained "abortion" and in 60.5% of women with two or more recurrent events.

Mild hyperhomocysteinemia was suggested in patients with recurrent early pregnancy loss *(122)*. In later studies, mild homocysteinemia was confirmed *(81,84)*. The odds ratio of both studies for mild hyperhomocysteinemia was 2.9 (95% CI: 0.9–10.2).

The common mutation C677T was found *(82)* in 16% of 185 Dutch women with unexplained recurrent early loss and in 5% of 113 case-controls (OR: 3.3; 95% CI:1.3–10.1) and 1250 population controls (OR: 2.0; 95% CI: 1.2–3.2)]. This was confirmed in a small French retrospective study *(123)*.

Homozygotes for the mutant gene of thermolabile MTHFR were sensitive to 0.5 mg of folic acid per day and normalized their plasma homocysteine concentrations *(71)*.

The higher prevalence of the C677T mutation in women with recurrent early pregnancy loss was confirmed in the Norwegian Hordaland study *(124)*.

It is important to realize that the possibly preventive effect of folic acid on the recurrence of early pregnancy loss cannot be investigated anymore in a placebo-randomized fashion because of the evidence-based prevention of NTD with folic acid, a preventive approach that has to start around conception. Therefore, more in-depth research is necessary into the mechanisms that interfere with embryonic development.

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8

Folic Acid and Homocysteine as Risk Factors for Neural Tube Defects

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THE ROLE OF FOLIC ACID IN PREVENTION OF NTD

It is now well known that maternal nutrition, specifically multivitamin supplementation during the periconceptional period, is a significant modulator of neural tube defect (NTD) risk. Initial efforts demonstrated that although not clinically low, serum levels of several micronutrients, particularly folic acid, were significantly decreased during the first trimester in mothers of NTD-affected infants when compared to mothers of healthy infants *(1)*. Subsequent nonrandomized trials demonstrated that periconceptional multivitamin supplementation was responsible for up to a 75% reduction in the recurrence risk for a NTD *(2–6)*. This observation was verified in a double-blind, placebo-controlled, randomized study, which observed a 72% reduction in NTD recurrence risk when the maternal diet was supplemented daily with 4 mg of folic acid *(7)*. These studies strongly support the hypothesis that periconceptional supplementation of the maternal diet with folic acid in the dose range of 0.4 *(3,4)* to 5 *(6)* mg/d is sufficient to overcome the majority of NTD recurrent risk.

More recent studies have generated evidence suggesting that supplementation with multivitamins containing folic acid also reduces the occurrence risk for NTD *(8–13)*. A reduction in NTD occurrence risk in the range of 35–74% has been observed in studies conducted in the United States, Australia, and Hungary. The only contradictory findings that have been reported were those of Mills and coworkers, who failed to find any association between periconceptional multivitamin supplementation and NTD risk among a sample of women from California and Illinois *(14)*.

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Data from clinical trials are generally in agreement with the hypothesis that this apparent reduction in NTD risk is specifically attributable to folic acid, although the mechanism underlying this effect is not well understood. Evidence of the complexity of the relationship between folic acid supplementation and NTD risk has recently been described in a California study, where it was determined that not all population subgroups derive the same beneficial effects of folate supplementation. In this study, Shaw and colleagues observed that college-educated women as well as Hispanic populations did not have a significant reduction in NTD risk in spite of folic acid supplementation *(13)*. This observation suggests several possibilities. The active factor in the multivitamin supplement could be a compound other than folic acid or it may, in fact, involve a behavior related to maternal vitamin use. It is also possible that different population subgroups maintain genetic variability within those genes regulating the absorption, transport, and metabolism of folic acid. Such variant alleles might well explain the observed variation in risk reductions among population subgroups receiving folic acid supplementation.

The possibility has also been raised that the mechanism by which supplemental folate acts to reduce NTD risk involves the reduction of serum homocysteine *(15–18)*. The inverse relationship between concentrations of folic acid and homocysteine in serum has been well characterized, wherein cases of folate deficiency invariably produce increased homocysteine concentrations. It is also known that hyperhomocysteinemia can result not only from an outright deficiency in folate but also in response to reduced activity on the part of one of several folate pathway enzymes *(19–21)*. In the chicken embryo model, exogenous homocysteine induced neural tube and heart defects in a concentration-dependent fashion. In these studies, supplementary folic acid was found to be protective only if its application to the embryos resulted in a reduced homocysteine concentration *(18)*.

The mechanism through which homocysteine was able to induce these abnormalities of neural tube and heart development has not yet been fully resolved. Although homocysteine in high concentrations can be cytotoxic, avian embryos with homocysteine-induced neural tube defects did not have fewer cells in the affected areas. Rather, there were frequent duplications of the notochord or the spinal cord, or both; and there appeared to be a hyperabundance of cells *(18)*. This apparent growth effect indicated the possibility of a receptor-mediated mechanism for homocysteine, which may have acted to either stimulate growth of the embryonic neural tube or to inhibit beneficial cell death. Homocysteine has the ability to act as an antagonist of the *N*-methyl-D-aspartate type of glutamate receptor

(NMDAR) *(22)*. Because the NMDAR is known to regulate several potentially relevant functions, including programmed cell death, it was hypothesized that homocysteine may induce NTD by acting as an NMDAR antagonist. This model of NTD etiology is supported by the finding that induction of NTD in the avian embryo was a general property of well-characterized NMDAR antagonists *(23)*. This hypothesis was further strengthened by evidence that NMDAR agonists were able to rescue the normal phenotype when given in conjunction with homocysteine. Glycine was observed to have the greatest ability to prevent homocysteine-induced NTD, presumably through the glycine activation site on the NMDAR *(24)*.

CANDIDATE GENES FOR FOLATE-SENSITIVE NTD RISK

In light of the facts that the underlying protective mechanisms attributed to folic acid supplementation remain unknown and that maternal folate supplementation is not universally effective in preventing NTD, it became apparent that genotypic differences between individuals may regulate the folate–NTD protective response. In order to explain individual sensitivity to the induction of NTD, it was necessary to identify candidate genes that might be involved in regulating this response. The genes involved in folate transport and metabolism were targeted for study because of compelling clinical and experimental evidence that folic acid is a significant modulator of NTD risk.

Folate Transport: Folate Receptor-α

Human folate receptor-α (FR-α) is a membrane-bound protein with a high binding affinity for 5-methyltetrahydrofolate (5-MeTHF). This protein works either in concert with unique plasma membrane structures known as caveolae *(25–28)*, or a traditional endosome *(29)* to transport folate across cellular membranes. The binding of 5-MeTHF to FR-α is the only known folate-specific step in the process of folate transport *(25)*, making this locus an excellent candidate gene for determining susceptibility to NTD. This is especially true given the high concentration of FR-α within the maternal placenta *(30,31)*, as well as the syncytiotrophoblast and fetal neuroepithelium. Because most embryonic cells do not express FR-α *(32)*, the expression of these receptors in neuroepithelial cells is suggestive of a critical role for folate in the normal morphogenetic events involved in neural tube closure (NTC).

Additional support for the involvement of *FR-α* in NTD etiology comes from studies of a murine model wherein the homolog of *FR-α* (*Folbp-1*) was knocked out by homologous recombination *(33)*. Embryos that are

nullizygous for the *Folbp–1* gene (–/–) die by gestational day 9.5 and all have exencephaly *(33)*. In these mice, supplementation of the dam with folate has been shown to rescue the normal phenotype with respect to neural tube closure *(33)*.

Investigation of this locus in human subjects has focused on the exons responsible for protein function and the promoter region. Barber and colleagues *(34)* conducted an exhaustive search for nucleotide polymorphisms within the coding region of *FR-α* utilizing three different molecular methodologies in a group of samples collected as part of a live-born Phenylketonuria (PKU) screening program in California. The NTD cases in this study consisted of infants affected with spina bifida. In the first experiment, exons 3, 5, and 6 were screened in over 1000 DNA samples using single-stranded conformational polymorphism (SSCP) analysis. The second experiment involved direct DNA sequence analysis of *FR-α* exons 5 and 6 in 50 NTD-affected individuals. Finally, the entire *FR-α* coding region was screened by dideoxy fingerprinting (ddF) in a sample of 219 individuals that was stratified by folate status and pregnancy outcome *(34)*. None of the analytical techniques employed were able to identify any polymorphic nucleotides within the coding region or intronic bases surrounding the intron–exon boundaries of the *FR-α* locus *(34)*. An explanation that was put forward by the authors for the lack of detectable polymorphism within the *FR-α* locus was that gene conversion had taken place within the FR gene family. As argued in that article by Barber et al. *(34)*, gene flow between homologous chromosomes would have the effect of cleansing mutations at the genomic level, including selectively neutral substitutions that normally accumulate within DNA over time. A total of 163 intronic bases immediately adjacent to the intron–exon boundaries within the coding region and 122 bp of the 3' UTR immediately downstream of the stop signal were screened in this study. The complete lack of observed nucleotide variation in these regions was cited as supporting the possibility of gene conversion. Additionally, the high degree of sequence identity between *FR-α* and the pseudogene suggested that recombination had occurred between these loci. Such recombination between active and null alleles has been well documented *(35–39)*, and forms the genetic basis of steroid 21-hydroxylase deficiency, which results in congenital adrenal hyperplasia *(38,40)*. The screening efforts of Barber and colleagues *(34)* would have necessarily failed to detect such a gene conversion event, as the primer sequences utilized were specifically designed to exclude the amplification of the pseudogene *(34)*. Studies of a folate-binding-protein knockout mouse *(33)* predicted that an individual lacking a func-

tional folate receptor allele would not be viable, thus any gene conversion event would occur in the heterozygous condition. Therefore, polymerase chain reaction (PCR) amplification of *FR-α* in these theorized heterozygous gene conversion mutants would have been restricted to the wild-type *FR-α* sequence, and the gene conversion event would have gone undetected. The possibility of gene conversion as a source of *FR-α* disease alleles has yet to be resolved.

In a separate investigation, Trembath et al. *(41)* screened the *FR-α* coding region by SSCP in a sample of 154 probands and relatives obtained from NTD clinics located in Iowa, Minnesota. and Nebraska. The only mutation observed in a case individual consisted of a single, silent mutation (TGA → TAA) within the stop codon *(41)*. This mutation was identified in a male, non-Hispanic Caucasian with a meningomyelocele. Analysis of parental DNA verified paternity and determined that the mutation was a *de novo* event. A separate population of 326 control individuals composed of Iowa newborn infants was also analyzed as part of this study. The only atypical sequence occurred in a phenotypically normal individual who possessed an amino substitution of serine to asparagine within exon 6 of *FR-α* and, thus, no significant association with NTD risk *(41)*.

In addition to studies of the protein-coding region, evaluations of *FR-α* as a candidate gene for NTD risk have included examination of the *FR-α* promoter region. The nucleotides encompassing a segment of the *FR-α* 5'UTR that has been shown to be necessary for maintenance of normal transcription rates *(42)* were screened for polymorphism by ddF in a sample of 185 case and 288 control women from the Rio Grande valley of Texas (Barber et al., unpublished data). Data analysis revealed three different polymorphisms within the promoter region of the *FR-α* gene (631T→C, 610A→G and 762 G→A). These three polymorphic sites formed only two variant alleles, as two of the substitutions (631T→C and 610A→G) were always observed together. No statistically significant association or trend was observed for either a risk, or a protective effect, for children or mothers with any of the different allelic forms of this gene ($p > 0.05$). Although the frequency of the polymorphic alleles showed significant variation between sampling locations, there was no significant association of either allele with NTD risk. These polymorphic promoter alleles appear to represent neutral variation within the promoter region of *FR-α*. Therefore, unlike the coding region of this gene, which showed very little variation, the promoter region does not appear to be under the same degree of selective pressure to remain invariant.

Folate Metabolism

5,10-Methylenetetrahydrofolate Reductase

The folate pathway enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is one of the principal means by which cells regulate intracellular concentrations of methionine and homocysteine. Although the enzyme methionine synthase catalyzes the final reaction, it is the enzymatic activity of MTHFR that represents the rate-limiting step in the cycle that generates methionine from homocysteine. A mutation in the MTHFR gene (677C→T) that codes for a thermolabile variant of the enzyme has been previously described *(43)*. This variant protein has reduced enzymatic activity *(44)* and has been linked to elevated serum homocysteine levels in individuals who are homozygous for the mutation *(43)*. Although a threefold to sevenfold elevation in NTD risk has been reported between homozygosity for this MTHFR mutation in Holland and Ireland *(45,46)*, several subsequent and larger studies have found no or a much smaller association with NTD *(47,48)*. The risk for individuals heterozygous for the mutation in the Dutch and Irish studies was only slightly elevated over those homozygous for the wild-type allele *(49)*. Interestingly, Weitkamp and colleagues *(50)* found a significant increase in heterozygotes in both normal male and NTD-affected female offspring in a recent study of transmission of the 677C→T mutation by heterozygous parents. These authors failed to offer a biologically sound explanation for this observation.

Shaw and coworkers *(48)* have also examined the MTHFR 677C→T mutation in a set of population-based, case-control samples that included 214 live-born spina bifida infants and 503 controls. In addition to ascertainment of the 677C→T mutation, information concerning maternal vitamin use during the periconceptional period was collected in order to detect gene–environment interactions that may have impacted NTD risk *(48)*. The frequency of the 677C→T mutation, in either a homozygous or heterozygous state, was found to be slightly higher among affected infants than it was among controls, although this difference was not significant *(48)*. The 677C→T mutation was observed significantly more often among control and affected Hispanic infants, relative to non-Hispanics ($p < 0.05$).

Barber and colleagues examined MTHFR 677C→T genotypes in a NTD case-control sample composed of Hispanic individuals from the Rio Grange valley of Texas. For the 677C→T polymorphism in the MTHFR gene, odds ratios (ORs) were 1.8 for heterozygosity and 1.8 for homozygosity relative to homozygosity for the wild-type allele. This study also involved a comparison of the frequencies of MTHFR genotypes among control populations

Table 1
Comparison of Reported MTFHR Genotype Frequencies

Genotypes	Barber, et al. 1999 (Hispanic—Texas)	Shaw, et al. 1998 (Hispanic—California)	Shields, et al. 1999 (Irish)
CC	35/186=19%	63/169=37%	114/242=47%
CT	120/186=64%	71/169=42%	108/242=45%
TT	31/186=17%	35/169=21%	20/242=8%
χ^2	0	40.37	63.9
p-Value (df)[a]	1	<0.005 (2)	<0.005 (2)

[a]df = degree of freedom.

of previously published reports (Table 1). Frequencies for the MTHFR 677C and 677T alleles were statistically significantly different between studies. In the Texas samples, Hispanic controls were more likely to be homozygous for either allelic form relative to control individuals from a California study *(48)*. Differences were also detected in a comparison of Texas and Irish control populations. The Irish samples *(51)* had a higher frequency of the homozygous genotype for the C allele and a higher frequency for the heterozygous genotype (TC) relative to the Texas samples (Table 1). In addition, the highest frequency of the mutant 677T allele was reported for the Texas control population (48.9%) compared to 41.7% in the California Hispanic *(48)*, and 30.6% in the Irish *(51)* control populations. Studies regarding the distribution of this polymorphism among different ethnic groups found the highest prevalence of the MTHFR 677T allele (54.5%) in a Spanish population *(52)*. Generally, European Caucasian populations are reported to have a frequency of the mutant allele ranging from 24% to 40%, followed by Asians whose frequencies range from 4.1% to 37.5%, and Africans with a range between 0% and 9.4% *(52,53)*. This last number is comparable to frequencies in African-American populations, which is approx 11% (*53*).

Papapetrou et al. *(47)* suggested that homozygosity for MTHFR 677T is only a risk factor for NTD in some ethnic groups, and not in others. They note that in populations where the 677T allele is found to be a risk factor for NTD, specifically the Dutch and Irish populations, the background frequency of this allele is low (Dutch = 26%, Irish = 28%). In populations with high background frequencies of the T allele, such as the British (36%, Papapetrou) or Mexican-American (49%, Texas), an association between

homozygosity for the T allele and NTD risk could not be established. Christensen et al. *(54)* found that low red blood cell folate (RBC folate) is an independent risk factor for NTD. The combination of low RBC folate and homozygosity for the 677T allele was found to act synergistically for children, but not for mothers. They concluded that an interaction between genotype and nutritional status is associated with a high risk for NTD in children.

A second mutation has also been described in exon 7 of the MTHFR locus (1298A→C) that results in a substitution of glutamate for alanine in the mature protein (*41,55*). This second mutant MTHFR allele encodes a protein that has reduced enzymatic activity, but unlike the 677C→T mutation, it is not associated with increased plasma levels of homocysteine or decreased levels of serum folate *(55)*. Although this second MTHFR mutation does not appear to affect plasma levels of homocysteine or folate when it occurs in isolation, there is evidence for an interaction between the 1298A→C and 677C→T mutations. When these two mutations are observed together in a compound heterozygote, MTHFR enzyme activity resembles that of an individual homozygous for the 677C→T mutation *(55)*. Additionally, compound heterozygotes have been observed to have slightly increased homocysteine levels and NTD risk *(55)*.

Methionine Synthase

The enzyme methionine synthase is responsible for catalyzing the transfer of a methyl group from 5-MeTHF to homocysteine, effecting the conversion of homocysteine to methionine. Vitamin B_{12} is a required cofactor in this reaction. Methionine, in the form of *S*-adenosylmethionine, is the single most important donor of methyl groups for the methylation of DNA and tRNAs *(56)*. Cells also utilize this reaction to generate tetrahydrofolate from 5-MeTHF, the plasma form of circulating folate. This conversion of 5-MeTHF to tetrahydrofolate is critical to folate metabolism, because polyglutamation of 5-MeTHF proceeds very slowly relative to tetrahydrofolate, and it is the polyglutamated folate that, for the most part, is acted upon by folate-metabolizing enzymes. The exception to this rule is methionine synthase. Individuals with a low level of methionine synthase activity present with high levels of plasma homocysteine, low levels of plasma methionine, and a clinical picture similar to that related to a deficiency of MTHFR activity *(43)*. Mills and colleagues *(15)* examined the plasma homocysteine levels of women who had previous NTD-affected pregnancies and compared these levels to those observed in control mothers. They observed that mothers who carried NTD-affected pregnancies had

significantly higher homocysteine levels, suggestive of a defect in either methionine synthase or MTHFR *(15,20)*.

Recently, Shaw and coworkers *(57)* examined the frequency of a known polymorphism within the methionine synthase gene *(58)* in a large sample of spina bifida-affected and matched-control infants from California. The variant allele under investigation is the result of a point mutation wherein adenine is replaced by guanine (A2752G). This nucleotide replacement produces an amino acid substitution of glycine for aspartate (D919G) *(58)*. In the Texas study, case and control infants were found to have a similar percentage of A2752G genotypes, in either the heterozygous or homozygous state, regardless of the ethnicity of the mother *(48)*. There was no indication of increased risk for spina bifida among infants who were either heterozygous or homozygous for this methionine synthase polymorphism, regardless of maternal ethnicity *(48)*.

The candidate genes that have been selected for their involvement in NTC defects are involved, in one way or another, with folate uptake, or with the interaction between folate and methionine metabolism, as discussed earlier. Elevated serum homocysteine is an inevitable sequel of folic acid insufficiency or of abnormal methionine metabolism, and hyperhomocysteinemia is said to be an independent risk factor for neural tube defects *(16,59)*. Thus, it is conceivable that hyperhomocysteinemia *per se* is the principal teratogen in the event of low folate intake or dysregulation of methionine metabolism. However, there is no consensus on a biological or molecular mechanism for this effect, as will be discussed next.

SPECULATION ON THE POSSIBLE ROLE(S) OF FOLATE IN NEURAL TUBE CLOSURE

One of the primary functions of folate in the cell is to provide single-carbon groups for the *de novo* synthesis of purines and thymidine *(60,61)*. The rapidly dividing cells of the developing neural tube are likely to require large amounts of nucleotides in order to facilitate DNA replication *(29)*. One model predicts that if neuroepithelial cells exhaust their internal supply of nucleotides, cellular replication will be slowed and the development of the neural folds retarded. Such a delay in the development of the neural folds has previously been implicated in the formation of NTD *(62)*.

It has been hypothesized that abnormal folate receptors would be less efficient at binding and internalizing available 5-MeTHF to the cytoplasm of the target cells at critical periods of development. Such decreased affinity would likely compromise receptor saturation in the syncytiotrophoblast and

neuroepithelium, thereby increasing the risk of embryonic folate deprivation. Periconceptional supplementation with multivitamins containing folic acid may overcome these deficiencies by increasing maternal blood folate levels. This could explain why folate supplementation prevents NTD; it stoichiometrically drives these reactions to improve transport across a poorly functioning receptor.

Following several years of investigation, it appears as if there is extremely little variation at the nucleotide level within the coding region of the *FR-α* gene. Only two mutations within the *FR-α* coding region have been detected after examination of nearly 2000 individuals. These observations were made in five separate studies, conducted in two laboratories, utilizing SSCP, ddF, or DNA sequence analysis. Of the two mutations discovered, only one occurred in a NTD-affected individual. This mutation was a synonymous polymorphism that arose *de novo* within the stop codon. The second mutation involved an amino acid substitution but was detected in an individual who was phenotypically normal with respect to neural tube closure.

In a similar manner, variation at the *FR-α* promoter region does not seem to be involved in NTD etiology. Although the promoter region seems to contain a higher degree of polymorphism than the coding region of *FR-α*, the *sequence* variation observed in this section of the locus does not appear to be related to increased NTD risk. This was evident upon examination of the statistical results, which revealed that the ORs for the polymorphic alleles were not elevated to a large degree relative to the wild-type allele and both of the 95% confidence intervals included one.

Several recent studies have reported an association between homozygosity for a mutation in the methylenetetrahydrofolate reductase gene (677C→T) and increased NTD risk *(44–46,49,63)*. Other studies, however, have either not observed this result *(47)* or observed it to a lesser degree when considered in conjunction with maternal folate intake *(48)*. It has been suggested that the 677C→T polymorphism may play only a small role in the reduction of NTD risk observed in response to maternal vitamin supplementation or that the 677C→T polymorphism segregates with NTD risk only in certain populations *(48)*.

A similar result was observed for methionine synthase. The presence of a known polymorphism in the methionine synthase gene, which has been linked to elevated levels of homocysteine and reduced enzyme activity, was screened in a group of case (spina bifida) and control infants. Regardless of maternal ethnicity, no association was observed between the mutant allele and the disease phenotype *(57)*. Because only the infant genotype was assayed, it is possible that the maternal genotype might have had an effect

on the risk for a spina bifida-affected pregnancy. This is unlikely, however, given the frequency (4%) of the mutant allele in the control population *(57)*. It is also possible that other, as yet unidentified, polymorphisms that are linked to spina bifida risk exist within this gene.

Although investigation of methionine synthase as a NTD candidate gene appears to be negative, the gene-encoding methionine synthase reductase (MTRR), which is the enzyme responsible for the reactivation of methionine synthase, may be related to increased NTD risk. Wilson and coworkers *(64)* have recently described a mutation in this gene (A66G) that appears to be significantly related to increased NTD risk when combined with low levels of cobalamin. Genotypes of the MTRR and MTHFR polymorphisms were determined and assayed in combination with cobalamin status in a sample of 56 spina bifida patients and 58 mothers of patients, as well as 97 control children and 89 control mothers. The mutant allele, which results in an amino acid substitution (I22M), was observed to occur in the general population at a frequency of 0.51. This common mutant allele was reported to significantly increase NTD risk when combined with either low cobalamin status or the mutant MTHFR 677T allele in either children or mothers *(64)*. The odds ratios for MTRR 66G in combination with low cobalamin represented a nearly fivefold increase in NTD risk in case mothers (OR 4.8; 95% CI 1.5–15.8) and 2.5 in NTD patients (OR 2.5; 95% CI 0.63–9.7). A trend for elevated NTD risk was also observed when the MTRR 66G allele occurred in combination with MTHFR 677T in mothers (OR 4.1; 95% CI 1.0–6.4) and children (OR 2.9; 95% CI 0.58–14.8).

These recent findings of an association between polymorphisms in homocysteine pathway enzymes and elevated NTD risk strengthen the support for the involvement of homocysteine in NTD occurrence. NTD etiology has been theorized to be related to elevated plasma homocysteine levels, which are a consequence of low folate concentrations *(15,16 20,55)*. Direct evidence of the involvement of homocysteine in NTD etiology has been obtained in experiments in which avian embryos were exposed to homocysteine *(18)*. When embryos were treated by injection of 200 m*M* D,L-homocysteine into the shell on incubation day 0, 1, and 2 and harvested at stage 14, after 53 h of incubation, 27% were observed to have defects of the neural tube. In addition, 11% of embryos treated with the same dose of homocysteine on incubation day 2, 3, and 4 showed neural tube defects when harvested on day 9. The teratogenic dose was determined to elevate serum homocysteine levels 15-fold, from 10 to 150 nmol/mL. More importantly, when 0.1 μg of supplemental folate was given concurrently with the homocysteine treatment, the normal phenotype was rescued and serum homocys-

teine levels did not rise above 45 nmol/mL. Although these experiments, as well as the epidemiological studies discussed earlier, appear to show that homocysteine may act directly to induce NTD, the mechanism is unknown. Homocysteine is cytotoxic at high concentrations, but may exert a growth-factor-like effect at lower concentrations, in some embryonic cell types *(65)*. Recent data show that homocysteine may act as an NMDA receptor antagonist *(22)*, and many compounds with this capacity are known to induce NTD *(23)*. In addition, NMDA receptor agonists significantly reduce the teratogenic effect of homocysteine *(24)*. Although the definitive mechanism of homocysteine-induced NTD has not been fully elucidated, it is clear that further investigation of the highly interrelated pathways of folate and homocysteine metabolism as related to NTD risk is warranted.

In summary, although numerous studies of folate pathway candidate genes have been conducted to date using DNA samples obtained from the United States, none have been able to conclusively identify a significant association between a genetic polymorphism and increased NTD risk. This is true of genes involved in both folate transport and metabolism. Although no marginal association between a polymorphism in any single gene and NTD risk in a US population has been observed, it is possible that some of these polymorphisms interact, requiring variants of several genes to be present to increase NTD risk. This situation would fit easily into the theoretical framework of a presumed oligogenic, multifactorial etiology of NTD, where several affected loci are required in conjunction with unidentified environmental factors to disrupt NTC and produce NTD in selected infants. The complexity of NTD etiology could also help to explain the conflicting data that has been reported for MTHFR genotype and NTD risk. Utilizing the multifactorial threshold model, MTHFR would represent one locus among many which contribute to NTD liability. In this model, it is possible that high background frequencies of a polymorphism associated with NTD risk (such as the MTHFR 677C→T polymorphism) could function to increase the overall susceptibility of a population to NTD risk while not actually segregating with the disease. In this hypothetical instance, alleles at additional loci that are associated with NTD risk would occur at low frequencies and represent the critical alleles that, along with key environmental factors, would push the individual over the disease threshold and determine the final incidence of a NTD. In this example, it would be these additional, rare alleles that would be observed to segregate with the disease.

In addition, as the intricate nature of folate metabolism implicates a large number of potential candidate genes, it is nearly certain that other loci, which have yet to be examined or even identified, are involved in determining the

NTD risk. Over the next few years, it should become easier to select and screen these candidate loci, as the number of genes which have been identified, mapped, and characterized increases through efforts associated with the human genome project.

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9

Developmental Toxicants Potentially Acting via Folate Perturbation

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INTRODUCTION

Periconceptional supplementation with multivitamins containing folic acid have been shown to decrease the incidence of neural tube defects (NTD) *(1,2)*. In addition, recent data have suggested that significant decreases in the incidence of orofacial clefting *(3–5)*, cardiovascular defects *(5–7)*, and urinary tract defects *(8,9)* may also occur. In a study from the Baltimore–Washington area, there was no difference in the rate of outflow tract cardiac defects among women in the four quartiles of folate intake, but there was a decreased risk for transposition of the great vessels *(10)*. Czeizel *(11)* reported no protection of periconceptional multivitamin use for limb deficiencies, but Yang et al. *(12)* did report a lower incidence of these defects, especially transverse limb deficiencies in the Atlanta area.

Presently, a great deal of research is investigating the role of genetic factors in the predisposition to these defects. In addition to the C677T *(13–26)* and other variants *(25,27,28)* in 5,10-methylenetetrahydrofolate reductase (MTHFR), the role of mutations in methionine synthase *(24,25,29–31)*, cystathionine β-synthase *(31–33)*, and folate receptor genes *(34,35)* is being examined for possible associations with these defects in human populations.

In addition to the genetic constitution of an individual, environmental factors may be involved. For example, there are several sites where compounds may interfere with folic acid to possibly produce birth defects. These include interference with hydrolysis and metabolism of dietary and supplemental folate, which could alter absorption. Within the liver and other tissues, compounds could interfere with the uptake, polyglutamation, and metabolism of folic acid, thereby altering intracellular concentrations of various folate derivatives. Finally, other vitamins and cofactors are neces-

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sary for efficient one-carbon metabolism, and compounds that alter the concentrations of these other cofactors could also have adverse effects on intracellular folic acid metabolism. In the pregnant female, alterations in placental transport of folate could also occur and produce adverse effects in the fetus. This topic was reviewed recently *(36)*, and information that was not included in that review will be presented here.

COMPOUNDS THAT MAY ALTER FOLATE LEVELS/ METABOLISM

Several compounds have been reported to alter plasma/serum folic acid levels, but there are no strong data to suggest that these compounds are developmental toxicants in humans. Because pregnancy increases the demand for folic acid, women having marginally adequate folate intake may be especially susceptible to adverse effects of these compounds. The diuretic drug triamterene and the organic solvent 2-methoxyethanol are included in this category, but they were previously reviewed *(36)* and will not be included here.

Trimethoprim

This antibiotic is able to block conversion of *p*-aminobenzoic acid to dihydrofolate, an enzymatic step that is present in bacteria but is not present in humans. It may also inhibit dihydrofolate reductase. The drug is listed in pregnancy category C. Trimethoprim has been reported to produce defects in rats, mainly cleft palate, and to increase fetal loss in rabbits, whereas, the combination of trimethoprim and sulfamethoxazole produced a variety of defects in rats but not in rabbits *(37)*. Trimethoprim alone produced resorptions, small fetuses, cleft palate, and skeletal abnormalities in rats at very high doses; no adverse effects were observed in rabbits *(38)*. A study examined the reproductive outcomes of the wives of a group of male pharmaceutical workers exposed to sulfonamides, including the combination of sulfamethoxazole and trimethoprim and compared these to the outcomes to those from a group of workers who were not exposed to sulfonamides. A significantly increased frequency of abortions and decreased frequency of live births were noted among the wives of men exposed to sulfonamides; however, there was exposure to sulfonamides other than the combination of sulfamethoxazole and trimethoprim as well as other factors that were not controlled *(39)*. The possible teratogenicity of the combination was examined using the Hungarian Case-Control Surveillance of Congenital Anomalies *(40)*. Cotrimoxazole (the combination of sulfamethoxazole and trimethoprim) use was significantly higher among women having children

with cleft lip with/without cleft palate or hypospadias compared to matched control cases; however, most of the usage of the antibacterial drug came after the first trimester, and there were no differences between cases and controls when considering exposure only during the first trimester *(40)*. In general, these studies do not support a strong teratogenic potential for trimethoprim in combination with sulfamethoxazole.

Sulfasalazine

Sulfasalazine is used to treat ulcerative colitis and Crohn's disease. Folate deficiency often occurs in patients with inflammatory bowel disease, and risk may be worsened by treatment with sulfasalazine. This drug inhibits the intestinal absorption of folate, as well as enzymatic activity of dihydrofolate reductase, MTHFR, and serine hydroxymethylase *(41)*. The drug is classified as pregnancy category B. Several case reports have suggested a teratogenic effect of the drug. A child with cleft lip and palate and hydrocephalus was born to a woman who had taken sulfasalazine prior to and throughout her pregnancy for treatment of ulcerative colitis *(42)*. Stillborn twins were born to a woman with Crohn's disease who had taken the drug throughout her pregnancy; one twin had a polycystic kidney on the left side and the other twin was missing both kidneys and ureters, had hypoplastic lungs and bladder, as well as undescended testes *(43)*. A second case reported by these authors *(43)* described a child born to a woman who had taken the drug for treatment of ulcerative colitis during her pregnancy; the child died at 10 d of age as a result of coarctation of the aorta and a ventricular septal defect. Another case report described an infant with macrocephaly, coarctation of the aorta, and a ventricular septal defect born to a woman with ulcerative colitis who had taken the drug her throughout pregnancy *(44)*. On the other hand, Mogadam et al. *(45)* surveyed patients with ulcerative colitis or Crohn's disease; 244 patients took neither a corticosteroid or sulfasalazine and 287 patients took one or both drugs. There was no difference in the incidence of birth defects between the two groups, suggesting no teratogenic effect of the drug. However, it is interesting to note that the defects present in the case reports are those which have been reported to be folate responsive in recent epidemiological studies.

Fumonisin B_1

Fumonisin B_1 is a mycotoxin that is present in the majority of corn products. Culture material containing fumonisin B_1 has been reported to be embryotoxic in hamsters *(46,47)*, mice *(48)*, and rats *(49)*. Purified fumonisin B_1 produced defects in hamsters in vivo *(47)* as well as to chick

(50) and rat embryos *(51)* treated in vitro. Recent experimental work in vivo has demonstrated that purified fumonisin B_1 can produce decreased fetal weight in rabbits as well as hydrocephalus, skeletal abnormalities, increased fetal death, and decreased fetal weight in rats *(52–54)*. However, these defects occur only at maternally toxic doses and might suggest that fumonisin B_1 does not cross the placenta *(52,55)*. Recent experimental work has suggested that fumonisin B_1 decreases the amounts of folate receptor and cellular uptake of 5-methyltetrahydrofolate in cell culture *(56)*. If fumonisin also decreases the number of folate receptors in vivo, this may be a factor in those women with marginal folate stores. This finding should be explored further.

Methanol

Methanol has been reported to produce defects in rats (*57*) and mice *(58–61)*. Although solvents in general may be associated with birth defects, a specific association between methanol exposure and birth defects in humans has not been made (reviewed in ref. *37*). Methanol is metabolized by a folate-dependent pathway to formaldehyde; species differences in metabolism may be the result of differences in hepatic folate concentrations *(62,63)*. Recently, Sakanashi et al. *(61)* observed that methanol-induced embryotoxicity was enhanced when mice were fed a low-folate diet. In particular, the incidences of cleft palate and exencephaly were increased in the low-folate groups. These findings suggest that marginal folate status may increase susceptibility to compound-induced developmental defects.

DEVELOPMENTAL TOXICANTS WHICH MAY NOT ACT VIA FOLATE PERTURBATION

Several compounds have been reported to produce birth defects in humans or animal models and have also been reported to possibly interfere with folate. However, folate deficiency has not been postulated as the mechanism for the embryotoxicity produced by the compound. The antimalarial agent pyrimethamine was reviewed previously *(36)* and will not be included here.

Carbamazepine

This anticonvulsant drug has been shown to be teratogenic in a variety of species. Various epidemiological studies in humans have suggested that the malformation rate is higher among epileptic women taking anticonvulsants than among either nonepileptic women or epileptics who are not taking anticonvulsants (reviewed in refs. *37* and *64*). One of the first reports of

possible adverse outcome following exposure to either monotherapy or polytherapy with carbamazepine came in 1989 with the description of a pattern of malformations, including craniofacial dysmorphology (upslanting palpebral fissures, epicanthal folds, short nose, and a long philtrum), fingernail hypoplasia, and developmental delay *(65)*. Many of these symptoms are similar to those reported following exposure to phenytoin. It is not clear if carbamazepine exposure produces a syndrome of malformations, but a number of studies have found an increase in the number of NTD among children prenatally exposed to this drug. Jones et al. *(65)* observed 1 infant with spina bifida in a total of 48 examined; Rosa *(66)* also observed approx a 2% incidence in a retrospective analysis of Medicare data from Michigan. Gladstone et al. *(67),* Little et al. *(68)*, and Kallen *(69)* have also reported NTD present in offspring. The overall risk of an NTD appears to be about 1% after exposure to carbamazepine. It is listed as pregnancy category C.

The drug is embryotoxic in mice in vivo *(70–72)* or in vitro *(73)*, and in rats *(74)*. The teratogenic compound has been suggested to be a metabolite of the parent drug *(75)*. The major metabolite, carbamazepine-10,11-epoxide, is teratogenic in mice in vivo *(76)* but is not teratogenic in either mice or rats in vitro *(73)*. More recent evidence has suggested that thioether metabolites may be responsible for the teratogenicity of the drug *(77)*.

There have been few studies examining the effects of carbamazepine on folic acid levels. In a study of 81 patients on carbamazepine monotherapy, serum folate levels were significantly decreased [10.1 ± 8.3 ng/mL in controls, 3.7 ± 1.7 ng/mL in carbamazepine treated; *(78)*]. In another study, plasma folic acid (17.4 ± 0.8 n*M* in controls matched for age and sex, 12.7 ± 2.0 n*M* in patients) and vitamin B_6 (66.2 ± 7.5 n*M* in controls, 37.1 ± 5.5 n*M* in patients) levels were decreased in 15 patients on carbamazepine monotherapy *(79)*. Mean plasma homocysteine levels were not different from controls among these 15 patients (9.5 ± 0.5 μ*M* in controls, 21.7 ± 9.6 μ*M* in patients); however, 6 of the 15 patients had plasma homocysteine levels above 15 μ*M*, which was significantly different from controls *(79)*. Plasma vitamin B_{12} levels were not different between the two groups. Another study *(80)* demonstrated a decreased serum folic acid level among patients on carbamazepine monotherapy (5.14 ± 1.88 ng/mL in 74 controls, 3.85 ± 1.02 ng/mL in 36 patients). These authors suggested that the mechanism for the effect of carbamazepine on folic acid levels was the result of enzyme induction because the anticonvulsants that they examined in this study that did not induce hepatic enzymes (valproic acid and zonisamide) did not reduce folate levels, whereas those anticonvulsants that did induce hepatic enzymes (phenobarbital and carbamazepine) also decreased serum

folate levels *(80)*. A significant inverse correlation was observed between plasma folic acid and total homocysteine levels among epileptics taking various anticonvulsants *(81)*. Carbamazepine also decreased plasma folate levels in rats *(82)*. Much work remains to be done to determine if carbamazepine decreases folate levels and if this is involved in the teratogenic activity of the drug.

Smoking

Recent reviews *(37,83)* have indicated that cigarette smoking during pregnancy is associated with lower birth weight, increased perinatal mortality, premature delivery, and abnormal placentation in humans. Less clear is the role of smoking in the production of various malformations in humans; these studies differ in design, size, and methodological aspects, and so it is not surprising that the conclusions differ. Exposure of rats or rabbits to cigarette smoking resulted in decreased fetal weight, but no congenital anomalies were produced *(37)*. In humans, several abnormalities such as neural tube defects, cardiac defects, limb reduction defects, urinary tract defects, and orofacial clefting that may be folate responsive have also been associated with maternal smoking, at least in some studies (*84–90*, and reviewed in ref. *83*) although neural tube defects in particular were not associated with smoking in two other studies *(91,92)*. Cigarette smoking has also been associated with decreased serum folic acid levels in some studies *(93–96)*. At this time, it is not clear what role, if any, decreased levels of folic acid may play in the developmental toxicity produced by smoking.

DEVELOPMENTAL TOXICANTS WHICH MAY ACT VIA FOLATE PERTURBATION

A number of compounds alter normal development either in humans or in animal models and may act via alterations in folate levels or metabolism. Several of these will be discussed below. The antimetabolites, aminopterin and methotrexate, were reviewed previously (*36*) and will not be included here.

Phenytoin

Anticonvulsant drugs, particularly phenytoin, were first reported to possibly produce birth defects in the mid-1960s. The defects observed included cleft lip with/without cleft palate, heart defects, and developmental delay (reviewed in ref. *97*). Later, nail hypoplasia was described as a part of the fetal hydantoin syndrome *(98)*. The drug also appears to alter neurobehavioral development in humans and animal models (reviewed in ref. *99*).

The drug causes orofacial clefting in mice (reviewed in refs. *37* and *100*), and is also embryotoxic in rats and rabbits (reviewed in ref. *37*). This drug is listed in pregnancy category C.

Phenytoin has been reported to lower plasma folate levels; the mechanism may be by impaired absorption, increased folate catabolism, or by an enzyme induction method (reviewed in ref. *36*). Recent work has demonstrated a possible inhibition of folate polyglutamation in rats *(101)*. Conflicting results have been obtained in animal experiments in which phenytoin and folate were administered together. When folic acid was given to pregnant mice, there were no differences in the rates of resorptions and malformations between the two groups *(102,103)*. Administration of folate decreased phenytoin-induced embryotoxicity in rats *(104)*. A nonlinear dose-response was observed when increasing doses of 5-formyltetrahydrofolate were administered with a teratogenic dose of phenytoin with low doses of the folate derivative having no effect on cleft incidence, high doses increasing the clefting rate, and intermediate doses decreasing the rate *(105)*. The route of administration of folate also appears to be important because 5-formyltetrahydrofolate increased phenytoin-induced clefting when it was added to the diet but had no effect when it was administered by gavage *(106)*. A single teratogenic dose of phenytoin was reported to decrease embryonic folate levels on gestestational day 11 (GD11) in mice *(107)*, but chronic dietary administration of the drug had no effect on embryonic folate levels on GD10, 12, or 14 *(108)*. Pharmacokinetic differences likely account for some of the discrepancies in results between these studies.

Although, overall, the data do not appear to support a major role for folic acid in phenytoin-induced embryotoxicity, more work is needed to determine this conclusively.

Valproic Acid

The antiepileptic drug valproic acid was first implicated as possibly producing birth defects in 1981, especially NTD (reviewed if ref. *37*), and the risk of having an infant with a neural tube defect is 1–2% when this drug is used during pregnancy *(109)*. The drug is embryotoxic in rats, mice, rabbits, hamsters, and primates (reviewed in ref. *37*). This drug is listed in pregnancy category D.

Valproic acid decreases plasma folate levels in rats *(82)*, but the mechanism for this effect is unknown. Prolonged treatment with antiepileptic drugs tends to decrease plasma folate levels in humans, but many of the studies are not large enough to examine this association with specific drugs. Also, polytherapy occurs frequently, making it difficult to examine the effect of a

single drug. For these reasons, it is not clear if valproate decreases plasma folate levels in humans. However, it has been suggested that intestinal absorption of folate may be decreased by valproic acid *(110)*.

Animal studies investigating the role of folate in valproate-induced birth defects have presented conflicting results. In vivo treatment with folinic acid by injection *(111)* or by osmotic mini-pump *(112)* was able to decrease the frequency of valproate-induced exencephaly in NMRI strain mice. However, this effect was not reproduced in in NMRI mice in another study *(113)* or in CD–1 mice in vivo *(114)* or in vitro *(115)*. There were no differences in fetal weight, length, or skeletal defects when comparing pregnant Wistar rats treated with the combination of valproic acid and folinic acid to rats treated with valproate only *(116)*. Additionally, various folate derivatives and other compounds involved in one-carbon metabolism, including methionine, were unable to prevent valproate-induced neural tube defects in vitro *(117)*.

The role that methionine may play in the abnormal development produced by valproic acid is not clear. In vivo treatment of NMRI mice decreased the number of fetuses with valproate-induced exencephaly and spina bifida occulta *(118)*. Administration of L-methionine to rat sera using a rodent whole-embryo culture system did not decrease the number of embryos with open neural tubes produced by valproic acid *(114,119)*. Nosel and Klein *(119)* did observe a decrease in the number of embryos with open neural tubes if the rat sera used for culture came from rats that had been pretreated with methionine. There was a further protective effect if the embryo donors used in the culture system were pretreated with the amino acid. This effect may relate to early methylation of proteins important in neural tube closure; Coelho and Klein *(120)* demonstrated decreased methylation in neural tube proteins from methionine-deficient rat embryos, and Moephuli et al. *(121)* demonstrated that methionine is important for methylation of actin, tubulin, and neurofilament L. Valproic acid has been reported to either decrease *(122,123)* or to have no effect *(119)* on the plasma concentration of methionine in rats. The drug has also been reported to decrease hepatic activity of methionine synthase *(116,123)*. Plasma homocysteine levels were increased by valproate treatment *(122)*. Because homocysteine has been reported to produce neural tube defects *(124)*, valproate may be acting by increasing plasma homocysteine levels. More work is needed to clarify the roles of methionine and homocysteine in valproate-induced defects.

Finally, recent work by Finnell's group has demonstrated that a teratogenic dose of valproic acid altered expression of folate binding protein I and MTHFR in the mouse *(125)*. There were differences in the gene expression

patterns of two strains of mice that differ in their susceptibility to valproate-induced neural tube defects. It appears that gene–environment interactions may be quite important in determining susceptibility to drug-induced defects.

Alcohol

Alcohol is clearly a developmental toxicant in humans and animal models (reviewed in ref. *37*). In humans, the fetal alcohol syndrome *(126)* consists of craniofacial, cardiovascular, and limb defects. Romitti et al. *(89)* found an association between cleft lip with/without cleft palate and alcohol use, but Shaw et al. *(91)* did not find such an association between neural tube defects and alcohol use. Folate deficiency is associated with chronic alcohol abuse *(127)*; however, the mechanism for the deficiency is unknown. Alcoholics tend to have poor dietary intake of folate *(128).* Alcohol has also been reported to decrease intestinal absorption of folate *(129,130)*, although other studies have disputed this effect *(131,132).* Increased urinary excretion of folate has also been reported *(133,134)*, although this effect does not appear to be the result of altered binding of folate by brush-border membranes in the rat kidney (*135*). Finally, alcohol has been reported to alter hepatic metabolism of folic acid (reviewed in ref. *136*).

Folate deficiency increases ethanol-induced embryotoxicity (reviewed in ref. *36*), possibly by altering embryonic folate metabolism *(137).* Chronic ethanol consumption has been reported to decrease methionine synthase activity in rats *(138)* and micropigs *(139)*, but acute exposure had no effect on this enzyme in mice *(140).* Acute administration of ethanol to pregnant mice resulted in fetal DNA hypomethylation *(141)*, suggesting that alterations in methyltransferase activities may play a role in ethanol-induced developmental toxicity.

Nitrous Oxide

Nitrous oxide is an anesthetic agent that is commonly used in dental offices and is less frequently used in surgical operating rooms. Previous epidemiological evidence has suggested that exposure to nitrous oxide during pregnancy was associated with an increased risk of spontaneous abortions but not with malformations (reviewed in refs. *97* and *142*). Chronic exposure to low levels or acute exposure to high concentrations of the gas produced defects *(143–147).* Other studies have not demonstrated adverse outcomes following exposure to the gas *(148,149).*

Nitrous oxide inhibits activity of methionine synthase presumably by oxidation of the vitamin B_{12} prosthetic group *(150).* Inactivation of the

enzyme leads to a loss of hepatic folates *(151)*. Treatment of pregnant rats with 50% nitrous oxide for 4 or 24 h decreased maternal hepatic methionine synthase activity by over 90% and decreased activity in GD14 embryos by almost 95% *(152)*. Maternal liver folate levels decreased and plasma levels increased with these treatments; the distribution of folate derivatives within the liver was also altered, in that 5-methyltetrahydrofolate was increased and formyltetrahydrofolate and tetrahydrofolate concentrations were decreased *(152)*. Although embryonic folate levels and distribution were not altered, the concentration of 5-methyltetrahydrofolate was slightly increased and tetrahydrofolate was decreased *(152)*. DNA synthesis and content were decreased by the 24-h treatment *(153)*. Fujinaga and Baden *(154)* observed a decrease in nitrous-oxide-induced defects with methionine, but not with folinic acid.

CONCLUSIONS

Alterations in folate uptake or metabolism may occur by a number of mechanisms, including genetic polymorphisms and environmental exposures. These exposures can be drugs used to treat various illnesses or contaminants such as fumonisin B_1. At this time, there is insufficient evidence to suggest that any developmental toxicant works via perturbation of folate. However, individuals with borderline folate status who are exposed to these compounds may be at an increased risk for having a child with a birth defect. Additionally, genetic polymorphisms may make certain individuals more or less sensitive to the folate-altering effects of some of these compounds. In an effort to determine if a compound acts via a folate-mediated mechanism, one must look beyond plasma folate levels and must try to determine what is happening within embryonic cells. In addition, more research is needed to determine the role of folic acid in embryonic cells and why such cells are sensitive to folate perturbations.

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10

Brain Development and Choline, the Other Methyl Donor

Steven H. Zeisel

THE RELATIONSHIP BETWEEN CHOLINE AND FOLATE METABOLISM

Choline is needed for synthesis of the phospholipids in cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling, lipid-cholesterol transport and metabolism *(1)* (Fig. 1). Cells require choline *(2)* and die by apoptosis when deprived of this nutrient *(3–6)*. When fed a choline deficient diet, humans and many species of animals deplete choline stores and develop liver dysfunction *(7–11)*. Animals fed a choline-deficient diet may also develop growth retardation, renal dysfunction and hemorrhage, or bone abnormalities *(10,12,13)*. The human diet must contain choline because the only endogenous pathway for synthesis of this nutrient [via the sequential methylation of phosphatidylethanolamine *(14)*] cannot meet the entire requirement for choline *(15,16)*. This pathway that forms choline moiety *de novo* from *S*-adenosylmethionine and the pathway in which the choline metabolite betaine donates a methyl group in the remethylation of homocysteine form the interfaces between choline metabolism and one-carbon metabolism (methylfolate and methionine metabolism). Because of this metabolic interrelationship, it is especially appropriate to consider the effects of choline on brain development whenever we think about the mechanisms that might underlie the effects of dietary folate on brain and neural tube development.

CHOLINE AND PREGNANCY

Given the essential role of choline in cell function, nature has developed a number of mechanisms to ensure that a developing animal gets adequate amounts of choline. In mammals, the placenta transports choline to the fetus

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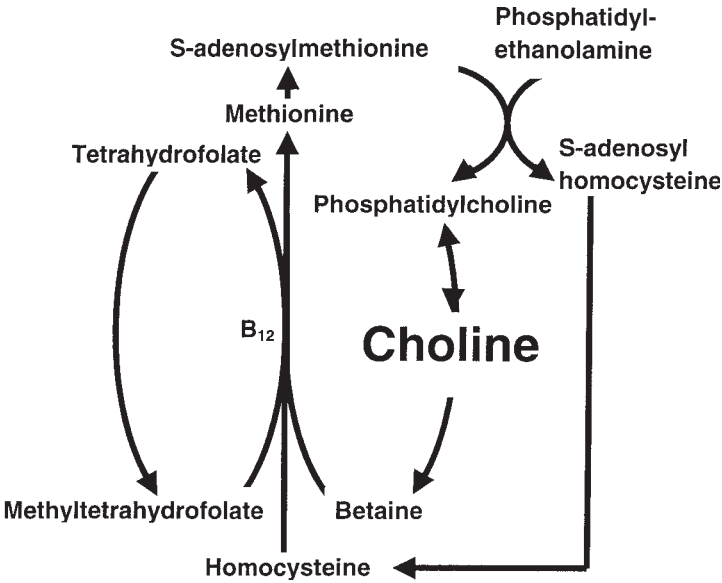

Fig. 1. Folate and choline metabolism are highly interrelated. Choline, via its oxidation product betaine, can donate a methyl-group to homocysteine, forming methionine. In parallel, methyltetrahydrofolate is an alternative donor of a methyl-group to homocysteine. Choline can also be formed *de novo* from methyl-groups (derived from *S*-adenoslymethionine) and phosphatidylethanolamine.

(17); human amniotic fluid choline concentration is 10-fold greater than that present in maternal blood (Zeisel, unpublished observations). This depletes maternal stores of choline *(18)*. Because so much choline must be transported to the infant, pregnancy may be a time when dietary supplies of choline are especially limiting. Though female rats are resistant to choline deficiency, pregnant rats are as vulnerable to deficiency, as are males *(19)*. The capacity of the brain to extract choline from blood is greatest during the neonatal period *(20)*. There is a novel phosphatidylethanolamine-*N*-methyltransferase (synthesizes choline *de novo*) in neonatal rat brain that is extremely active *(21)*. This special enzyme is not present in the adult brain. In the brains of newborn rats, *S*-adenosylmethionine concentrations are 40–50 nmol/g of tissue *(22)*. These levels are probably sufficient to enable the neonatal form of phosphatidylethanolamine-*N*-methyltransferase to maintain high rates of activity. At birth, humans and other mammals have plasma choline concentrations that are much higher than those in adults *(23)*. The supply of choline to the infant after birth is maintained, as mammary epithelial cells are capable of concentrative uptake of choline from maternal blood *(24)*. Human and rat milk provide large amounts of choline to the neonate *(25–27)*. Because so much choline is transferred in milk, lactating rats are more sensitive to choline deficiency than are nonlactating rats *(19)*. These

multiple mechanisms, which have evolved for ensuring the availability of choline to the fetus and neonate, suggest that choline supply must be crucial during the perinatal period.

CHOLINE AND BRAIN DEVELOPMENT

Choline availability during embryogenesis and perinatal development are especially important for brain development. There are two sensitive periods in rat brain development during which treatment with choline (about 1 mmol/d administered to the mother during a critical period during pregnancy, or 300 mg/kg administered subcutaneously 2 wk after after birth) produces long-lasting enhancement of spatial memory that is lifelong *(28–36)*. The first critical period occurs during embryonic d 12–17 (rats give birth on d 21) and the second occurs during postnatal days 16–30. Choline supplementation during these critical periods elicits a major improvement in memory performance at all stages of training on a 12-arm radial maze. The two sensitive periods for memory responsiveness to supplemental choline correlate with the formation of cholinergic neurons (neurogenesis; prenatal) and with the formation of nerve–nerve connections (synaptogenesis; prenatal and postnatal) in the hippocampus and basal forebrain. Neurogenesis of cholinergic cells of the basal forebrain occurs between embryonic d 12 and 17 (E12, E17) in the rat. In the hippocampal formation, the majority of cells are produced prenatally, but production continues into adulthood, particularly in the dentate gyrus (e.g., hippocampus–Ammon's horn: E15–E17; dentate gyrus: E19–postnatal d 5 (P5); cortex: E15–E19) *(37,38)*. The choline-related memory changes were correlated with altered distribution and morphology of septal neurons in the adult animals *(30)*, including larger cell bodies *(35)*, and with changes in the electrophysiological properties of the hippocampi in supplemented or deficient animals *(39,40)*. The choline-supplementation memory changes are also correlated with changes in the neurotransmitter-related enzymes present in the adult brains *(34,41)*.

POTENTIAL MECHANISMS FOR CHOLINE'S EFFECTS ON THE BRAIN

The mechanisms whereby choline availability affects brain development and cognitive functions are just beginning to be investigated. Because cholinergic neurotransmission is important in physiology of memory, choline (or choline-containing esters) has been used as a possible memory-improvement drug *(42–45)*. Choline's relationships to acetylcholine synthesis have been thoroughly reviewed elsewhere *(46)*. Our initial hypothesis was that the effects of neonatal choline supplementation on memory were mediated

by increased brain choline with subsequent increased acetylcholine release. However, the amounts of choline that accumulate in fetal brain after treatment of the pregnant dam *(47)* do not appear to be of sufficient magnitude to enhance acetylcholine release, as hypothesized. Rather, supplementing choline to dams resulted in significantly greater accumulation of phosphocholine and betaine in fetal brain than in fetuses of controls *(47)*. Although these biochemical changes may be important in themselves, we feel that the likely mechanism explaining differences in memory involves choline-mediated alterations in the birth, migration, and death of cells in the memory centers of brain during critical periods in the development of these centers *(48,49)*.

CHANGES IN CELL BIRTH AND MIGRATION

Cell birth and migration in the hippocampus and septum initially involve progenitor cells residing in the neuroepithelial layer adjacent to these brain regions. In the developing hippocampus, thymidine-labeling studies established the existence of three distinct developmental domains of neurogenesis in the ventricular neuroepithelium: One region provides cells for the development of Ammon's horn (i.e., pyramidal cells); a second region provides cells for the development of the dentate gyrus (i.e., granule cells); a third region provides cells destined to populate the fimbria *(50–53)*. The multipotent progenitor-type cells generated by this proliferative activity in the neuroepithelial layer ultimately give rise to the neurons (and glial cells) able not only to form neural networks within the hippocampus but also to innervate functionally related brain regions (e.g., basal forebrain, amygdala) *(54)*. We found that feeding a choline-deficient diet for E12–E18 reduced mitosis and the birth of cells in the progenitor neuroepithelial ventricular zone in E18 hippocampus *(48,49)*. Feeding a choline-supplemented diet increased the birth of cells in the neuroepithelial layer of the hippocampus on E16 and resulted in an increased number of cells populating subregions of the hippocampus on E18 *(48,49)*. In the lateral septum, choline-deficient diet altered the genesis and migration of cells born on E16 and E17 *(49)*. Individual progenitor cells in the ventricular neuroepithelium are capable of giving rise to the full array of neuronal and glial cell types in the developing brain *(55)*. However, we do not know the identity (e.g., glial vs neuronal) and developmental lineage (i.e., cholinergic, GABAergic) of migrating cells for which choline influences development. We reported that decreased availability of choline in the diet increased the expression of the TOAD-64 protein *(49)*. The TOAD-64 protein is an early embryonic postmitotic marker of neuronal differentiation that is expressed for a finite period of time in the

fetal rat brain *(56)*. Recent studies also indicate that TOAD-64 has homology to proteins involved in axonal pathfinding *(57)*.

CHOLINE AND CELL CYCLING

The brain is not the only organ in which choline increases mitosis. The availability of choline alters cell proliferation in the adult rat liver *(58)*, and in cultured liver cells choline availability influences cell cycling by affecting the availability of phosphatidylcholine in membranes *(53,59)*. Choline deficiency alters the expression of growth factors and cell cycle regulatory factors (e.g., TGFβ1, $p27^{Kip1}$) known to alter mitotic activity *(60,61)*. During fetal brain development, correct cell cycle control appears to be essential for correct timing of differentiation. In most tissues, the onset of differentiation occurs upon exit from the cell cycle during the G_1 phase *(62)*. Inhibition of the G_1 to S phase transition is under the control of Cdk–cyclin complexes, the activities of which are regulated by cyclin-dependent kinase inhibitors (CDKIs). These CDKIs have been assigned to the CIP/KIP (p21, p27, and p57) or Ink4 (p15, p16, p18, and p19) families based on their preferred Cdk–cyclin targets, subcellular localization, and other factors *(63)*. Members of the INK4 family inhibit the activity of Cdk4/Cdk6–cylcin D complexes, whereas all G_1 Cdk–cyclin complexes are inhibited by members of the CIP/KIP family *(64)*. We have just completed studies on cyclin inhibitors in the brain (*64a*). In choline-supplemented animals compared to controls, the number of cells with nuclear immunoreactivity for the p15Ink4b CDKI protein was decreased twofold to threefold in neuroepithelium of the dentate gyrus and Ammon's horn regions of fetal hippocampus. In contrast, maternal dietary choline deficiency significantly decreased nuclear p15Ink4b immunoreactivity in dentate gyrus neuroepithelial layer. Maternal dietary choline supplementation decreased the cytoplasmic immunoreactivity for p27Kip1 throughout the fetal hippocampus compared to control animals. Choline deficiency increased the expression of p27Kip1 throughout the hippocampus in association with increased expression of MAP-1 and vimentin proteins. These results link maternal dietary choline availability to CDKI protein expression and commitment to differentiation during fetal hippocampal development.

CHOLINE AND APOPTOSIS

Choline deficiency also resulted in an increase in apoptosis in a specific region of the hippocampus, namely the dentate gyrus. We observed an inverse correlation between apoptosis rates in the brains of rat fetuses and dietary intake of choline by their mothers *(65)*. Others have reported

apoptosis in the hippocampus and cortex of rats during the perinatal period *(66,67)*; we were the first to report an effect of maternal nutrition. Apoptosis is usually inducible when cells are dividing and then undergo checkpoint arrest *(68)*; cells in the resting phase are not susceptible *(69)*. Why do choline-deficient cells die by apoptosis? Inhibition of transmethylation reactions by pharmacologic inhibitors *(70,71)* or by folate deficiency *(72,73)* can induce apoptotic cell death. However, because methyl supplementation with betaine, methionine, folate, or vitamin B_{12} did not prevent apoptotic death induced by choline deficiency in hepatocytes, it must be that depletion of intracellular choline moieties rather than depletion of methyl groups was the critical parameter involved in choline deficiency's induction of apoptosis *(4)*. Synthesis of phosphatidylcholine is needed for progression of the cell cycle *(53,59,74)*. Cells cultivated in choline-deficient medium are arrested in the G_1 phase *(53)* and many studies have suggested that events during G_1 can trigger apoptosis *(75)*. Inhibition of phosphatidylcholine synthesis by pharmacological inhibitors induces apoptosis *(76–81)*. This apoptotic cell death could be partially prevented by phosphatidylcholine or lysophosphatidylcholine supplementation. All of these data suggest that phosphatidylcholine is the critical molecule that is involved in protection from apoptosis. This hypothesis was supported by an observation that cells that were incapable of phosphatidylcholine synthesis by the CDP–choline pathway died by apoptosis *(82)* and by reports that a genetic defect in phosphatidylcholine biosynthesis triggers apoptosis *(83)*. We did not observe diminished phosphatidylcholine concentrations within fetal hippocampi from choline-deficient dams compared to choline-sufficient or supplemented groups *(65)*. We did observe a significant increase in hippocampal phosphocholine concentrations in the supplemented group *(65)*. Phosphocholine may be the important active derivative of choline in our brain model; in NIH 3T3 cells, generation of phosphocholine from phosphatidylcholine by phospholipase D and choline kinase is required for the induction of DNA synthesis (*84*).

Phosphatidylcholine is a precursor of another important phospholipid, sphingomyelin, and, in turn, sphingomyelin is a precursor for formation of ceramide. Ceramide is an important mediator of apoptosis *(85)* and we believe that it is an important intermediate in choline-deficiency-induced apoptosis. Ceramide concentrations increased prior to the increase in apoptosis in primary neuronal cells and in PC12 cells (refs. *6* and *85a*). In PC12 cells, we previously demonstrated that exogenous ceramide induced apoptosis and that inhibition of choline-deficiency-induced apoptosis was associated with correction of intracellular ceramide levels *(6)*. In several

described paradigms of apoptosis, ceramide generated from sphingomyelin hydrolysis mediates apoptosis induced by stimuli such as tumor necrosis factor α, ultraviolet radiation, and activation of the CD95 receptor *(86,87)*. Also, an increase in *de novo* ceramide synthesis may induce apoptosis *(88,89)*.

BALANCING MITOSIS AND APOPOSIS

Apoptosis and cell proliferation are two interrelated processes that regulate cell number during fetal brain development *(66,67,90,91)*. The significance of perturbation in the balance of these events in brain development is not well understood. This is a critical issue for brain regions that are no longer completely self-renewing in the adult, because any transitory increase in apoptosis during development would lead to a long-lasting change in brain cytoarchitecture. Our studies show that a decreased availability of choline during E12–E18 window of brain development leads to an increase in apoptosis that is focused primarily in the dentate gyrus. However, our results also suggest that the effects of choline availability on cell proliferation, migration, and commitment to differentiation are quantitatively more significant for brain development than is apoptosis during this period of gestation.

SUMMARY

Are these findings in rats likely to be true in humans? We do not know. Human and rat brains mature at different rates; comparable time frames in human brain to E12–E18 in the rat would be approx 20–30 wk of gestation. Although the thought that maternal nutrition could dramatically alter infant development would have been received with skepticism a decade ago, the experience with folate and neural tube closure helped us to understand the importance of maternal nutrition during critical periods during pregnancy. This concept is reconfirmed by the effects of choline on the developing hippocampus and septum, which appear to of significant consequence for cognitive function later in life.

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11

Nutrient Interactions Between Folate and Zinc or Copper

Their Possible Implications to Pregnancy Outcome

Tsunenobu Tamura

INTRODUCTION

The objectives of this chapter are to review (1) the relationship between pregnancy outcome and nutriture of folate, zinc, and copper, (2) the interactions of these three nutrients, particularly folate metabolism in zinc or copper deficiency, and (3) these interactions in possible relation to pregnancy outcome. Extensive reviews on the role of zinc as well as micronutrients, in general, to pregnancy outcome and the interaction between zinc and folate have recently been published *(1–4)*.

FOLATE NUTRITURE AND PREGNANCY OUTCOME

Folate Deficiency and Pregnancy Complications

Folate deficiency is believed to be one of the most common nutrient deficiencies worldwide and is common during pregnancy, possibly the result of increased demand for fetal growth and poor dietary folate intake in certain countries. Folate may be one of the most widely studied vitamins in relation to pregnancy outcome. It is well known that plasma and red cell folate concentrations decline throughout pregnancy, although the exact mechanism is unknown *(5)*. In addition to megaloblastic anemia during pregnancy, a result of severe folate deficiency, various pregnancy complications, such as abruptio placentae, spontaneous abortion, and preterm delivery have been associated with folate deficiency *(6–8)*; however, the findings of these complications are not always unequivocal *(9–11)*. To prevent pregnancy-induced anemia and to overcome these pregnancy complications, prenatal folic acid (pteroylglutamic acid) supplementation has been practiced in Europe and the United States since the 1960s *(5)*.

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Table 1
Trials to Evaluate the Effect of Prenatal Folic Acid Supplementation on Birth Weight

Investigators (ref.)	Year	Country	Folic acid given (mg/d)	Mean birth weight increase (g)
Baumslag et al. *(12)*	1970	South Africa	5.0	370
Giles et al. *(17)*	1971	Australia	5.0	None
Fleming et al. *(18)*	1974	Australia	0.5	None
Iyengar and Rajalakshmi *(13)*	1975	India	0.2–0.5	200
Rolschau et al. *(14)*	1979	Denmark	5.0	407
Tchernia et al. *(15)*	1982	France	0.35	157
Agarwal et al. *(16)*	1991	India	0.5	290

Folate Nutriture and Fetal Growth

Many investigators evaluated the effect of folate nutriture on fetal growth. As shown in Table 1, several groups of researchers have reported that folic acid supplementation resulted in the increase in birth weight of infants born to women with inadequate dietary folate intake during pregnancy *(12–16)*. On the other hand, two Australian groups did not observe such a positive effect of folic acid supplementation on birth weight, probably the result of adequate folate nutriture of the subjects before the trials began *(17,18)*.

We examined the association between folate nutriture, as assessed by the determination of serum folate concentrations during pregnancy, and various measures of pregnancy complications and outcome measures, including birth weight *(19)*. In this study, the subjects were participants of a large-scale study to identify risk factors for having infants with fetal-growth restriction (FGR). They were from a medically indigent population in the vicinity of Birmingham, Alabama and were offered daily supplementation of folic acid (1.0 mg) throughout pregnancy starting at the first prenatal visit (about 16 wk gestation). Blood samples were collected at averages of 18 and 30 wk gestation, and serum folate was measured by microbiological assay. Based on serum folate concentrations, we estimated the compliance of taking folic acid supplements to be about 50%. We found that the mean birth weight was significantly higher among newborns who were born to mothers with higher serum folate concentrations than those with lower concentrations at either 18 or 30 wk gestation. The groups of mothers with higher serum folate concentrations had a less prevalent rate of FGR and maternal

infections than those with lower folate concentrations *(19)*. Our findings are consistent with those by other investigators *(20–23)*. Furthermore, we evaluated a psychosocial profile in these women, including measures of depression, anxiety, self-esteem, mastery, stress, and social support, and compared the scores of the psychosocial profile to serum folate concentrations and pregnancy outcome *(24)*. These analyses indicated that lower serum folate concentrations were associated with poorer psychosocial scores, and higher serum folate concentrations were associated with higher infant birth weights regardless of maternal psychological scores. These findings suggest that pregnant women with better psychological scores are more likely to demonstrate higher compliance in taking folic acid, which is related to higher birth weight of infants and a lower risk of FGR *(24)*.

All findings combined, it may be safe to conclude that adequate folate nutriture during pregnancy is important for fetal growth. Furthermore, folic acid supplementation during pregnancy is beneficial for fetal growth, if women are at risk of folate deficiency as a result of inadequate dietary intake.

Folate and Neural Tube Defects

During the last decade, one of the most significant findings in the area of folate in terms of disease prevention and health maintenance is the beneficial effect of periconceptional folic acid supplementation in reducing the rate of pregnancies complicated with neural tube defects (NTD). Two large-scale European studies clearly demonstrated that the recurrence or occurrence of NTD-affected pregnancies can be prevented, to a certain extent, by folic acid supplementation before the time of neural tube closure at about the fourth week of gestation *(25,26)*. However, the mechanisms are unknown as to how the excess amount of folic acid supplementation prevents the development of NTD. As a possible mechanism, several groups of investigators have proposed the association between elevated homocysteine concentrations in maternal plasma/serum and amniotic fluid and the development of NTD as well as other pregnancy complications *(27–29)*. Homozygous or heterozygous thermolabile genotypes of 5,10-methylene tetrahydrofolate reductase have also been associated with the increased risk for the development of NTD *(30–32)*. Further research is needed to identify how folate is involved in the development of these devastating malformations. To reduce the rate of pregnancies complicated with NTD, folic acid fortification of cereal grain products in the United States was mandated in early 1998 *(33)*. The report on the effect of this fortification on the rate of pregnancies complicated with NTD should be available in a few years.

ZINC NUTRITURE AND PREGNANCY OUTCOME

Zinc plays vital roles in normal fetal growth and development during pregnancy. Many investigators have shown that zinc deficiency results not only in FGR but also malformations in animals *(34)*. In human pregnancy, results of numerous studies have indicated the significant association between various measures of pregnancy outcome and zinc nutriture; however, the conclusions are not always consistent, as reviewed previously *(1)*.

Zinc Nutriture and Fetal Growth

To date, over 40 human studies have been carried out to evaluate the association between maternal zinc nutriture during pregnancy and fetal growth. Approximately one-half of the studies indicates that a one-time assessment of maternal zinc nutriture during pregnancy is a suitable predictor of fetal growth, but the other half rejects such a notion. The majority of these studies were previously reviewed *(1)*. It is likely that the major reason for such a dispute is because of the lack of a method to accurately assess zinc nutriture of pregnant women. In most of these studies, zinc concentrations in maternal blood, particularly serum or plasma, were used to assess zinc nutriture during pregnancy. It has been well established that serum/plasma zinc concentrations decline steadily throughout pregnancy and this decline makes it very difficult to use serum/plasma concentrations as a dependable index for zinc nutriture among women with different gestational ages at the time of blood drawing *(1)*.

We have recently analyzed the information of plasma zinc concentrations and various measures on pregnancy outcome including complications during pregnancy and delivery, as well as anthropometric measures and Apgar scores of neonates in a total of 3448 medically indigent women (85% African-American and 15% Caucasian) *(35)*. Each of these subjects gave a blood sample at about 16 wk gestation for the screening of eligibility to participate in a randomized double-blind clinical trial to evaluate the effect of zinc supplementation during pregnancy on fetal growth *(36)*. The association was evaluated after plasma zinc concentrations were adjusted for gestational age. We found no significant association between plasma zinc concentrations and any measures of pregnancy complications (Table 2) as well as birth weight. Therefore, we conclude that a single determination of plasma zinc concentrations during the late first trimester to the early third trimester may not be suitable for predicting pregnancy outcome in women of a low-socioeconomic background. For the zinc-supplementation trial, we selected a total of 580 subjects who had plasma zinc concentrations below the 50th percentile from the entire population of 3448 pregnant women *(36)*. In this trial, we

Table 2
Relationship Between Prevalence (%) of Maternal Complications and Quartiles of Plasma Zinc Concentrations

	All subjects (n = 3448)	Lowest quartile (n = 826)	Upper three quartiles (n = 2586)
Fetal growth restriction	4.5	4.8	4.4
Preterm delivery (< 37 wk gestation)	14.7	15.3	14.5
Early preterm delivery (< 32 wk gestation)	4.0	3.4	4.2
Hypertension	7.9	7.7	7.9
Amnionitis	4.2	3.8	4.4
Postpartum infection	6.2	6.1	6.2

Note: None of the differences between the lowest quartile and the upper three quartiles were significant.

Source: ref. *35*.

found a positive effect of zinc supplementation during pregnancy on fetal growth, indicating that at least one-half of the entire population had suboptimal zinc nutriture. If plasma zinc concentration at about 16 wk gestation is a reliable indicator of zinc nutriture, we should have found an association(s) between plasma zinc concentrations and various measures of pregnancy outcome. The lack of such an association suggests that plasma zinc concentration during the second trimester is not a dependable indicator of zinc nutriture.

Effect of Zinc Supplementation on Fetal Growth

In addition to the conflicting findings on the relationship between maternal zinc nutriture and fetal growth found by many investigators, the consensus on the effect of zinc supplementation during pregnancy on various aspects of pregnancy outcome has not been achieved *(1)*. To the author's knowledge, a total of 12 trials have been performed worldwide, as shown in Table 3 *(36–48)*. Among these, only one double-blind clinical trial indicated a positive effect on fetal growth *(36)*. This trial was carried out between 1991 and 1994 to evaluate the effect of zinc supplementation on pregnancy outcome among 580 women with a low-socioeconomic background in the Birmingham area. As described in the preceding subsection, over 3400 women were screened for their plasma zinc concentrations at the mean of 16 wk gestation. In this study, only African-Americans with plasma zinc concentrations below the 50th percentile of the population were selected. One-

Table 3
Trials to Evaluate the Effect of Zinc Supplementation During Pregnancy on Fetal Growth

Investigators (ref.)	Country	No. of subjects (zinc/placebo)	Dose of elemental zinc (mg/d)	Effect of zinc
Jameson *(37)*	Sweden	64/248	45	Increased rate of uneventful delivery
Hunt et al. *(38)*	USA	107/106	20	Reduced rate of pregnancy-induced hypertension
Hunt et al. *(39)*	USA	70/68	20	Reduced rate of low serum zinc
Ross et al. *(40)*	South Africa	32/33	4.6 – 12.9	None
Kynast and Selig *(41)*	Germany	170/345	(20; zinc asparate)	None
Mahomed et al. *(42)*	UK	246/248	20	None
Cherry et al. *(43)*	USA	266/288	30	Reduced rate of prematurity
Simmer et al. *(44)*	UK	30/26	22.5	None
Goldenberg et al. *(36)*	USA	294/286	25	Increased birth weight and head circumference
Jonsson et al. *(45)*	Denmark	585/621	44	None
Caulfield et al. *(46,48)*	Peru	488/469	15	Improved fetal neurobehavioral development
Osendarp et al. *(47)*	Bangladeshi	269/290	30	None

half of the subjects was given zinc supplementation (25 mg of elemental zinc/d) between about 19 wk gestation and delivery; the other half received placebo. We found a positive effect of prenatal zinc supplementation on birth weight and head circumference of the infant *(36)*. In contrast, the investigators found no positive effect of zinc supplementation on fetal growth in other studies, including three recent trials using a large number of subjects conducted in Denmark, Peru, and Bangladeshi *(45–47)*. In the study carried out in Peru, however, zinc supplementation was associated with the improvement in neurobehavioral development of fetuses *(48)*. Caulfield et al. *(46)* presented an extensive discussion on possible reasons for the outcome differences between the studies in Peru and Birmingham. It remains to be seen whether our finding of the positive effect of zinc supplementation on fetal growth in indigent African-Americans with low plasma concentrations in mid-pregnancy can be applied to other populations *(1,36)*.

Zinc Deficiency and Malformations of the Central Nervous System

It is well established that prenatal zinc deficiency produces fetal malformations, particularly of the central nervous system in experimental animals *(34,49)*. However, the results are far from conclusive in humans *(50–52)*. Therefore, at present, it is difficult to draw a conclusion as to whether zinc deficiency during pregnancy results in the development of malformations of the central nervous system in humans *(1)*.

COPPER NUTRITURE AND PREGNANCY OUTCOME

Copper is involved in numerous biochemical reactions, and its importance in normal growth and development of the fetus is well established in animals *(34,53–55)*. Copper deficiency during pregnancy is known to induce neurological abnormalities, skeletal malformations, and abnormal development of the heart and lung in offspring of various animals *(34,55)*. Although the findings of these abnormalities are clear in experimental animals, the consensus on the effect of copper deficiency in humans has not been achieved.

Copper Deficiency and Fetal Growth

Many investigators have tried to identify the association between copper nutriture of either pregnant women or newborns and fetal growth. As shown in Table 4, to the author's knowledge, a total of 16 investigations have been performed to date showing contradicting findings *(56–71)*. The determination of copper concentrations in maternal or cord blood was mainly used to assess the nutriture of the subjects. In 5 of the 16 investigations, researchers

Table 4
Relationship Between Copper Indices and Fetal Growth

Investigators (ref.)	Year	Location	No. of subjects	Pregnancy outcome measure	Indices
Dawson et al. *(56)*	1969	USA	48	Fetal growth retardation	No difference in placental copper
Krishnamachri and Rao *(57)*	1972	India	40	Birth weight	Positive correlation with cord ceruloplasmin
Bogden et al. *(58)*	1978	USA	25	Low birth weight	No difference in maternal and cord copper
Anand et al. *(59)*	1981	India	39	Fetal growth	No difference in maternal serum copper
Goel and Misra *(60)*	1982	India	20	Fetal growth retardation	Low cord plasma copper
Ette and Ibeziako *(61)*	1985	Nigeria	90	Birth weight	Positive correlation with cord and maternal copper
Mameesh et al. *(62)*	1985	Kuwait	57	Birth weight	Positive correlation with maternal copper
Fehily et al. *(63)*	1985	Egypt	23	Birth weight	Positive correlation with maternal copper
Bro et al. *(64)*	1988	Denmark	47	Fetal growth retardation	Elevated serum copper
Okonofua et al. *(65)*	1989	Nigeria	58	Birth weight	No association with maternal plasma copper
Borella et al. *(66)*	1990	Italy	16	Fetal growth retardation	Elevated plasma copper
Okonofua et al. *(67)*	1990	Nigeria	26	Birth weight	Positive correlation with cord and maternal copper
Pro-Jordanova and Bogdanova *(68)*	1992	Yugoslavia	79	Birth weight	No association with cord serum copper
Wasowicz et al. *(69)*	1993	Poland	67	Birth weight	Negative correlation with maternal copper
Arnaud et al. *(70)*	1994	Zaire	166	Birth weight	Positive association with cord serum copper
Grebremeskel et al. *(71)*	1994	UK	79	Birth weight and head circumference	Negative association with cord serum copper

Table 5
Studies Showing Associations Between Copper Indices and Pregnancy Complications

Investigators (ref.)	Year	Location	No. of subjects	Complications	Indices
O'Leary et al. *(72)*	1966	USA	12	Preeclampsia	Elevated serum copper
Schenker et al. *(73)*	1969	Israel	28	Preeclampsia	Elevated serum copper
O'Leary (74)	1969	USA	2	Hypertension and fetal death	Low serum copper
Friedman et al. *(75)*	1969	Israel	7	Premature labor	Low serum copper
			10	Preeclampsia	Low serum copper
			118	Threatened abortion	No difference in serum copper
Schenker and Jungreis *(76)*	1970	Israel	107	Missed abortion	Low serum copper
Schenker et al. *(77)*	1972	Israel	38	Preeclampsia	Elevated serum copper
Ylöstalo and Ylöstalo *(78)*	1973	Finland	114	Preeclampsia	Elevated serum copper
Fattah et al. *(79)*	1976	Egypt	10	Severe preeclampsia	Elevated serum copper and ceruloplasmin
El-Shazly et al. *(80)*	1976	Egypt	10	Preeclampsia	Low serum copper and ceruloplasmin
Bassiouni and Refei *(81)*	1979	Egypt	24	Spontaneous abortion	Low plasma copper and ceruloplasmin
Artal et al. *(82)*	1979	USA	11	Premature rupture of membrane	Low plasma copper
Singhal et al. *(83)*	1982	India	29	Abortion	Low serum copper and ceruloplasmin
Kiilholma et al. *(84)*	1984	Finland	60	Premature rupture of membrane	Low serum copper and ceruloplasmin
Brophy et al. *(85)*	1985	USA	8	Preeclampsia	Elevated placental copper
Kalra et al. *(86)*	1989	India	75	Anemia	Elevated serum copper and ceruloplasmin
Borella et al. *(66)*	1990	Italy	24	Preeclampsia	Elevated plasma copper

found no association between copper nutriture and fetal growth *(56,58,59,65,68)*. A negative association between copper nutriture and fetal growth was found in four studies *(64,66,69,71)*. Therefore, only seven investigations provided a positive association between copper nutriture of mothers or newborns and fetal growth *(57,60–63,67,70)*.

Copper Nutriture and Pregnancy Complications

A total of 20 articles have been published on the association between copper indices and pregnancy complications, as summarized in Tables 5 and 6 *(56,66,72–89)*. In the majority of the studies, serum/plasma copper or ceruloplasmin concentrations were also used to assess copper nutriture during pregnancy. In 16 studies, some associations were found between maternal copper nutriture and pregnancy complications including preeclampsia, threatened abortion, and hyperemesis gravidarum (Table 5) *(66,72–86)*. However, as shown in Table 6, the data in the remaining four investigations indicated no association between copper nutriture and pregnancy complications *(56,87–89)*. Based on the information summarized in Tables 5 and 6, it is difficult to draw a reasonable conclusion. For example, in 8 of the 16 studies in which some associations were found, women with pregnancy complications had *elevated* copper or ceruloplasmin concentrations *(66,72,73,77–79,85–87)*, whereas in the remaining eight, the investigators concluded that *decreased* copper or ceruloplasmin concentrations are associated with pregnancy complications *(74–76,80–84)*.

Copper Deficiency and NTD

To date, a total of six groups of researchers have conducted studies to associate copper nutriture and various malformations, including NTD *(52,64,90–93)*. In these investigations, three presented positive associations between poor copper nutriture and the development of NTD, whereas the other three failed to show such an association (Table 7). Thus, unlike the animal studies *(34,55)*, it is difficult to draw any conclusion on the effect of copper nutriture during pregnancy on the development of malformations in humans.

In summary, there are several possible reasons for such inconclusive findings in regard to the relationship between copper nutriture and pregnancy complications or outcome measures including fetal growth and the development of malformations. First, plasma copper and ceruloplasmin concentrations increase steadily during pregnancy, probably as a result of hormonal changes *(73,94)*; therefore, the validity of these parameters is unknown for accurately assessing copper nutriture in pregnant women. Second, copper

Table 6
Studies Showing No Association Between Copper Indices and Pregnancy Complications

Investigators (ref.)	Year	Location	No. of subjects	Complications	Indices
Borglin and Heijkenskjöld *(87)*	1967	Sweden	43	Threatened abortion	Serum copper
Dawson et al. *(56)*	1969	USA	30	Preeclampsia	Placental copper
Prema *(88)*	1980	India	26	Preeclampsia	Serum copper
El Tabbakh et al. *(89)*	1989	Egypt	20	Hyperemesis gravidarum	Serum copper

Table 7
Relationship Between Copper Indices and Malformations

Investigators (ref.)	Year	Location	No. of subjects	Complications	Indices
Morton et al. *(90)*	1976	UK		NTD	Negative correlation with copper in tap water
Wald and Hambidge *(91)*	1977	UK	18	NTD (anencephaly)	No difference in maternal serum copper
Buamah et al. *(92)*	1984	UK	9	NTD (anencephaly)	Low maternal plasma copper
Bro et al. *(64)*	1988	Denmark	17	Malformations	No change in serum copper
Jiang *(93)*	1991	China	50	NTD	Low maternal serum copper
McMichael et al. *(52)*	1994	Australia	69	NTD	No association with maternal serum copper

concentrations and ceruloplasmin (either enzyme activity or immunoreactive protein) may not be a reliable and dependable indicator of copper nutriture, even in a nonpregnant state, unless the individual is severely copper deficient *(94)*. No other determination of copper indices has been tried to evaluate the relationship between copper nutriture and pregnancy outcome, which may include superoxide dismutase, cytochrome-*c* oxidase and peptidylglycine α-amidating mono-oxygenase *(94,95)*. However, at present, it is unknown whether these are influenced by pregnancy. The identification of a specific and sensitive indicator(s) of copper nutriture is needed.

FOLATE–ZINC INTERACTION AND PREGNANCY OUTCOME

In 1984, Mukherjee et al. *(96)* reported that high maternal plasma folate concentrations during pregnancy are associated with an increased rate of unfavorable pregnancy outcome measures, including complications during pregnancy and delivery, birth weight, and neonatal conditions. They identified the significant association between fetomaternal complications and the lowest quartile of plasma zinc and albumin concentrations with the highest quartile of plasma folate concentrations. Based on these findings, they suggested that folic acid supplementation during pregnancy negatively affects zinc absorption and impairs maternal zinc nutriture, hence increasing the rate of fetomaternal complications *(96)*.

We evaluated the relationship between serum folate and zinc concentrations at 18 and 30 wk gestation from medically indigent pregnant women *(19)*. There was a weak, but significant, positive correlation between folate and zinc concentrations at 30 wk gestation, whereas the relationship was not significant at 18 wk. Furthermore, no systematic associations between the mean birth weight or Apgar score of infants and serum folate or zinc concentrations were found. Our findings do not support the data by Mukherjee et al. *(96)* that folic acid supplementation during pregnancy adversely affects pregnancy outcome. An extensive review on nutrient interaction between folate and zinc has been published *(4)*.

FOLATE–COPPER INTERACTION AND PREGNANCY OUTCOME

The information on the interaction between folate and copper is limited *(97)*. To the author's knowledge, there has not been a report describing the relationship between folate–copper interaction and pregnancy outcome.

Table 8
Various Parameters in Zinc-Deficient and Copper-Deficient Rats

	Zinc-deficient group	Pair-fed, zinc-sufficient	Copper-deficient group	*Ad libitum*-fed control group
Dietary zinc/copper content	2/100	100/100	100/1	100/100
Body weight	34	42	92	100
Plasma zinc concentration	33[a]	95		100
Plasma copper concentration			6[a]	100
Hepatic methionine synthase activity	171[a]	129	79[a]	100
Plasma folate concentration	54[a]	87	113	100
Red cell folate concentration	109	105	123	100
Hepatic folate content	117	113	100	100
5-Methyltetrahydrofolate of total hepatic folate	82[a]	122	150[a]	100
Plasma homocysteine concentration	25[a]	34	161[a]	100

Note: Values of the *ad libitum*-fed control group were set at 100.
[a]Values in zinc-deficient group significantly different from those in the pair-fed, zinc-sufficient group and the values in the copper-deficient group were significantly different from the *ad libitum*-fed control group. *Source*: Data from refs. *98* and *99*.

FOLATE METABOLISM IN ZINC- OR COPPER-DEFICIENT RATS

In order to evaluate the effect of zinc deficiency on folate metabolism, we produced zinc-deficient male rats by feeding a low-zinc diet for 6 wk *(98,99)*. As shown in Table 8, various parameters relating to folate metabolism were measured and compared with two control groups (pair-fed or *ad libitum*-fed zinc-sufficient groups). Hepatic methionine synthase activities were increased in zinc-deficient rats compared to the controls. This increase resulted in a decreased 5-methyltetrahydrofolate in the liver of zinc-deficient rats, although the total liver folate concentrations were similar in all groups. Furthermore, plasma folate and homocysteine concentrations were lower in zinc-deficient rats than the control groups *(99)*. A metabolic scheme of folate and homocysteine in zinc deficiency is presented in Fig. 1.

Recently, we also studied folate and homocysteine metabolism in copper-deficient rats *(97)*. After 6 wk of treatment, hepatic methionine synthase activity in the copper-deficient group was significantly lower than that in

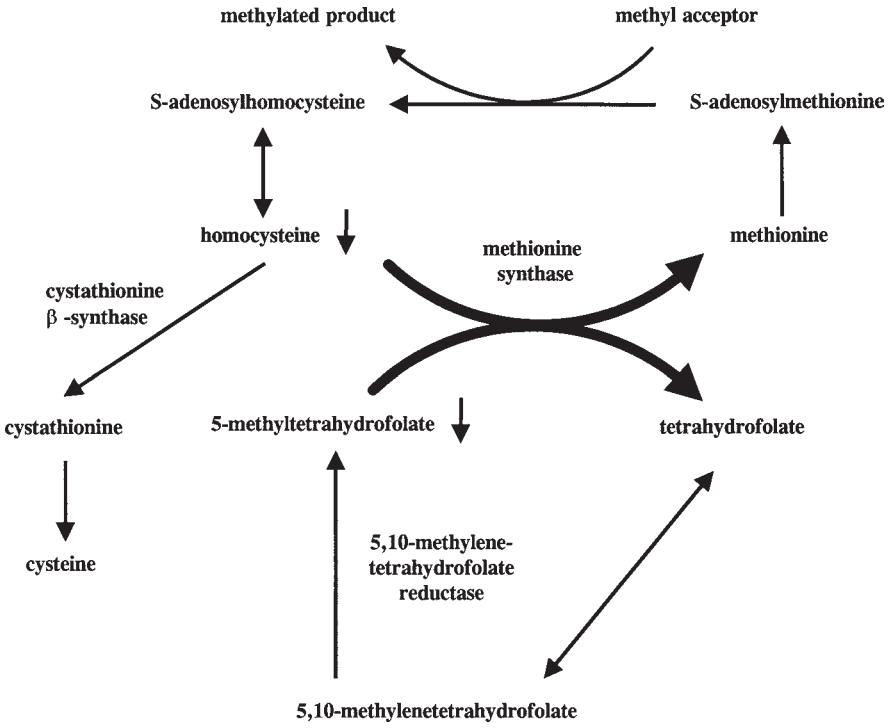

Fig. 1. Folate and homocysteine metabolism in zinc deficiency.

the control group (Table 6). Both plasma and red cell folate concentrations in the copper-deficient group were slightly higher, although not significant, than those in the controls. The percentage of 5-methyltetrahydrofolate in total hepatic folates in the copper-deficient group was significantly higher than in the control group; however, total hepatic folate concentrations were similar in both groups. The mean plasma homocysteine concentrations were significantly higher in copper-deficient rats than in the controls. Folate and homocysteine metabolism in copper deficiency is proposed in Fig. 2.

All of these changes in folate metabolism in zinc or copper deficiency are likely to be secondary to the alteration in methionine synthase activity. As depicted in Figs. 1 and 2, the alterations in folate metabolism observed in zinc deficiency are the reverse of those in copper deficiency, which is analogous to vitamin B_{12} deficiency *(100)*. Although the mechanism(s) of decreased hepatic methionine synthase activity in copper deficiency is unknown, it is possible to offer a few possible explanations. First, copper may be required for a structural part of the enzyme or as a cofactor for its

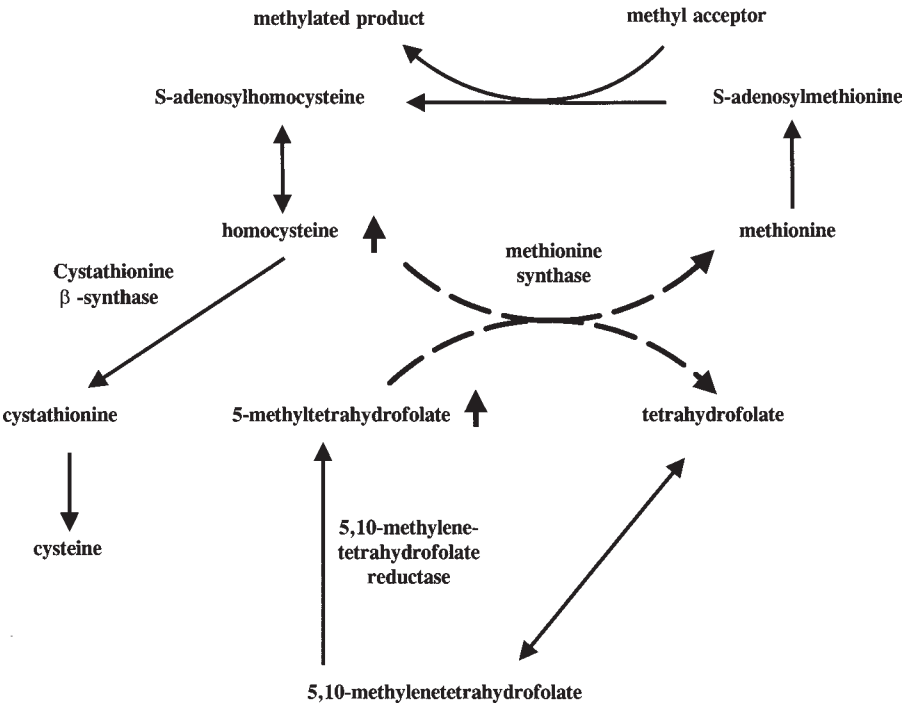

Fig. 2. Folate and homocysteine metabolism in copper deficiency.

activity. Second, copper may control the synthesis or degradation of the enzyme. Third, copper deficiency may cause alterations of an unidentified factor(s) that controls the activity or the synthesis (or degradation) of the enzyme. Considering the findings of studies of both zinc deficiency and copper deficiency in rats, it may be reasonable to postulate that a zinc–copper interaction (or the ratio of zinc to copper) regulates the activity or synthesis of methionine synthase.

What are the implications of these findings of altered folate metabolism observed in either zinc- or copper-deficient pregnant rats? Although we are not certain whether the data in this animal model can be extrapolated into humans, it may be reasonable to offer the possible implications of our findings. As for folate–zinc interaction, decreased folate concentrations in the maternal circulation in zinc deficiency may result in decreased placental folate transfer; thus, compromised folate nutriture may lead to fetal growth retardation or malformations of the central nervous system. With regard to folate–copper interaction, increased homocysteine in the maternal circulation, secondary to decreased methionine synthase, may be detrimental to

normal fetal growth and development. Increased homocysteine concentrations in the maternal circulation as well as in amniotic fluid are associated with the development of NTD *(27–29,101)*. Therefore, the alterations of folate metabolism caused by one of these two trace mineral deficiencies during pregnancy may be detrimental to fetal growth and development. Furthermore, the synergistic effect of the deficiencies of these three nutrients may play an important role in influencing pregnancy outcome.

SUMMARY

There is strong evidence that adequate folate nutriture is important for normal fetal growth and development in humans. It appears that adequate zinc nutriture during pregnancy is important for fetal growth as well. However, it is not clear whether compromised copper nutriture during pregnancy, the condition that is extremely rare among women of childbearing age, is detrimental to pregnancy outcome, although sufficient evidence is available that it induces malformations in animal models. In rats, either zinc or copper deficiency induces alterations in folate metabolism secondary to the changes in hepatic methionine synthase activity. The nutritional status of these nutrients may synergistically play an important role in fetal growth and development. Future investigations on the effect of nutrient interactions between folate and these two trace minerals on pregnancy outcome are warranted.

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12

Regulation of Folate Metabolism by Iron

A. Katherine Herbig and Patrick J. Stover

INTRODUCTION

Iron and folate are essential nutrients for mammals and share an overlapping clinical and basic research history that suggests these two nutrients may interact. Simultaneous and independent deficiencies of these nutrients are common and are more prevalent during states of increased physiological demand and utilization, including pregnancy and lactation *(1–5)*. Maternal folate or iron deficiency during gestation impacts both the mother and the developing embryo with elevated risks for anemia, mortality, premature delivery, and low birth weight *(2–9)*. Clinically, nutritional imbalances of either nutrient are associated with anemia, neutrophil hypersegmentation, certain cancers, and cognitive decline *(6,10–12)*. At the molecular level, both serve as coenzymes or cofactors for enzymatic reactions and are essential for the synthesis of deoxyribonucleotides and, therefore, for DNA replication and repair *(13–15)*. Finally, both nutrients have been the targets of public health intervention policies and are often coadministered as iron–folate supplements *(16)*.

Although many studies have demonstrated that folate and iron deficiencies can occur independently, other human clinical and experimental animal studies have indicated that folate-dependent biosynthetic reactions are impaired and perhaps regulated by alterations in iron metabolism. Iron deficiency may trigger clinical and biochemical manifestations of folate deficiency without evidence for frank folate deficiency. However, results from these studies have been inconsistent, and molecular mechanisms that may account for this interaction have only been put forward recently. Therefore, a direct biochemical relationship between iron and folate has yet to be established. In 1991, O'Connor *(17)* reviewed the evidence supporting an iron–folate relationship, highlighting the discrepancies that existed within

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the literature. This chapter focuses on recent molecular studies that demonstrate that iron can directly influence folate metabolism at the level of gene expression and attempts to reevaluate the historical clinical and animal literature in light of the emerging biochemical mechanisms.

IRON AND FOLATE IN MAMMALIAN DEVELOPMENT

Impairments of folate metabolism are commonly found in human populations and can result from inadequate dietary intake, intestinal malabsorption, increased rates of folate turnover, and single nucleotide polymorphisms in genes that encode key folate-metabolizing enzymes *(6,9,18,19)*. In the cell, folate serves as a family of at least six distinct one-carbon-substituted cofactors that accept, carry, and donate one-carbon units for enzymatic reactions associated with purine and thymidylate biosyntheses and with the remethylation of homocysteine to methionine (Fig. 1) *(13–15)*. Methionine can be converted to *S*-adenosylmethionine (SAM), a cofactor that is required for the methylation of numerous substrates, including DNA, RNA, proteins, and many metabolites *(20)*. The one-carbon units carried by tetrahydrofolate can be present at one of three oxidation states: that of formate in the form of 10-formyltetrahydrofolate, formaldehyde in the form of methylene tetrahydrofolate, or methanol in the form of 5-methyltetrahydrofolate (Fig. 1). Each folate-dependent biosynthetic pathway requires a specific folate one-carbon derivative, although the one-carbon forms of folate can be interconverted.

Results from several studies have indicated that folate-dependent deoxyribonucleotide biosynthesis (purine and thymidylate biosyntheses) and homocysteine remethylation (leading to SAM biosynthesis) vie for a limiting pool of folate cofactors and that SAM-dependent reactions have a "metabolic priority" over DNA precursor synthesis *(21–23)*. This metabolic competition is possible because the concentration of cellular folate-binding proteins exceeds the cellular concentration of folate, and folate cofactors are, therefore, limiting for folate-dependent biosynthetic reactions *(24–26)*. Limiting concentrations of intracellular folate cofactors might serve a regulatory function: Under limiting conditions, increasing the flux of folate one-carbon units through a particular biosynthetic pathway compromises other folate-dependent pathways, and these alterations in folate metabolism result in changes in the relative steady-state one-carbon distribution of intracellular folate cofactors *(27)*.

Thymidine biosynthesis and homocysteine methylation are highly sensitive to compromised folate status. Impaired thymidylate biosynthesis increases the rate of uracil misincorporation into DNA *(28)*, whereas com-

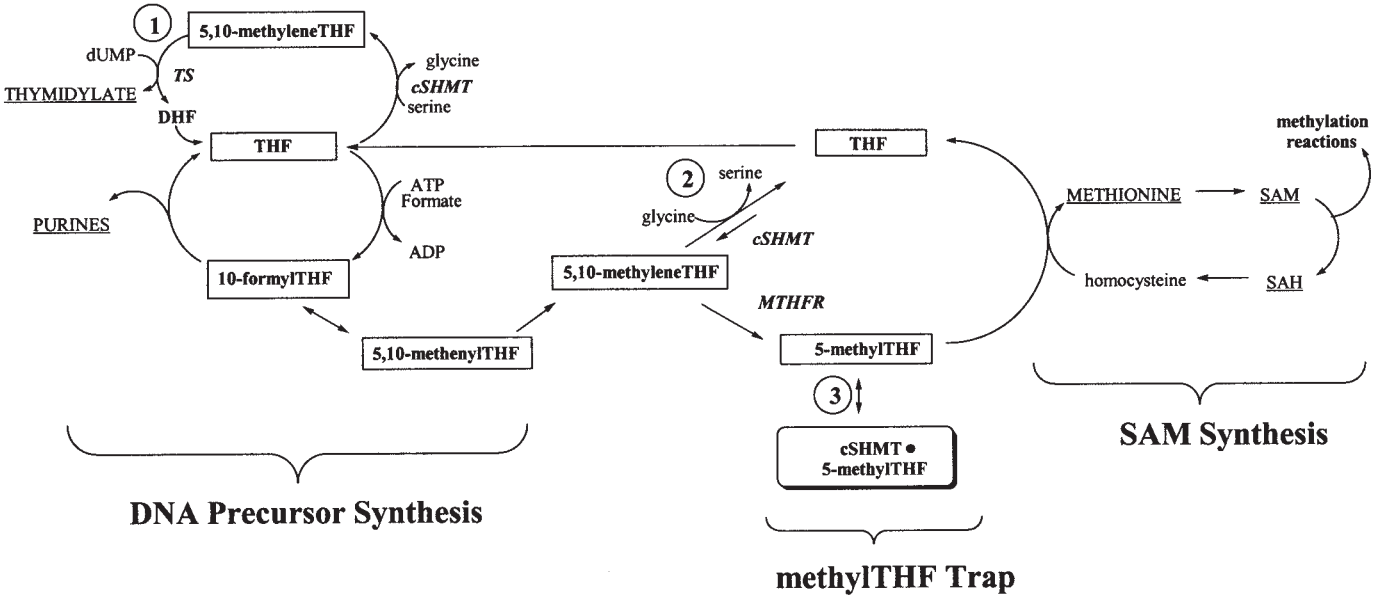

Fig. 1. Cytoplasmic folate metabolism. Folate metabolism in the cytoplasm is required for the synthesis of DNA precursors (purines and thymidine) and for the remethylation of homocysteine to methionine. The cytoplasmic serine hydroxymethyltransferase (cSHMT) enzyme plays at least three roles in folate metabolism. (1) It enhances the flux of methylenetetrahydrofolate through the thymidylate synthase (TS) reaction; (2) it competes with methylenetetrahydrofolate reductase (MTHFR) for methylene tetrahydrofolate; (3) it binds 5-methyltetrahydrofolate and creates a folate methyltrap. In this manner, cSHMT can enhance DNA precursor synthesis and inhibits homocysteine remethylation.

promised methionine synthesis results in elevated concentrations of serum homocysteine *(6)* (Fig. 1). Inhibition of these biosynthetic pathways leads to increased risk for specific diseases. Maternal folate deficiency in humans increases risk for bearing a child with neural tube defects (NTD), including spina bifida *(29,30)*, and maternal folate supplementation in mice can rescue NTD associated with genetic mutations in *Pax3* and *Cart1* mice *(31,32)*. For *Axd1* mice, risk for NTD can also be ameliorated with increased dietary methionine *(33)*, whereas thymidine, but not methionine, can rescue NTD in *Pax3* mice *(31)*. Human epidemiological studies have implicated impaired folate metabolism in other birth defects as well, including craniofacial defects and Down syndrome *(34,35)*. Folate deficiency is also associated with increased risk for certain cancers *(11)*. Increased uracil content in DNA leads to chromosome instability *(28)*, whereas elevated homocysteine is associated with impaired SAM synthesis and DNA hypomethylation, leading to subsequent alterations in the expression of methylation-sensitive genes that may be involved in neoplasic transformation *(11)*.

In contrast to the effects observed with folate deficiency, moderate maternal iron deficiency is not normally associated with an incidence of congenital malformations. Infants delivered by iron-deficient mothers normally do not display any symptoms of iron deficiency up to 1 yr of age *(36)*. The human fetus actively sequesters maternal iron, and the levels of serum iron and ferritin in humans are typically at their highest values at birth. However, maternal iron deficiency can result in low birth weight and premature delivery, outcomes that may have negative long-term consequences for the infant *(36,37)*.

BIOCHEMICAL MECHANISMS FOR IRON–FOLATE INTERACTIONS

Iron Metabolism Influences Folate Status

Regulation of intracellular folate concentrations is complex and is influenced by dietary folate intake, intestinal and cellular transport systems, polyglutamylation of the cofactor, and folate turnover, including folate catabolism *(19,24,38)*. Folate catabolism is defined as the oxidative and irreversible scission of the *p*-aminobenzoyl(poly)glutamate moiety of the cofactor from the quinazoline ring system, a reaction that destroys folate as a metabolic cofactor *(24,39,40)*. Certain physiological states are associated with increased rates of folate catabolism and simultaneous frank folate deficiency despite adequate dietary folate intake. These states of increased folate catabolism include cancer *(41,42)*, antiepiletic drug therapy, and pregnancy

in rodents (43,44) and perhaps humans *(45–48)*, as evidenced by elevated concentrations of *p*-aminobenzoylglutamate in urine *(38)*.

The in vivo catabolism of folate cofactors has been assumed to result from the nonenzymatic, oxidative degradation of labile folate cofactors, including dihydrofolate *(44)* and 10-formyltetrahydrofolate *(24)*. Each of these cofactors is readily oxidized in vitro *(39,40)*. However, observations that increased rates of folate catabolism occur during defined physiological states indicate that folate catabolism may be a regulated, enzyme-mediated process *(24)*. Recently, a protein was purified from crude rat liver homogenates that effectively generated *p*-aminobenzoylglutamate from 5-formyltetrahydrofolate *(49)*. The protein was identified as ferritin, a multisubunit protein that sequesters and stores intracellular iron *(50)*. Ferritin is a 24-mer composed of heavy-chain (HCF) and light-chain (LCF) subunits. Expression of the rat HCF cDNA in Chinese hamster ovary cells results in increased rates of folate turnover and decreased intracellular folate concentrations, even when cells are cultured in the presence of pharmacological levels of folic acid *(49)*. Therefore, increased rates of ferritin-mediated folate catabolism can affect intracellular folate concentrations in cell cultures, indicating that changes in iron metabolism may influence cellular folate concentrations in vivo.

Iron Status Influences Folate Metabolism

Clinical manifestations of folate deficiency during iron deficiency can occur in the absence of depleted tissue folate *(17)*. This observation indicates that catabolism-mediated folate deficiency cannot, in itself, fully account for the iron–folate relationship. Iron status is influenced by three pools of cellular iron: the functional pool that is bound by iron-requiring proteins, the storage pool that is ferritin-bound, and the labile, or regulatory, pool that exists free in solution. Elevated expression of HCF in the absence of increased iron availability lowers intracellular regulatory iron concentrations and triggers the cellular iron-deficiency response, which includes increased expression of the transferrin receptor and increased rates of iron uptake *(51)*. A recent study demonstrated that increased expression of HCF, but not increased LCF, in cell cultures alters the relative distribution of folate one-carbon-substituted cofactors, indicating that HCF expression alters the flux of one-carbon units through folate-requiring anabolic pathways *(52)*. HCF contains a ferrioxidase activity that catalyzes the oxidation of cytoplasmic Fe^{2+} to Fe^{3+}, a reaction that is associated with cellular iron chelation and storage within the ferritin polymer, whereas LCF does not contain this activity and does not function as an active iron chelator *(52)*. Therefore,

decreases in the cellular regulatory iron pool may mediate the changes in folate-dependent pathways observed with increased HCF expression in cell culture. The alterations in folate metabolism associated with increased HCF expression are the result, at least in part, of increased activity of the folate-dependent enzyme cytoplasmic serine hydroxymethyltransferase (cSHMT). The increases in cSHMT activity occur without alterations in cSHMT mRNA levels, but rather are caused by elevated cSHMT protein levels resulting from increased rates of cSHMT mRNA translation. Increased expression of the LCF cDNA does not affect cSHMT protein levels, indicating that increases in cSHMT expression respond either directly to increases in HCF protein or are responsive to HCF-induced decreases in the regulatory iron pool *(52)*.

The role of cSHMT in folate metabolism has been elusive. The serine hydroxymethyltransferase (SHMT) enzyme catalyzes the reversible interconversion of serine and tetrahydrofolate to glycine and methylene tetrahydrofolate:

$$\text{serine} + \text{tetrahydrofolate} \longleftrightarrow \text{glycine} + \text{methylenetetrahydrofolate}$$

Serine is the primary source of the one-carbon units that are required for folate-dependent biosynthetic reactions, indicating that SHMT plays an important role in folate metabolism *(13–15)*. There are two cellular isozymes of SHMT that are encoded by separate genes; one isozyme resides in the cytoplasm (cSHMT) and the other resides in the mitochondria (mSHMT) *(53,54)*. Loss of mSHMT activity in Chinese hamster ovary cells results in a glycine auxotrophy and cellular deficits in one-carbon units, indicating that mSHMT is responsible for the catabolism of serine to glycine and a single carbon and that cSHMT cannot compensate for loss of mSHMT function *(55)*. Several studies have indicated that SHMT activity in the cytoplasm favors serine synthesis, and cSHMT enzyme is expressed at high levels in the liver and kidney *(53,56,57)*. These observations may reflect glycine's role as an important gluconeogenic amino acid in these tissues. The definitive metabolic function of cSHMT in other cell types is less certain.

Changes in cSHMT expression influence both thymidylate synthesis and homocysteine remethylation in cell cultures, but in a reciprocal manner *(52,58)* (Fig. 1). cSHMT activity has been shown to be rate limiting in thymidine biosynthesis in MCF–7 cells, and elevated cSHMT expression in these cells increases the flux of one-carbon units through the thymidylate synthesis pathway *(52)*. Simultaneously, increased cSHMT expression inhibits the homocysteine remethylation pathway by two distinct mechanisms *(58)* (Fig. 1). Evidence suggests that the cSHMT enzyme competes

with methylenetetrahydrofolate reductase (MTHFR) for one-carbon units in the form of methylenetetrahydrofolate; that is, cSHMT-catalyzed serine synthesis competes with 5-methyltetrahydrofolate synthesis and ultimately homocysteine remethylation *(58)*. The cSHMT enzyme can also inhibit homocysteine remethylation by a second mechanism. The cSHMT enzyme is a high-affinity 5-methyltetrahydrofolate-binding protein *(59)*, and increased expression of the cSHMT cDNA or induction of endogenous cSHMT expression by increasing HCF expression greatly increases intracellular 5-methyltetrahydrofolate concentrations through sequestration of the cofactor *(58)*. This decrease in intracellular 5-methyltetrahydrofolate availability impairs homocysteine remethylation and results in markedly decreased cellular concentrations of *S*-adenosylmethionine. Collectively, these studies suggest that the cytoplasm contains two pools of cSHMT, one that interacts with and perhaps channels one-carbon units to the enzyme thymidylate synthase and a second "free" pool that competes with MTHFR for methylenetetrahydrofolate *(58)*. Furthermore, although previous studies have indicated that homocysteine remethylation has a higher priority compared to deoxyribonucleotide biosynthesis, increased expression of cSHMT reverses that priority by accelerating folate-dependent thymidylate synthesis while simultaneously inhibiting homocysteine remethylation. This "switch" is influenced by HCF expression *(52)*, retinoic acid, and developmental stage *(60)*.

Effect of Chemical Iron Chelators on cSHMT Expression

Chemical iron chelators inhibit cell proliferation in some but not all tumor cell lines *(61–63)*. The iron chelators mimosine and deferoxamine have been demonstrated to alter and deplete intracellular deoxyribonucleotide pools, presumably by inhibiting the iron-dependent enzyme ribonucleotide reductase *(64–66)*. It has been suggested that iron chelators inhibit the cell cycle by this mechanism. However, this suggestion has remained controversial because depletion of cellular deoxyribonucleotide concentrations would be expected to inhibit the elongation phase of DNA replication, whereas mimosine inhibits the formation of replication bubbles, or the initiation phase of DNA replication *(67,68)*. Additionally, neither mimosine nor deferoxamine effectively inhibits cell cycle progression in cells of embryonic origin; therefore, other mechanisms must be considered. Mimosine alters folate metabolism in human MCF-7 cells, a cell line known to be growth arrested by iron chelators, but mimosine does not influence folate metabolism or cell proliferation in human neuroblastoma *(62)*. Mimosine inhibits cSHMT expression in MCF-7 cells at the level of transcription, presumably

by activating a transcriptional silencing factor whose activity is increased by chemical iron chelators *(62)*. This factor appears to be tissue-specific, as it has only been found in MCF-7 cells. However, it is unlikely that inhibition of cSHMT expression is the primary mechanism whereby iron chelators inhibit cell cycle progression because characterization of MCF-7 mutants that are resistant to mimosine has revealed that they also fail to express cSHMT. Therefore, it has been proposed that activation of this silencing factor may regulate other proteins necessary for cell cycle progression.

At first glance, the transcriptional silencing effect of chemical iron chelators on cSHMT expression appears to contradict the effect of HCF-induced activation of cSHMT expression *(52)*. However, these two chelators have very different effects on cellular iron stores. HCF is only capable of influencing the regulatory iron pool, whereas chemical iron chelators are capable of depleting both the free and functional iron pools. Therefore, the effect of chemical iron chelators on cSHMT transcription, an effect not observed when HCF expression is increased, likely results from chelation of the functional iron pool.

Indicators of Impaired Folate Metabolism

Impairment of folate metabolism is made manifest by several clinical and biochemical outcomes that serve as indicators, or proxies, of whole-body folate status *(17,69)*. As indicated in Fig. 2, short-term folate deficiency lowers serum folate concentrations; therefore, this measurement is a good indicator of recent folate intake. Longer-term folate depletion results in increased segmentation of peripheral blood neutrophils. Peripheral neutrophils isolated from folate-replete subjects normally contain no more than three or four lobes (average = 3.2 lobes), but during folate deficiency, they can become hypersegmented and contain as many as six lobes or average greater than 3.5 lobes. This hypersegmentation is believed to result from impaired DNA synthesis. Longer-duration deficiency results in increased excretion of formiminoglutamate (FIGLU), an intermediate in the folate-dependent catabolism of histidine. Finally, severe depletion of whole-body folate stores is evidenced by low red blood cell (RBC) folate and megaloblastic bone marrow and anemia. RBC folate is a good indicator of long-term folate status because the RBC accumulates folate during erythropoiesis, and these folate levels are retained throughout the RBC life span. RBC folate correlates well with liver folate in the absence of pernicious anemia. Impairment of folate metabolism is also quantified by the deoxyuridine (dU) suppression assay, which measures the efficiency of *de novo* thymidine biosynthesis *(69)*. Thymidine can be synthesized *de novo* by a folate-dependent

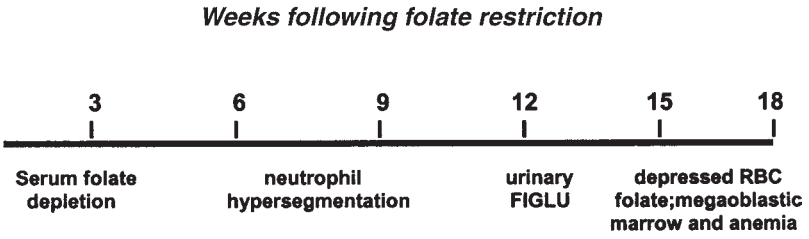

Fig. 2. Indicators of folate status and metabolism.

biosynthetic pathway or it can be recycled through a salvage pathway that is folate independent. The dU suppression assay measures the efficiency of *de novo* thymidine biosynthesis relative to synthesis through the salvage pathway.

CLINICAL AND ANIMAL STUDIES: REINTERPRETATION IN LIGHT OF MOLECULAR DATA

Studies of clinical populations and animals have indicated that iron deficiency alters folate-dependent one-carbon metabolism. Specifically, iron deficiency can influence all of the biochemical indicators listed in Fig. 2. However, marked discrepancies exist within the literature, primarily resulting from different patient populations, indicators of folate status or metabolism, experimental protocols, as well as insufficient measurements and lack of experimental controls *(70)*. These discrepancies led one author in 1966 to conclude that "…the causal relationship of folate and iron deficiencies cannot be satisfactorily resolved on the basis of data available today" *(71)*. Although this statement is still accurate at the present time, new avenues for investigation are now emerging. Recent mechanistic studies suggest that iron metabolism is capable of influencing cellular folate metabolism by two distinct mechanisms: by altering the expression of cSHMT and by influencing folate catabolism leading to frank folate deficiency *(52,58)*. These effects will be considered individually next, and potential biochemical mechanisms that account for these effects will be postulated based on the aforementioned biochemical studies of iron deficiency conducted in cell culture models.

Effects of Iron on Folate-Dependent DNA Synthesis

Interpretations of clinical data that support or dismiss an iron–folate relationship must be viewed with caution considering what is known about this relationship at the molecular level. HCF-mediated increases in cSHMT ex-

pression can affect DNA precursor synthesis in two opposing ways: It can facilitate DNA synthesis by increasing the rate of *de novo* thymidylate biosynthesis, and it can impair DNA synthesis by sequestering intracellular folate as 5-methyltetrahydrofolate, thereby creating a "functional" folate deficiency. This functional folate deficiency is the result of the depletion of other folate cofactor forms that results from 5-methyltetrahydrofolate accumulation, as occurs during vitamin B_{12} deficiency *(27)*. These two opposing effects of cSHMT expression on DNA synthesis might account for the discrepancies that exist within the clinical literature.

Iron Deficiency Induces Functional Folate Deficiency

Megaloblastosis, which occurs during folate deficiency, has been hypothesized to occur as a result of thymidylate "starvation" *(69,72)*. Folate deficiency induced by gastrointestinal disorders *(73)* results in neutrophil hypersegmentation, elevated urinary FIGLU, and giant metamyelocytes in bone marrow despite normal or elevated RBC folate. Studies have demonstrated that iron therapy alone can improve the symptoms of folate deficiency *(73)*. Similar observations of apparent folate deficiency were seen in patients with intestinal parasitism *(74)*. These patients presented with megaloblastic anemia and elevated neutrophil lobe count without evidence for inadequate folate intake or absorption. For these patients, iron repletion alone also reversed the symptoms of folate deficiency. A more recent case-control study supports these observations that iron deficiency can inhibit DNA synthesis by inducing a symptomatic and functional folate deficiency *(75)*. In this study, all patients who had evidence for vitamin B_{12} or folate deficiency, infection, or who were undergoing chemotherapy were excluded because these factors are known to promote neutrophil hypersegmentation by impairing folate-dependent DNA synthesis. Fifty patients with iron-deficiency anemia were matched with 50 control patients. The authors found that 62% of patients with iron-deficiency anemia exhibited neutrophil hypersegmentation compared to only 4% of control patients. The symptomatic folate deficiency in this study occurred despite patients displaying significantly increased RBC folate concentrations relative to control patients, and this elevation in RBC folate during iron-deficiency anemia is consistent with findings from other studies *(76,77)*. The clinical observation that iron deficiency causes symptoms of folate deficiency, which are caused by impaired DNA synthesis, is consistent with cell culture models. Specifically, decreases in the regulatory iron pool increase cellular levels of cSHMT and result in trapping of cellular folate as 5-methyltetrahydrofolate *(58)*. Furthermore, the response of the hypersegmentation to iron supplementa-

tion suggests that iron therapy rescues a cSHMT-induced methyl trap of folate cofactors (Fig. 1). Therefore, cSHMT joins vitamin B_{12} as a factor that can induce a folate methyl trap.

Iron Deficiency Masks Functional Folate Deficiency

Although cSHMT is capable of sequestering or trapping cellular 5-methyltetrahydrofolate, cSHMT has also been demonstrated to enhance thymidylate biosynthesis and thereby support DNA synthesis. The degree to which these two opposing mechanisms affect overall DNA synthesis would be dependent on the magnitude of increase in cSHMT expression and on cellular folate status. High levels of cSHMT would be expected to create a severe methyl trap and inhibit DNA synthesis; this effect would be compounded by folate deficiency. Alternatively, moderate levels of cSHMT expression would not be sufficient to trap folate cofactors but would be expected to enhance thymidylate biosynthesis and thereby promote DNA synthesis (Fig. 1). This suggestion is supported by a study of iron- and folate-deficient patients by Das et al., who were among the first to suggest that iron deficiency can mask apparent folate deficiency *(78)*. In this study, patients were chosen who presented uncomplicated iron-deficiency anemia as evidenced by apparent normal RBC folate concentrations and normal bone marrow dU suppression tests. Iron supplementation alone resulted in the appearance of hypersegmented neutrophils, abnormal dU suppression in the bone marrow, and megaloblastic transformations, all symptoms of folate deficiency. These indicators of folate deficiency emerged only after iron therapy, whereas folate values continued to read in the normal range throughout the entire study.

Emerging mechanisms described earlier may account for this apparent masking of functional folate deficiency by iron deficiency. Moderate increases in cSHMT expression that are observed with increased HCF expression may also occur during iron deficiency *(52)*. Increased cSHMT expression at levels that do not produce a methyl trap would be expected to stimulate thymidylate biosynthesis and thereby mask marginal functional folate deficiency, because folate deficiency normally impairs thymidylate biosynthesis *(72)*. Therefore, increases in cSHMT activity compensate for folate deficiency and thereby yield a normal dU suppression test. According to this model, this stimulation is lost by iron repletion, which is expected to restore cSHMT levels to normal. It is not likely nor is it supported in the literature that iron deficiency can mask severe folate deficiency.

In conclusion, the discrepancies among clinical studies that suggest an iron–folate relationship in DNA synthesis pathways may be the result of subtle differences in their patient populations. These differences include the

severity of iron deficiency, which, in turn, may influence the extent of increased cSHMT expression. According to the above model, the level of cSHMT expression will determine if DNA synthesis is stimulated by increasing thymidylate biosynthesis or inhibited by 5-methyltetrahydrofolate trapping. Furthermore, this model indicates that the masking of folate deficiency by iron deficiency will only occur in marginally folate-deficient populations and that megaloblastosis will occur during severe folate deficiency and iron deficiency. In other words, the severity of both iron and folate deficiencies will work in combination to determine the final metabolic effect on DNA synthesis.

Effects of Iron on Folate Status and Folate Catabolism

Changes in cellular folate concentrations independent of dietary folate intake may be influenced by iron status as well as alterations in HCF expression *(49)*. Iron-related changes in cellular folate concentrations can occur by two distinct mechanisms: by increased rates of folate turnover mediated by HCF *(49)* and by increased sequestration of cellular folates caused by increased expression of cSHMT, a major folate-binding protein in the cytoplasm of some cell types *(26,49)*. In fact, polymorphic variants of cSHMT in human populations significantly influence RBC folate concentrations *(79)*. Therefore, some reevaluation of the clinical literature that relates iron status to actual folate status is warranted.

Patients with iron-deficiency anemia often display symptomatic folate deficiency yet tend to exhibit significant increases in RBC folate *(73,75,76)*, indicating that a folate methyl trap may be operative. RBC folate concentrations fall markedly following iron repletion, with simultaneous increases in plasma folate *(76)*. However, at least one study failed to see this association *(80)*. Another study of nonanemic pregnant women noted that subjects with depleted iron reserves tend to be less responsive to folate supplementation, consistent with the occurrence of a folate methyl trap *(81)*. This effect of folate accumulation during iron deficiency is also observed in the liver of rodents *(17,82)* and is reversed upon iron repletion, although other studies have failed to see this effect *(83)*. These discrepancies in the animal literature have been previously discussed *(17)*. Iron depletion does not influence folate polyglutamate processing or absorption in the intestine, indicating that the effect occurs at the cellular level *(17)*. The decreases in RBC folate and increases in serum folate that occur following iron repletion have been proposed to result from increased demand for folate following iron repletion *(73)*. However, sequestration and trapping of 5-methyltetrahydrofolate

resulting from increased cSHMT expression would be an alternative explanation for elevated folate content that can be readily tested experimentally.

Iron may also influence rates of folate turnover. Increased expression of heavy-chain ferritin occurs in physiological states associated with increased rates of folate catabolism and folate deficiency. Tumor cells exhibit increased rates of folate uptake while displaying cellular folate deficiency *(11,84,85)*. Mice with ascitic tumors have greatly increased concentrations of urinary *p*-aminobenzoylglutamate, an indicator of increased rates of folate catabolism *(41)*. Similarly, the folate content of neoplastic cells is significantly lower than surrounding normal cells in patients with colorectal adenomas *(86)*. Consistent with studies of cell culture models that demonstrate that increased HCF expression increases rates of folate catabolism, HCF is elevated in most tumors and its expression is markedly increased by the oncogene c-*myc (86–91)*. Therefore, changes in iron metabolism independent of iron status can influence intracellular folate concentrations. Pregnancy is also associated with folate deficiency, and increased rates of folate catabolism have been observed in rodent studies, but such observations have been inconsistent in human studies *(24)*. In rodents, HCF was found to be induced by progesterone, and HCF expression was induced 8- to 10-fold in the endometrial stromal cells of pregnant rats *(92)*. Although definitive whole-animal studies that conclusively demonstrate a role for HCF in catalyzing folate catabolism and regulating intracellular folate concentrations are lacking, in vitro and cell culture studies support a role for alterations in iron metabolism influencing intracellular folate concentrations.

Other studies indicate a reciprocal relationship between iron and folate status. Iron overload occurs in patients with folate deficiency, presumably because iron is preferentially deposited in the parynchemal cells during folate deficiency, predisposing that individual to iron overload *(93)*. Folate deficiency has been observed in hemochromatosis without evidence for defective intestinal malabsorption of folate. The authors of this study concluded that hemochromatosis impairs folate storage in liver *(94)*. However, no study has been reported that systematically investigated the effects of iron overload on folate catabolism in humans or experimental animals.

Maternal Iron Deficiency and Lactation

Maternal iron deficiency in rodent animal models dramatically decreases milk folate content (35–47% reduction from moderate to severe iron depletion), an effect that is independent of maternal folate status *(82)*. However, the folate status of iron-deficient and iron-replete pups is similar at birth, indicating that pups are at risk for folate deficiency only after parturition

(95,96). Pups nursed by folate-sufficient, iron-depleted mothers become folate deficient by d 18 and display growth retardation *(96)* despite increased milk consumption relative to pups nursed by iron-replete dams *(97)*. Additionally, pups nursed by iron-deficient dams do not have impaired intestinal folate absorption, indicating that the folate-related growth retardation is the result of inadequate folate intake. Similar results are seen in piglet models *(98)*. The decreased milk folate content in iron-deficient dams occurs without differences in maternal RBC or tissue folate concentrations compared to iron-replete dams throughout lactation, indicating that the effect of iron on milk folate may be a mammary-specific effect in this animal model *(99)*. The activities of enzymes associated with folate retention, including methionine synthase and formlylpolyglutamate synthetase, are unaffected in the mammary gland of iron-deficient dams, and the content of milk folate-binding protein is not influenced by maternal iron status *(100)*. Therefore, it was proposed that iron deficiency specifically targets mammary tissue in lactating dams and impairs the ability of the mammary epithelial secretory cells to accumulate folate *(99,100)*. Previous biochemical studies have indicated that small-molecule chemical iron chelators, which induce cellular iron deficiency by sequestering both the functional and regulatory iron pools, inhibit cSHMT transcription specifically in mammary carcinoma cells *(62)*. This relationship should be tested in a rodent model. If cSHMT is a major folate-binding protein in the secretory mammary cell, as has been documented in other cells, and iron deficiency inhibits cSHMT transcription, as seen with chemical iron chelators in cultured cells, this would at least partially account for the inability of the mammary cells to accumulate folate.

CONCLUSIONS

This review attempts to reconcile an extensive, controversial, and contradictory literature concerning the effects of iron status on folate metabolism and folate status in humans. As outlined in this chapter, animal studies that have attempted to clarify the conflicting clinical associations also proved to be contradictory themselves, leaving the issue confused and unproven to the present day. Even the best validated association, that iron deficiency impairs milk folate content in animal models, has been known for nearly 15 yr, yet has not been reported for humans. Therefore, it seems unlikely that additional associational studies will clarify or validate the iron–folate relationship. Recent molecular studies from our and other laboratories show that iron or the iron-storage protein ferritin can influence folate metabolism and folate status. The enzyme cSHMT is a primary conduit through which iron impacts folate metabolism. Armed with this knowledge, we have attempted

to reinterpret results from the most consistent clinical and animal studies that suggest an iron–folate relationship exists. This reinterpretation is not meant to validate prior associational studies, but rather to offer plausible metabolic mechanisms that can guide and inform future study designs that seek to examine or prove this relationship. Future clinical and animal studies must be carefully designed and controlled, as in vitro and cell culture studies indicate that the underlying mechanisms that support this association are complex. Additionally, as reviewed elsewhere, animal models must be carefully selected for studying the iron–folate relationship. Some models may be inadequate for certain experiments. For instance, iron-deficient rats deliver pups with iron-deficiency anemia, an occurrence that is rare in humans or pigs *(17)*. Additionally, milk iron concentration in pigs and humans is not affected by maternal iron status, but is in rats. However, continued study of the biochemical mechanisms whereby iron impacts folate metabolism will likely allow the measurement of more focused outcomes in clinical and animal studies, and results from these studies will hopefully clarify the existence of an iron–folate metabolic relationship.

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13

A Differential Role for Folate in Developmental Disorders, Vascular Disease, and Other Clinical Conditions

The Importance of Folate Status and Genotype

Mark Lucock and Zoe Yates

INTRODUCTION

Since the 1980s there has been considerable attention focused on the B vitamin folic acid. This interest accelerated when, in 1991, it was shown beyond any doubt that this vitamin had a protective role against neural tube defects (NTD) such as spina bifida *(1)*. Since then, interest in its relationship with homocysteine (Hcy) and, consequently, a potentially beneficial role it might play in occlusive vacular disease (OVD) has also emerged *(2–5)*.

As if an impact on these two major clinical conditions were not enough, the recent emergence of molecular biological data on genes coding for folate-dependent enzymes, particularly on common single-nucleotide polymorphisms (SNPs), indicates that folate has a sphere of influence that extends to other disease processes. These include other birth defects *(6)*, several types of cancer *(7–11)*, affective disorders *(12)*, Down syndrome *(13,14)*, dementia *(15),* and serious conditions affecting pregnancy outcome *(16,17)*.

What makes these genetic findings so important is that as an essential nutrient, dietary folate is likely to interact with allelic variants of folate genes that have a high frequency in the population. In so doing, dietary folate may play a modulatory role in disease formation by influencing folate-dependent one-carbon transfer reactions leading to the biosynthesis of important biological molecules, particularly methionine as well as purine and pyrimidine nucleotides. This chapter will explore the important interaction between folate nutrition, gene polymorphisms, and health.

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DISCOVERY

The name *folate* is derived from the latin for leaf, *folium*, and is the generic term for a large group of water-soluble B vitamins. Its name stems from the fact that the vitamin is found in leafy green vegetables, the source from which it was originally isolated. Liver, yeast extract, and citrus fruits are also good natural sources of the vitamin. The story of this vitamin's beneficial properties first began in 1931 when Lucy Wills reported that injections of yeast or liver autolysate were effective in treating tropical macrocytic anemia, which was common during late pregnancy in India *(18)*. Although she did not know it at the time, the biologically active principle was folic acid. Later, the endeavors of several researchers *(19–22)* led to the discovery of a nutritional hemopoietic factor that was eventually identified as *N*-(4-{((2-amino-4-hydroxy-6-pteridiny1)methyl)amino}benzoyl) glutamic acid. In 1941, Mitchell et al. proposed the alternative name of "folic acid" *(21)*.

During the intervening years, it has been established that the various coenzyme analogs of folic acid facilitate the transfer of one-carbon units from donor compounds into important biosynthetic pathways leading to purines, pyrimidines, and methionine. They are also involved in the interconversion of serine and glycine as well as histidine catabolism.

STRUCTURE OF NATIVE FOLATES

It has been estimated that well over 100 forms of folic acid occur in nature. This large number is the result of variation at several sites in the basic molecular structure of the parent folic acid compound, which is often referred to as pteroylmonoglutamic acid. Native folates differ in (1) the oxidation state of the pteridine ring, (2) the character of the one-carbon substituent at the N5 and N10 positions, and (3) the number of glutamic acid moieties conjugated one to another via a series of γ-glutamyl links to form an oligo-γ-glutamyl tail. Figure 1 shows the structure of 5-methyltetrahydrofolic acid ($5CH_3$-H_4folate).

The existence of many folyl-coenzyme analogs has led to tremendous difficulties in characterizing folates in biological materials. The problems are compounded by their low levels and extreme lability. Although modern analytical methods such as high-performance liquid chromatography (HPLC) with fluorescence *(23,24)* or multichannel coulometric electrochemical detection *(25,26)* offer a way forward in the analysis of folates from clinical samples and foods, the complexity inherent in folate biochemistry still remains a major challenge for the bioanalyst to overcome.

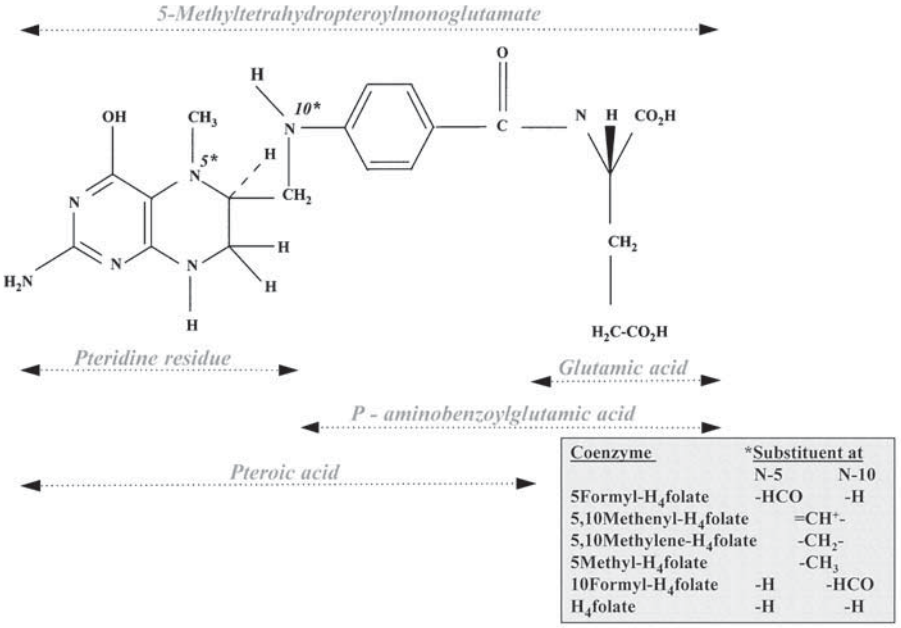

Fig. 1. The molecular structure of monoglutamyl $5CH_3$-H_4folate and related congeners.

ABSORPTION, TRANSPORT, AND METABOLISM OF DIETARY FOLATES

Because man is unable to manufacture folate, he depends on a variety of exogenous sources for the vitamin. In addition to the rich sources mentioned earlier, moderate sources such as bread, potatoes, and dairy products are consumed in large amounts and thus contribute a significant proportion to our total folate requirement.

If one takes into account all folyl oligo-γ-glutamyl forms of the various one-carbon and unsubstituted oxidation states of the vitamin, folate metabolism becomes complex. However, it is generally agreed that food folate exists largely in $5CH_3$-H_4folate and formyltetrahydrofolate (formyl-H_4folate) forms (*27*). (Figure 1 gives the structure of all reduced folate derivatives.) The predominant natural dietary folate is $5CH_3$-H_4folate *(28,29)*, which is readily oxidized to 5-methyl–5,6-dihydrofolate ($5CH_3$-5,6-H_2folate) *(27)*. In this oxidized form, it may add up to 50% of the total food folate *(30)*.

At a mildly acidic postprandial stomach pH, $5CH_3$-5,6-H_2folate is rapidly degraded via cleavage at the C9-N10 bond, while $5CH_3$-H_4folate remains

relatively stable. Fortunately, ascorbate secreted into the stomach lumen can salvage acid-labile $5CH_3$-5,6-H_2folate by reducing it back to acid-stable $5CH_3$-H_4folate and, thus, may be critical for optimizing the bioavailability of food folate *(31)*. It has recently been shown that dietary formyl-H_4folates may also utilize the natural pH of the gastrointestinal tract to isomerize and yield biologically useful forms of the vitamin *(32)*.

During the absorption process, folates are transported across the enterocyte membrane by a saturable anion-exchange mechanism enabled by the transmembrane pH gradient. At the intraluminal pH, folate is anionic and is exchanged for a hydroxyl anion. Absorption occurs in the jejunum, being most efficient proximally. All dietary folylpolyglutamate derivatives are hydrolyzed (deconjugated) to folylmonoglutamates by pteroyl-γ-glutamylhydrolase and metabolized within the enterocyte into $5CH_3$-$H_4folate_1$. This monoglutamyl coenzyme is the major plasma form of the vitamin (*see* below), which is transported to peripheral tissues for use in one-carbon transfer reactions.

In order for the cell to utilize $5CH_3$-$H_4folate_1$, it must be demethylated by vitamin B_{12}-dependent methionine synthase (MetSyn), which converts it into monoglutamyl $H_4folate_1$. $H_4folate_1$ is the optimal substrate for folylpolyglutamate synthase (FPGS) which generates oligo-γ-glutamyl H_4folate by conjugating glutamate moieties via a γ-glutamyl peptide linkage. With $H_4folate_1$ as the substrate, the main product of this reaction is hexaglutamyl-H_4folate *(33)*, which, along with folylpentaglutamates, are the optimal oligo-γ-glutamyl chain lengths for cellular one-carbon transfer reactions based on reported kinetic data *(34)*. The conversion of $5CH_3$-$H_4folate_1$ into $H_4folate_1$ by vitamin B_{12}-dependent MetSyn is therefore considered to be an essential step in converting extracellular folate from dietary sources ($5CH_3$-H_4folate) into a biologically more useful intracellular form of the vitamin (H_4folate) that can be used in nucleotide biosynthesis *(35)*.

In order to contribute to the five major one-carbon transfer reactions that occur within the cell (*viz.* conversion of serine to glycine, catabolism of histidine, and synthesis of thymidylate, methionine and purine), folate must be transported into the cell. Only then do these reactions take place through various electron-transfer steps facilitated by specific enzyme systems and coenzymes such as $FADH_2$ and NADPH. Two classes of cellular transport systems have been identified. The first involves membrane carriers. Tissue culture cells, cancer cells and fetal tissue contain one of the best studied transporter systems. The mechanism in normal adult tissue differs and exhibits a wide range of transporters with a differential affinity for the vari-

ous folate analogs *(36)*. Folate transport in hepatocytes is energy dependent with both saturable and low-affinity nonsaturable components *(37)*. Rat and human liver basolateral membrane have an electroneutral folate H^+ cotransporter *(38,39)*, whereas the basolateral membrane of the small intestine has an anion-exchange folate transporter *(40)*. Mitochondria are also known to possess specific folate transporters.

The second class of cellular folate transporter is a specific folate binding protein (FBP) that is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. This FBP is generally confined to the apical membrane of certain epithelial cells *(41,42)*, where it binds a variety of folylcoenzymes with high affinity. The FBP–folate complex internalizes bound folate by a non-clathrin-mediated endocytotic pathway that does not involve lysosomes *(43)*. The phrase "potocytosis" has been coined for the recycling of a binding protein via vesicular structures known as caveolae *(44)*. Lowering of the pH releases anionic folate from the carrier prior to its subsequent liberation from the vesicle into the cytosol. The FBP then cycles back to the plasma membrane. Tissues rich in this transport system include the choroid plexus where plasma $5CH_3\text{-}H_4folate_1$ is transported into the cerebrospinal fluid (CSF), the vas deferens, renal proximal tubules, erythropoietic cells, ovary, and placental trophoblasts *(42)*. In humans, four isoforms of what has become known as the folate receptor (FR) are recognized. Human FR-α and FR-β are analogous to FBP-1 and FBP-2 in murine tissue and are attached to the cell membrane via a GPI anchor; the other two FRs are not.

Once folate has been internalized, the length of the polyionogenic oligo-γ-glutamyl chain conjugated to folate aids cellular retention and regulates reaction rates that control biosynthetic pathways. Our understanding of the complex regulatory mechanisms involved in partitioning of one-carbon units donated by the various oligo-γ-glutamyl folate coenzymes into the production of metabolites critical for life processes like methionine, purine, DNA-thymine, and so forth is still being refined, largely because, until recently, in vivo models and analytical strategies lacked sensitivity and specificity. Perhaps, at least in part, as a result of logistical problems in the routine characterization of so many labile, low-abundance native folyl coenzymes, considerable interest has arisen in Hcy as a simple indicator of folate status. Figure 2 shows the interrelationship between dietary folate and the various biosynthetic pathways that it serves. It also shows how these pathways might influence human health. A more detailed description of one-carbon biochemistry and metabolism in humans is provided elsewhere in this volume.

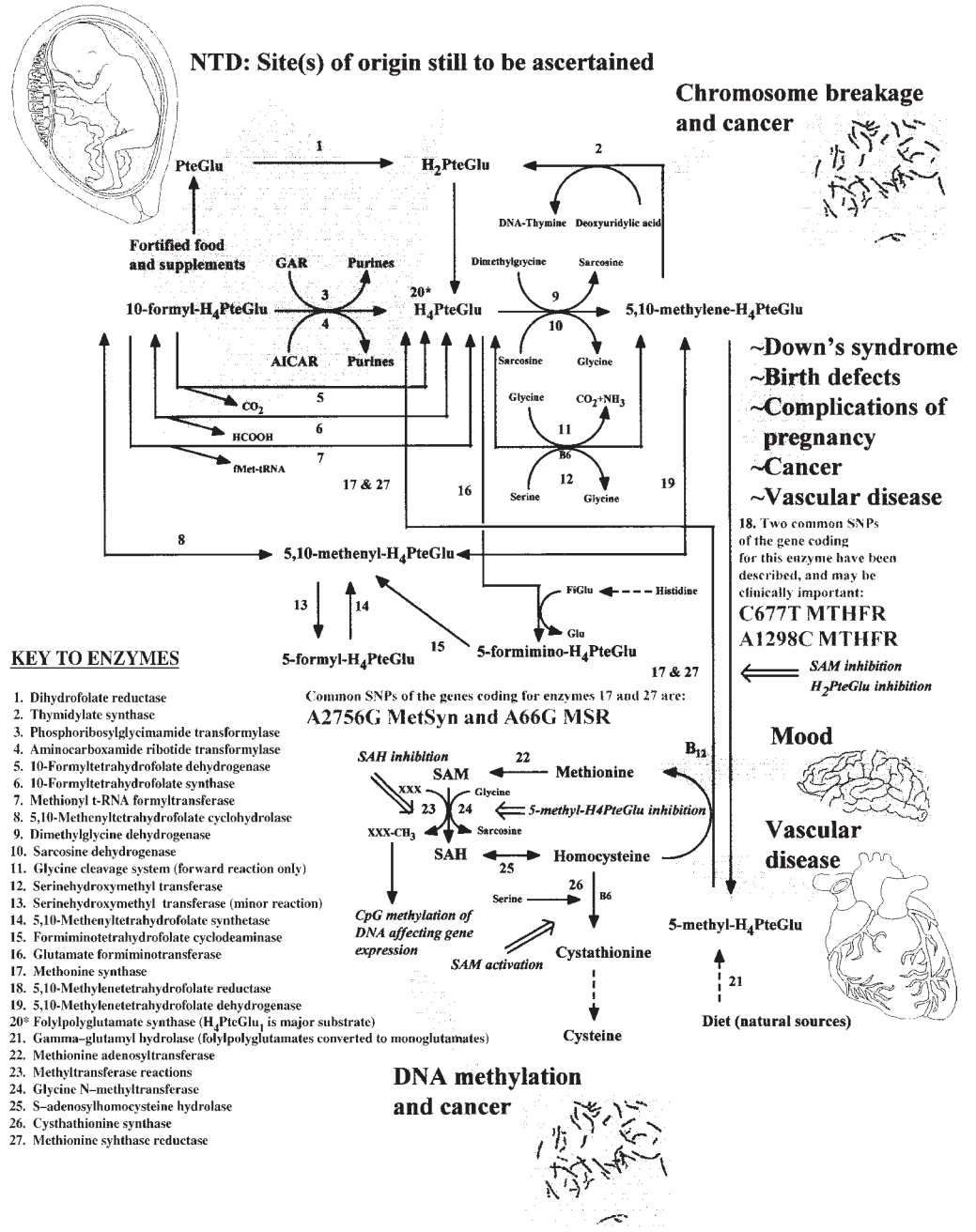

Fig. 2. Interrelationship between dietary folate and the various biosynthetic pathways that it serves. Arrows direct the reader to sites thought to underpin disease formation.

FOLATE STATUS: THE NATURE OF FOLATE IN PHYSIOLOGICAL FLUIDS

What Do We Really Mean by Folate Status

Given that folate has so many forms in which it can exist and because both dietary and genetic factors influence the disposition of cellular folate, it is probably fair to conclude that no simple answer can address this question adequately. However, if we look beyond conventional nonspecific radioassay measures of blood folate and focus on what happens at the individual cellular folyl-coenzyme level, it is possible to gain some understanding of the issues involved. In one study, it was found that 1 mo supplementation with folate (400 µg pteroylmonoglutamate/d) caused a 29% increase in total formylfolate and a 35% drop in total methylfolate (n = 13) *(45)*. However, the folate response varied according to the number of glutamate moieties conjugated to the pteroic acid. Most loss within the methylfolate pool was the result of a decline in hexaglutamates, with a minor loss of pentaglutamates. By contrast, monoglutamates to tetraglutamates of methylfolate actually became more abundant. This shows that the oligo-γ-glutamyl chain length is sensitive to folate status in humans. In vitro studies support these findings and the idea that the glutamate chain length decreases as a response to increased folate supply and vice versa *(46)*. The benefit of increasing the folyl oligo-γ-glutamyl chain length in folate depletion probably arises from an increased avidity (lower K_m) for folate-dependent enzymes. By responding in this way, folate-dependent biosynthetic pathways critical for life processes could theoretically be maintained despite perturbations in folate availability.

Plasma and Cerebrospinal Fluid

Following absorption in the proximal jejunum, $5CH_3$-$H_4folate_1$ is released into the portal circulation, where it is taken up by the liver, although some is released into the bile, where it is recirculated by the enterohepatic cycle, not necessarily in the $5CH_3$-$H_4folate_1$ form. The plasma $5CH_3$-$H_4folate_1$ level is in the region of 3–30 ng/mL, with the final value depending on which proprietory (radioassay) or bespoke (HPLC/*Lactobacilius casei*) assay is used.

The liver plays a central role in folate homeostasis *(47)*. Following short periods of folate deprivation, supply is maintained by folylmonoglutamate pools within the cell and enterohepatic cycle. Decreased tissue uptake leads to reduced cellular folylpolyglutamate synthesis and increased folylpolyglutamate hydrolysis to monoglutamate forms. This process increases the

available extracellular plasma $5CH_3$-$H_4folate_1$ level. 5CH3-$H_4folate_1$ is also reabsorbed in the proximal tubule, further contributing to the available circulating $5CH_3$-$H_4folate_1$ level. Plasma contains pteroyl-γ-glutamylhydrolase, and any folylpolyglutamates released into plasma should be hydrolyzed to their monoglutamate forms.

Much of the circulating $5CH_3$-$H_4folate_1$ is bound to proteins in the plasma. Around 40% of endogenous plasma folate is associated with low-affinity binding proteins, mainly albumin (K_d ~ 1 m*M*). Other binding proteins include α2 macroglobulin and transferrin. The binding equilibrium increases in folate deficiency, with a shift in binding from α2 macroglobulin to transferrin during pregnancy *(48–52)*. Plasma also contains a low-abundance, high-affinity folate-binding protein (K_d ~ 1 n*M*), which increases at times of poor folate status *(53,54)*, as well as in pregnancy *(55)*, leukemia *(56)*, uremia *(57)*, liver disease *(58)*, and in the serum from umbilical cord blood *(59)*. This high-affinity binding protein would seem to be homologous with the cellular FBP/FR, which is normally attached to the cell membrane by a GPI anchor (*see* previous section) *(60–62)*.

Cerebrospinal fluid contains approximately three times the level of folate that plasma does. The form found in CSF is exclusively $5CH_3$-$H_4folate_1$, which is concentrated in the spinal fluid from the blood by a transport system localized in the choroid plexus. The increased CSF level of $5CH_3$-$H_4folate_1$ may reflect the demand of neuronal tissue for methyl groups (*see* below), although it also undoubtedly acts as a buffer protecting the brain from the fluctuating dynamics of plasma levels of the vitamin.

Red Blood Cell Folate

The developing erythroblast incorporates folate during erythropoiesis in the bone marrow. It is reported that erythrocytes contain predominantly folylpolyglutamates with four to seven glutamyl residues, which is in complete accord with our own findings *(24,45)*. The folate level normally encountered in erythrocytes is 180–500 ng/mL. The routine tests employed for measuring erythrocyte folate are nonspecific and are really only suited to diagnosing folate deficiency. In order to establish how diseases might influence folate metabolism, techniques like HPLC, which can resolve the various folate analogs found in the erythrocyte, are required.

Early studies used the selective growth response of micro-organisms to investigate folate status. One of the first indications of the nature of erythrocyte folate came from Iwai et al. *(63)*, who demonstrated that human erythrocyte folate showed almost equal growth activity for both *Pediococcus cerevisiae* and *Streptococcus faecalis*, suggesting to them that one of the

folates may be an N^5,N^{10}anhydroformyl-H_4folate or 10CHO-H_4folate coenzyme. In support of this, even earlier studies indicated the presence of oxidized 10-formylfolate among the degradation products of the red cells folate content *(64)*. Moreover, in their study, Iwai et al. *(63)* also discovered an elevated *Lactobacillus casei* activity, suggesting the presence of $5CH_3$-H_4folate. Interestingly, the microbiological activity to erythrocyte folate (high *L.casei* response coupled with low activity for *S. faecalis* or *P. cerevisiae*) can be drastically altered in favor of enhanced activity for *P. cerevisiae* by treatment with high levels of ascorbate. This offers the strongest possible evidence that native erythrocyte folates exist at the formyl oxidation level, because *P. cerevisiae* growth is specific for 10CHO-H_4folate, 5CHO-H_4folate and H_4folate, but not the oxidatively degraded 10-formylfolate or, indeed, $5CH_3$-H_4folate. However, upon degradation of 10CHO-H_4folate, the product 10-formylfolate would exhibit *L. casei* activity and therefore explain these early findings *(65)*.

Formyl folates are perhaps the most difficult coenzymes to characterize because several interconversions are possible. These are under the influence of both matrix pH and enzyme catalysis. It has been proposed that a nonenzymatic pathway exists for conversion of 5,10-methenyltetrahydrofolate (5,10CH-H_4folate) into the transient intermediate coenzyme, (11*S*)-hydroxymethylene-H_4folate, and, subsequently, into either 10CHO-H_4folate or 5CHO-H_4folate, depending on the protonation of the N-5 or N-10 sites. Moreover, it has also been suggested that both facile and enzyme (serine hydroxymethyltransferase (SHMT)) catalyzed formation of 5CHO-H_4folate from 5,10CH-H_4folate occurs. In this scenario, (11*R*)-hydroxymethylene-H_4folate (anhydroleucovorin B) formed from the 11*S* isomer by isomerizsation (or by an enzyme activity still to be discovered) breaks down into 5CHO-H_4folate *(66)*. The increased avidity of anhydroleucovorin B for SHMT, compared with 5,10CH-H_4folate, suggests that this 11*R* isomer may be the preferred in vivo substrate for SHMT hydrolysis to 5-CHO-H_4folate *(66)*.

Although nonspecific radioassay continues to be the way that most routine and some research laboratories assess red cell folate, other groups are turning to HPLC to provide a more detailed explanation of the true character of red cell folate that takes into account oxidation state, 1-C substituent, and glutamate tail length.

Although HPLC is fairly selective when coupled to fluorescence detection (λ_{exc} = 310 nm, λ_{emm} = 352 nm), tissues do vary in the distribution and level of both folates and interfering material, potentially causing problems where identification is based solely on retention time. HPLC–diode array

Table 1
λ_{max} for Detecting Native Folate Coenzymes by Spectrophotometric Methods

Folate congener	λ_{max}(nm) at pH 3.0
p-Aminobenzoylglutamate	273
H_4folate	267,290
$5CH_3$-H_4folate	265,290
$5CH_3$-5,6-H_2folate	277
10CHO-H_4folate	260
5,10CH-H_4folate	355
5CHO-H_4folate	286
$5,10CH_2$-H_4folate	300
Non-native pteroylmonoglutamic acid	282

detection provides useful spectral data but is limited to simple matrices with supraphysiological folate levels (Table 1 gives λ_{max} for several folate species). HPLC–amperometric detection is more sensitive but does little to help characterize a peak.

Twelve-channel coulometric electrochemical detection (CED) combines sensitivity with qualitative information on native folates. A preliminary assessment of CED in our laboratory suggested that it had the potential to characterize individual red cell folates *(26)*. We were able to demonstrate homology between native folylpolyglutamates in two major red cell folate groups; methylfolates and formylfolates.

The dominant oxidation voltage for the homologous series of red cell formylfolates was 560 mV, with lesser responses at 640 mV and 480 mV [acidic protocol seemed to favor an equilibrium in the direction of the 5,10CH-H_4folate isomer *(25)*]. The homologous series of red cell methylfolates were identified by their voltage-response patterns at 0 and 880 mV. Homology was clearly demonstrated within each folylpolyglutamate group.

We concluded that CED should make it easier to analyze folates in many complex matrices such as red cells where coextracting material causes problems. However, given efficient chromatography, fluorescence detection is still an excellent, although less information-rich, form of HPLC detection, and one we use routinely for measuring red cell folate *(23,24,45)*.

Despite the interconvertability, lability, and multiplicity of form that folates exhibit, it is now widely accepted that erythrocyte folate is largely $5CH_3$-H_4folate and formyl-H_4folate *(24)*, with most studies agreeing that

the majority of erythrocyte folate is in the form of folylpolyglutamates with pentaglutamates and hexaglutamates predominating *(24,67)*.

Red cell folate has no known metabolic role and is considered to be a storage reservoir and long-term buffer for maintaining folate homeostasis. Unlike plasma folate levels, it is unaffected by recent dietary intake. Folate is salvaged from senescent red cells, by the reticuloendothelial system. It is then transported to the liver and reappears in the bile for redistribution to peripheral tissues via the enterohepatic cycle.

THE GENERAL INFLUENCE OF FOLIC ACID IN HEALTH AND DISEASE HAS MANY FACETS

Despite the fact that folate is gaining a reputation as a simple panacea for several clinical conditions, its mechanisms of action are many-fold. So what provides a link between folate nutrition/biochemistry and a diverse list of disorders such as OVD, NTD [and other mid-line defects such as cleft palate *(6)*], several cancers (cervical, bronchial, colon, breast, and leukemia) *(7–11)*, dementia *(15)*, mood disorders *(12)*, Down's syndrome *(13,14)*, unexlained recurrent early pregnancy loss and preeclampsia *(16,17)*? The answer would seem to be that these conditions can be modulated by either (1) folate nutritional status, (2) common allelic variation in genes coding for folate-dependent enzymes (particularly common SNPs of the Hcy remethylation (methionine) cycle), (3) altered gene expression, or (4) impaired nucleic acid elaboration linked to folate metabolism.

A detrimental permutation of these and as-yet undiscovered factors probably elicits a threshold effect on the occurrence of many of these serious conditions.

Birth Defects

Neural tube defects are among the commonest of all congenital malformations. The predominant types of NTD are spina bifida and anencephaly which have a prevalence of about 0.1% and 0.5% in the United States and the United Kingdom, respectively *(68)*. Randomized trials *(1,69,70)* verified early work that showed periconceptional folic acid prevents NTD pregnancy *(71,72)*, although the exact nature of the protective mechanism remains unclear. It has become well established that the etiology of NTD is multifactorial, with both gene and environment playing critical roles. Folate is the best known environmental factor, although low vitamin B_{12} levels have also been implicated in NTD pregnancy *(73,74)*.

At the gene level, molecular biological studies also implicate a number of SNPs in the aetiology of these devastating birth defects. In particular,

Table 2
Hcy Values (Median and IQR) for Each Female Group (Thromboembolic and Nonthromboembolic Vascular Patients, NTD Mothers and Matched NTD Controls) on the Basis of C677T Genotype

	CC	CT	TT
NTD control mothers			
Median homocysteine (μmol/L)	6.8	7.1	6.4
QR	5.7–10.1	6.4–7.4	6.0–10.6
NTD affected mothers			
Median homocysteine (μmol/L)	7.6	8.2	11
QR	6.5–8.7	6.6–9.3	9.2–12.8
Thromboembolism controls			
Median homocysteine (μmol/L)	10.6	9.3	12.5
QR	8.0–12.8	8.8–11.1	8.3–13.9
Thromboembolism patients			
Median homocysteine (μmol/L)	8.3	8.6	13.7
QR	8.1–8.5	8.1–10.2	10.5–16.1
All non-thromboembolism subjects			
Median homocysteine (μmol/L)	8.0	7.7	10.6
QR	6.4–10.6	6.6–9.3	6.4–12.8

Note: The table also gives the same information on the basis of thromboembolic vs all nonthromboembolic female patients.

enzymes involved in *de novo* methionine biosynthesis have been identified as risk factors *(73,75)*. 5,10-Methylene tetrahydrofolate reductase (5,10MTHFR) is a folate-dependent enzyme of the Hcy remethylation pathway responsible for the generation of $5CH_3$-H_4folate from 5,10-methylene tetrahydrofolate ($5,10CH_2$-H_4folate). $5CH_3$-H_4folate produced by this enzyme is used by vitamin B_{12}-dependent MetSyn in the *de novo* biosynthesis of methionine from Hcy (*see* Fig 2). Considerable interest exists in a common allelic variant of 5,10MTHFR in which a C-to-T substitution at nucleotide 677 converts alanine into valine (C677T MTHFR) *(76)*. Several reports describe a moderate elevation in plasma Hcy and /or increased frequency of C677T MTHFR in NTD *(45,77,78)*.

Approximately 10% of the population possess the recessive TT genotype and roughly half the population are either heterozygous or wild type. Research has shown that this common SNP exhibits a tiered reduction in 5,10MTHFR activity between genotypes *(76)*. Despite its high frequency and clear effect on folate metabolism, this SNP only accounts for a small

Table 3
Combined Hcy Data for All Subjects

	CC	CT	TT
Median homocysteine (μmol/L)	8.1	8.15	11.5
QR	6.5–10.1	6.6–9.3	8.1–13.4

Note: A Mann–Whitney test on this combined data shows that TT Hcy is significantly higher than combined CT and CC Hcy ($p = 0.026$).

proportion of all NTD *(79)*, with subsequent studies not always being able to confirm it as a risk factor at all *(24,80,81)*. The lower blood folate and, particularly, the moderately elevated plasma Hcy levels associated with the C677T MTHFR SNP are also associated with OVD *(2–5)*. Our own C677T MTHFR data from several female populations is given in Tables 2 and 3 and Fig. 3 [NTD data first published in Lucock et al. *(24)* with attendant vascular data in press *(82)*]. Clearly, there is little from our data to indicate that our Yorkshire mothers who experienced NTD pregnancy have an increased frequency of the mutant T allele. This comparison *(82)* therefore further validates the conclusions presented in our previous report *(24)*.

Despite mixed findings for C677T MTHFR as a risk factor in NTD pregnancy, seminal work by Guenther et al. showed that folate coenzymes can stabilize the polymorphic C677T MTHFR enzyme by preventing loss of its flavin cofactor *(83)*. This mechanistic explanation supports the treatment of elevated Hcy with folate supplements and provides one of the most elegant examples of a nutrient–gene relationship with significant ramifications in maintaining health and well-being. The influence of this SNP on other clinical conditions along with the metabolic implications of C677T MTHFR are discussed later.

The disposition of B vitamins and Hcy in blood of NTD affected individuals has led to a general focus on the methionine cycle, and particularly it's MetSyn locus as areas where the underlying metabolic problem(s) in NTD might reside *(73,75)*. However, as yet, no mutations of the MetSyn gene represent an increased risk for NTD. One of the most common mutations of MetSyn is A2756G MetSyn *(84)*. Although A2756G MetSyn appears not to be a risk factor for NTD *(85)*, the A → G transition at bp 2756, converting an aspartic acid (D919) into glycine, occurs near the crucial vitamin B_{12}-binding site and therefore might influence the enzymes secondary structure with possible functional consequences. Despite this interesting fact, the A2756G SNP frequency is similar in NTD and controls in a number

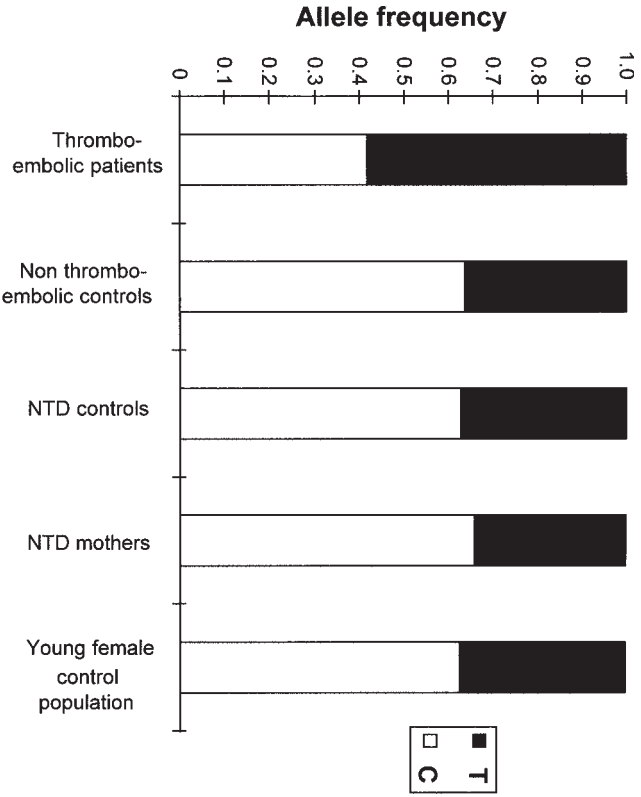

Fig. 3. Comparison of T allele frequency in female thromboembolic and nonthromboembolic vascular patients, NTD mothers and matched NTD controls. A small population of young, healthy females representative of the general population is also included ($n = 92$).

of recent reports *(24,84,85)*. Nevertheless, it has been suggested that this seemingly benign mutation may make a moderate but significant contribution to other clinical conditions that are associated with elevated Hcy *(86)*.

Other than the C677T mutation, a second SNP of MTHFR (A to C substitution at bp 1298) *(87)* and a SNP coding for methionine synthase reductase (MSR), an auxilliary protein for MetSyn that leads to an A to G substitution at bp 66 *(88)*, are the only other recognized folate-gene risk factors for an affected pregnancy at the present time. Despite this, C677T MTHFR only accounts for some NTDs *(79)*, whereas A66G MSR increases the risk of NTD only when vitamin B_{12} is low or in the presence of C677T MTHFR *(88)*. Combined heterozygosity for A1298C MTHFR and C677T MTHFR

was found in 28% of NTD vs 20% of controls, giving a significant odds ratio and accounting for a proportion of folate related NTDs not explained by C677T MTHFR alone *(87)*.

Other gene mutations of folate metabolism that could potentially account for NTD have been studied, but to date, none have been found causal for the majority of NTD pregnancies. These include both 5,10-methylene tetrahydrofolate dehydrogenase (MTHFD) (*89*), FR-α and FR-β *(90,91)*, and SHMT (in press). In our laboratory, we have attempted to examine the intracellular folate composition in NTD and control mothers *(24)* and to see how it is affected by any altered equilibrium in the methionine cycle as a result of the four common polymorphisms of folate/vitamin B_{12} metabolism mentioned earlier *(76,84,87,88)*. In particular, we were interested in seeing if it could provide any clue to the precise origin of any major new folate locus that might be causal for NTD pregnancy. Some of our most interesting findings showed that in a cell lysate model, NTD samples were less able than controls to produce long-chain polyglutamyl forms of formyl-H_4folate from an exogenous 5,10CH-H_4folate$_1$ substrate, the difference being significant. In addition, NTD samples were less able than controls to expand their 5,10CH2-H4folate pool following treatment with exogenous $5CH_3$-H_4folate$_1$ substrate. These latter effects were SNP independent. Taking polymorphisms into account, lysate from NTD–MTHFR wild types utilized significantly less exogenous $5CH_3$-H_4folate$_1$ substrate than control–MTHFR wild types. Commensurate with this latter effect, the initial production of $5,10CH_2$-H_4folate resulting from exogenous $5CH_3$-H_4folate$_1$ substrate was significantly reduced in the NTD–MTHFR wild type. These two MTHFR wild type effects imply that the C677T SNP is certainly not the only mutation affecting folate metabolism in NTD mothers.

In a separate study (*91a*), we found that all four remethylation cycle SNPs (two MTHFRs, MetSyn, and MSR) seemed to influence the circulating red cell folate-coenzyme profile in a way predicted by theoretical considerations. For instance, MTHFR variants increase formylfolates but reduce methylfolates, whereas SNPs at the MetSyn locus had the reverse effect. Figure 4 illustrates this SNP effect in a large group of women who have either never been pregnant or who have had a previous normal pregnancy (n = 32). By using a radar plot, the SNP effect on the relative size of the methylfolate and formylfolate pools is clearly apparent.

In addition to preventing NTD, Czeizel showed that periconceptional multivitamin supplementation (0.8 mg folic acid daily) reduced both NTD occurrence and the rate of other major congenital abnormalities *(92)*. Although major abnormalities like conotruncal cardiovascular malforma-

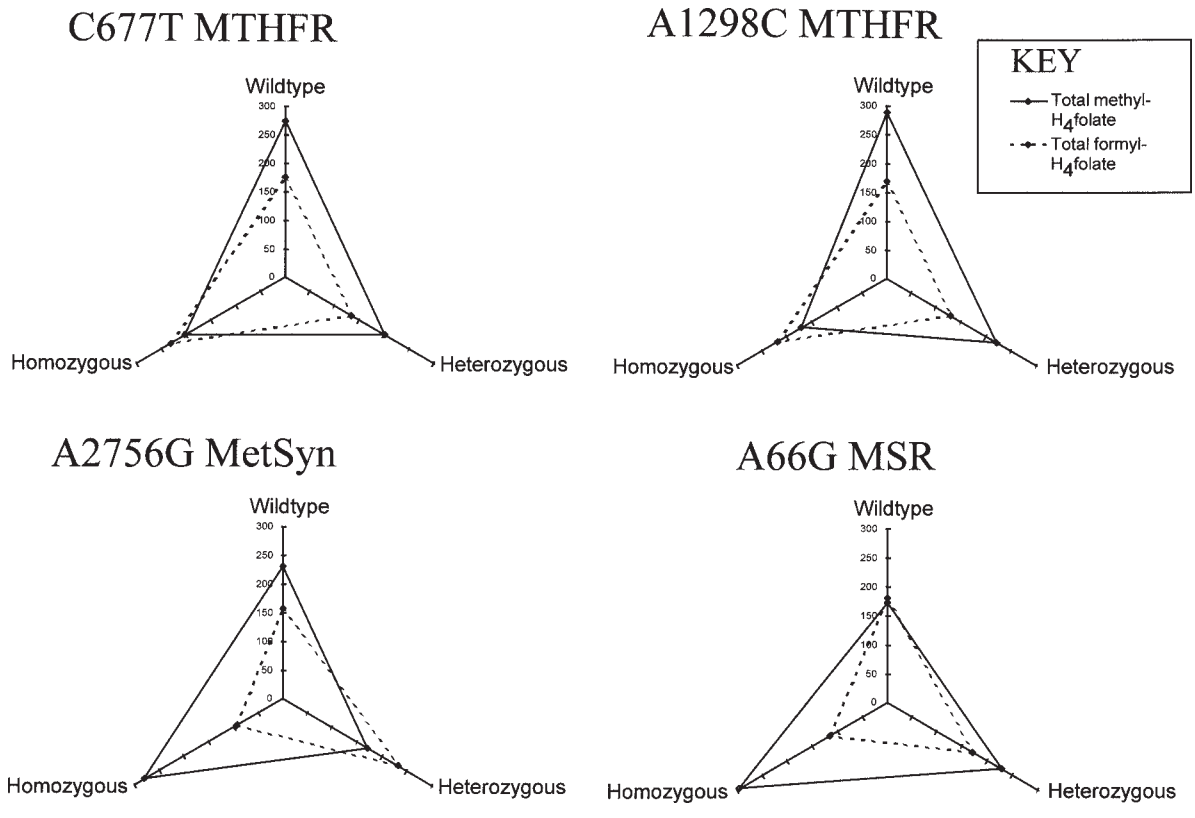

Fig. 4. The effect of C677T MTHFR, A1298C MTHFR, A66G MSR, and A2756G MetSyn polymorphisms on the cellular folate profile. MTHFR variants increase formylfolates relative to methylfolates, whereas variants at the MetSyn/MSR locus increase methylfolates relative to formylfolates. This behavior is what might be expected on theoretical grounds. The study group (n=32) consisted of women who have either never been pregnant or have had a previous normal pregnancy.

tions, defects of the urinary tract, congenital hypertrophic pyloric stenosis, and congenital limb deficiencies were diminished by multivitamin supplements containing folic acid, the rate of cleft lip and palate were not reduced *(93)*. Despite this, more recent work showed that recessive homozygosity for C677T MTHFR was more frequent in subjects with isolated cleft palate, and the authors indicated this could be etiologically important *(6)*. In a separate study, periconceptional supplementation with multivitamins containing folic acid (10 mg/d) was, in fact, shown to be effective in reducing the recurrence rate of orofacial clefts *(94)*. Although the precise biochemical basis of many of these disorders, for the most part, still lack clarity, the simple elegance of being able to prevent such a devastating malformation as spina bifida with an inexpensive safe vitamin is one of the finest success stories of modern preventative medicine.

Folate, Homocysteine, and Occlusive Vascular Disease

At present, there is considerable interest in Hcy. Several inherited disorders of Hcy remethylation and transsulfuration pathways elevate plasma and urinary Hcy well beyond the normal range, which is in the 7 to 24 μmol/L range for both fluids. As an example, the effect of cystathionine β-synthase (CBS) deficiency is to impair transsulfuration and greatly elevate Hcy. Homozygotes for this inborn error of metabolism exhibit mental retardation, thromboembolism, and premature OVD presenting at any age *(95)*.

Studies now clearly link the plasma Hcy level with vascular disease; sustained Hcy exposure in primates duplicates changes observed in early human arteriosclerosis *(96)*. Human studies support animal data and are consistent in their findings that indicate patients with OVD have higher blood Hcy levels than individuals with no disease. Interestingly, there is a narrow range of tolerance, as most vascular patients have values within what had, at one time, been considered to be the normal range *(97,98)*.

An inverse linear relationship exists between plasma Hcy and blood B vitamins (particularly folate). It has therefore been suggested that supplemental folate, especially in combination with vitamins B_6 and B_{12}, may offer a protection against OVD *(99,100)*. In fact, it has been determined that 9% of male and 54% of female coronary artery deaths in the United States (around 50,000 deaths/yr) could be prevented by fortification of grain products on a mandatory basis with non-native pteroylmonoglutamic acid *(101)*.

The therapeutic effects of folate in OVD are far simpler to comprehend than those involved in NTD. Hcy sits at the intersection of two important pathways (*see* Fig. 2) and is regulated by several enzymes, some of which are polymorphic. The partitioning of Hcy between *de novo* methionine bio-

synthesis and transsulfuration to cystathionine is allosterically regulated by *S*-adenosylmethionine (SAM) at the level of cystathionine β-synthase (stimulates) and 5,10MTHFR (inhibits) (ref. *102* provides a recent review on Hcy regulation).

In the Hcy remethylation (methionine) cycle, $5,10CH_2$-H_4folate is reduced to $5CH_3$-H_4folate by the polymorphic flavoprotein 5,10MTHFR. This is the only reaction capable of producing $5CH_3$-H_4folate and, in vivo, it is irreversible *(103)*. Vitamin B_{12}-dependent MetSyn then methylates Hcy, a process that uses $5CH_3$-H_4folate and yields H_4folate and methionine *(104)*. This latter step also requires a recently discovered enzyme, MSR, that reductively activates MetSyn *(105)*. The use of betaine for conversion of Hcy into methionine involves the vitamin B_{12}-independent enzyme betaine: homocysteine methyltransferase. Methionine-synthesized *de novo* can then be activated by ATP and methionine adenosyl transferase to yield the methyl donor SAM, which methylates a variety of important biomolecules such as adrenalin, phosphatidylcholine, and carnitine. During this process, SAM is converted to *S*-adenosylhomocysteine (SAH), which is then hydrolyzed back to Hcy to recommence a new remethylation cycle *(102)*. This is the sole route for Hcy production in vertebrates.

Transsulfuration involves the condensation of Hcy with serine to form cystathionine, a vitamin B_6-dependent step catalyzed by CBS. Cystathionine is then hydrolyzed to cysteine and α-ketobutyrate by another B_6-dependent enzyme, γ-cystathionase.

The coordinated regulation of remethylation and transsulfuration pathways is under the influence of SAM, which controls the utilization of Hcy *(102)*. SAM allosterically inhibits 5,10MTHFR while activating CBS. Therefore, when SAM levels drop, $5CH_3$-H_4folate formation is unrestricted while cystathionine formation is reduced. In this situation, Hcy is conserved for methionine production. By contrast, elevated SAM stimulates the transsulfuration of Hcy resulting from allosteric activation of CBS. Therefore, the SAM/SAH ratio, concentration of the *de novo* methyl group acceptor Hcy, and dietary factors, particularly folate and methionine, but also vitamins B_{12} and B_6,are all important determinants of one-carbon metabolism and the metabolic balance between remethylation and transsulfuration pathways *(102,106–112)*.

Clearly, folate nutrition is intimately tied into Hcy status. Although a few studies have been unable to find an association between plasma Hcy and OVD *(113,114)*, ample evidence now exists to uphold such an association *(2–5)*. Even modest elevations in plasma Hcy have a pathological effect on vascular endothelium. It is now accepted that Hcy is an independent risk

factor for arteriosclerosis and venous thrombosis, although the precise cellular mechanisms involved are unresolved. Among the possibilities is that the pro-oxidant activity of this thiol inhibits production of an endothelin-derived relaxation factor and activates quiescent vascular smooth muscle cells. At physiological concentrations, Hcy may inhibit the vascular endothelial cell cycle at or before the G_1-S junction. This inhibition seems to be mediated by a drop in carboxyl methylation, membrane association, and activity of $p21^{ras}$, a G_1 regulator.

Particular interest exists in Hcy because of its association with a gene that encodes an important folate-dependent allosteric enzyme (5,10MTHFR) that may link folate to OVD via regulation of plasma Hcy levels *(76)*. It is clear that an association exists between elevated homocysteine, and both TT C677T MTHFR and vascular disease. However, any association between TT genotype and vascular disease remains controversial despite Kang et al., showing that the variant C677T thermolabile enzyme is also a risk factor for OVD *(115)*. For this reason, we recently examined T allele frequency and Hcy in four female populations; thromboembolic (TE) and nonthromboembolic (NTE) vascular patients, NTD mothers, and matched controls. These clinical groups were selected because TE events are the principal vascular signature of homocysteinuria and the major cause of morbidity and mortality in this condition and also because it allows comparison with another clinical group (NTD) reportedly having both elevated Hcy and T allele frequency. The results are presented in Tables 2 and 3 and Fig. 3 *(82)*.

We examined 92 women in total. Twelve had had TE events, 21 were vascular controls treated for NTE vascular problems, 19 were NTD mothers, and 31 matched NTD controls. For completion, five young healthy females representative of the general population were also included.

In our comparative study, T allele frequency was constant at around 36% in all groups except TE patients, where it rose to 58.3% (Fig. 3). When data were combined (TE vs all NTE), allele numbers gave a significant odds ratio indicating that the T allele is a risk factor for TE (OR = 2.46, 95% CI 1.03 – 5.90).

The Hcy levels were assessed by genotype and TE vs NTE event (Table 2). Combined, Hcy data for all subjects shows TT Hcy is significantly higher than combined CT and CC Hcy ($p = 0.026$) (Table 3).

We believe that our data suggest C677T MTHFR is a more important factor in TE events than it is in NTD pregnancy. As mentioned earlier, Guenther et al. showed that folate coenzymes stabilize C677T MTHFR by preventing the polymorphic enzyme from relinquishing its flavin cofactor

(83). It could well be an abberation of this nutrient–enzyme interaction that explains our TE findings.

Our findings on TE *(82)* are consistent with other recent reports linking C677T genotype to thromboembolic disease *(116,117)*. Some of the most recent findings in this general area suggest that another remethylation cycle SNP, A66G MSR, is also a risk factor for vascular disease—specifically the development of premature coronary artery disease, although. in this case, by a mechanism independent of elevated Hcy *(118)*.

Folate and Cancer

Several types of cancer have been attributed to folate status and or metabolism: Butterworth showed a link between folate and premalignant cervical dysplasia *(7)* and Kamei et al. showed that folate could reduce squamous metaplasia of the bronchial epithelium *(8)*. More recently, research into folate and cancer linked low levels of the vitamin to a risk for breast cancer when associated with high alcohol consumption *(10)*. It has also been shown that a threshold effect exists for vitamin B_{12} and enhanced risk for breast cancer among postmenopausal women *(119)*. Interestingly, a familial association exists between NTD and gastric cancer, with a parallel decline in these two conditions indicating a possible common etiology *(120)*, which might be folate related *(31)*. At the present time, considerable interest is focused on folate nutrition and C677T in the etiology of colon cancer *(9)*. It is possible that by increasing levels of $5,10CH_2$-H_4folate, a coenzyme required for nucleotide biosynthesis, C677T MTHFR might actually protect against cancer *(121)*. However, under poor folate nutritional status, the same SNP might augment uracil misincorporation and promote carcinogenesis. A similar principle may occur in leukemia. Skibola et al. *(11)* have shown that individuals with the C677T MTHFR TT genotype and both homozygous recessive and heterozygous A1298C MTHFR genotypes have a decreased risk of acute lymphoblastic leukemia (but not acute myeloid leukemia), indicating folate depletion may play a role in the development of this form of malignancy.

Down's Syndrome

Evidence exists that abnormal folate and methyl-group metabolism can lead to genomic hypomethylation (see below) and abnormal chromosome segregation. On this premise, James et al. *(13)* hypothesized that the C677T MTHFR SNP may be a risk factor for maternal meiotic nondisjunction and Down's syndrome (trisomy 21) in young mothers. Their study showed that folate metabolism is abnormal in mothers of these children and that this may

be explained in part by the 677 nucleotide substitution. This finding has since been repeated *(14)*. These same workers showed that homozygous recessive A66G MSR mothers were also at higher risk, and that, in combination, these two SNPs were associated with increased risk (OR: 4.08; 95% CI; 1.94–8.56).

Folate and the Brain

In Alzheimer's disease, it is possible that Hcy may interact with the cerebral microvasculature in a negative way *(15)*, thereby introducing folates into yet another completely different, yet nonetheless important clinical condition.

Parenteral treatment with pharmacological $5CH_3$-H_4folate has been used as therapy for affective disorders *(12)*. However, it is unclear whether the folate effect is mediated via a direct impact on neuronal membranes or if $5CH_3$-H_4folate acts indirectly through an alteration in neurotransmitter metabolism. Methyltransferases such as catechol-*O*-methyltransferase, hydroxyindole-*O*-methyltransferase, and phenylethanolamine-*N*-methyltransferase are crucial for the synthesis of neuronal products. SAM, produced *de novo* from $5CH_3$-H_4folate, is the methyl donor for all these important enzymes. Although plasma $5CH_3$-H_4folate is taken up at the choroid plexus and concentrated in the CSF, it is not known exactly why this should be and if it is related to the above.

A Probable Role for Folate in Preeclampsia, Recurrent Miscarriage, and Low Birth Weight

Normal cell growth and division relies upon an adequate supply of folate and it is, thus, an important vitamin during pregnancy. Studies show an inverse correlation between birth weight and maternal Hcy *(122,123)*. Also, there is a direct association between birth weight and both maternal serum folate and red cell folate *(124,125)*. Whether the link between moderately elevated Hcy levels in mother or fetus and low birth weight is mediated through defective placental function is unclear. Reports also describe increased Hcy in women with placental abruption or infarction when compared to normal controls *(16,123,126)*. Moreover, preeclampsia, a condition characterized by defective placentation, is also linked to elevated Hcy levels *(16,17)*.

All of the above are pathological conditions associated with notable maternal and fetal morbidity and mortality. In fact, even low birth weight itself is associated with OVD in later life *(127)*.

It seems that C677T MTHFR may be clinically significant in these conditions. Women with preeclampsia exhibited a higher carriage of the mutant T allele and frequency of the recessive genotype *(128)*. This association is also seen in women with unexplained recurrent early pregnancy loss *(129)*. On the basis of these findings, it seems that folate nutrition combined with specific common SNPs of genes coding for folate-dependent enzymes may be critical determinants of pregnancy outcome. Recent work carried out in this laboratory by Ovadia *(130)* showed that C677T MTHFR allele frequency determined from blood spots on archived Guthrie cards was similar in low-birth-weight babies (2340 g [Interquartile range (IQR) 2205 g–2430 g], $n = 125$) and control babies (3483 g [IQR 3400g–3588 g], $n = 50$); the mutant T allele frequency is 0.27 and 0.26 for low vs normal birth weight, respectively (OR: 1.06; 95% CI: 0.63–1.80). However, when the TT genotype was examined as a proportion of the population, a trend seemed to emerge because the calculated OR approached significance: TT = 11% and 4% for low versus normal birth weight, respectively (OR: 2.97; 95% CI: 0.91–9.66). Therefore, maternal genotype may not be the only issue; the extent to which fetal genotype influences pregnancy outcome could also be an interesting and important question and one that requires more detailed examination with a larger population.

The importance of MTHFR in life processes is illustrated by the almost complete absence of more than two mutated alleles when the C677T and A1298G SNPs are examined in combination. In a large separate study of vascular patients, we observed that of 157 adults examined, 21 had no mutant alleles, 73 had 1 affected allele, 62 had 2 affected alleles, and only 1 individual had 3 affected MTHFR alleles. No individuals were homozygous recessive for both MTHFR SNPs (Yates and Lucock, unpublished data). This data might suggest that three or more MTHFR alleles are selected against by a reduced fetal/embryonic viability. This, however, also requires further examination.

PUTATIVE MECHANISMS INVOLVING FOLATE METABOLISM THAT MAY EXPLAIN THE ABOVE DISORDERS

As explained in some detail earlier, folate nutritional status and common allelic variants of Hcy remethylation cycle enzymes, particularly C677T MTHFR, are implicated in several disorders. The mechanistic link between C677T MTHFR (and possibly other SNPs) and these disease processes may involve the following mechanisms.

Abnormal Methylation of Genomic DNA

The cytosine base of 5'-CG–3' DNA sequences is methylated to yield a 5-methylcytosine residue, representing a biologically important covalent modification that modulates the expression of genes (including important housekeeping genes) and reinforces developmental processes in mammalian cells. Folate-regulated *de novo* methionine metabolism (*see* Fig. 2) probably plays a critical role in CpG methylation, given the direct role of SAM in this process. Because methylation of CpG clusters associated with promoter regions tends to silence the expression of genes, it is possible that impaired folate-dependent *de novo* methionine biosynthesis may lead to a methyl-group deficit, modifying normal regulation of proto-oncogene expression and predisposing individuals to neoplastic change *(131)*. Clearly, any reduced channeling of one-carbon units between $5,10CH_2$-H_4folate and $5CH_3$-H_4folate because of an impaired enzyme activity of MTHFR resulting from carriage of the mutant T allele could influence *de novo* methionine biosynthesis, SAM levels, and, therefore, genomic methylation. Figure 4 shows the reduced methylfolate pool that occurs in C677T MTHFR.

Chromosome Breakage

A further mechanism involving folate in malignant disease is thought to involve damage to the structural integrity of the chromosome. Inadequate folate nutrition can precipitate misincorporation of uracil into DNA, leading to chromosome breakages. Uracil misincorporation is particularly sensitive to the $5,10CH_2$-H_4folate coenzyme level, as this folate is required by thymidylate synthase for converting dUMP to dTMP *(132)* (*see* Fig. 2). It has been suggested that where folate nutrition is replete, the C677T MTHFR mutation affords greater protection against misincorporation compared to wild types, but it augments the process if folate nutrition is poor *(11,121)*. Therefore, optimal nutritional conditions and the presence of C677T MTHFR elevates $5,10CH_2$-H_4folate and could theoretically improve the potential for nucleotide biosynthesis, but, as a consequence, may restrict $5CH_3$-H_4folate synthesis and, therefore, *de novo* methionine production.

Chromosome breakage and DNA methylation are both thought to be factors in the development of certain cancers. Those with an etiology linked to C677T MTHFR include colon cancer *(9,121)* and acute lymphoblastic leukemia *(11)*. As mentioned earlier, other cancers have also been associated with folate status. These include premalignant cervical cancer *(7)* and squamous metaplasia of the bronchial epithelium *(8)*. The most recent work on folate and the development of cancer suggests low levels of the vitamin are a risk for breast cancer when associated with high alcohol intake *(10)*, with

a threshold existing for vitamin B_{12} and enhanced breast cancer risk among postmenopausal women *(119)*. The extent to which nutrition interacts with a genetic component or the role of specific mechanisms in many of these malignancies still remains to be ascertained.

C677T MTHFR and Folate Status in Nonmalignant Disorders

The C677T MTHFR SNP is thought to disrupt the folate-dependent Hcy remethylation cycle. This elevates potentially atherogenic Hcy and impairs regeneration of H_4folate and SAM. As described earlier, C677T MTHFR is a risk factor for several nonmalignant disorders, including NTD *(78)*, cleft palate *(6)*, Down's syndrome *(13,14)*, vascular disease *(76,82,115–117)*, preeclampsia *(128)*, and unexplained recurrent early pregnancy loss *(129)*. Although it is probable that folate nutritional status is an issue in all these disorders, only in OVD is there a reasonably clear understanding of the mechanism of action via a damaging effect of Hcy on the vascular endothelium *(133)*. However, even then, the precise cellular interaction elicited by Hcy that precipitates OVD is still not fully resolved. There is also a lack of clarity as to how folates influence mood disorders. SAM, produced *de novo* from $5CH_3$-H_4folate, is the methyl donor for methyltransferases such as catechol-*O*-methyltransferase, hydroxyindole-*O*-methyltransferase, and phenylethanolamine-*N*-methyltransferase, all of which underpin the production of neurotransmitters. This metabolic relationship probably represents the best explanation for the reported successful treatment of affective disorders with pharmacological $5CH_3$-H_4folate *(12)*. Similarly, one can speculate that although no mechanisms have as yet been postulated for the relationship between dementia and B vitamin status, a link to the vascular effects of Hcy would not be surprising *(15)*.

Although some of the explanations put forward to explain the beneficial role of folate in the above conditions are clear enough, others are, at best, gray and fairly hypothetical. None, however, is more difficult to explain than the role of folate in NTD and other mid-line defects. Hcy is slightly elevated in NTD *(24)*, and C677T MTHFR is a risk factor in some reports *(77,78)* (but not all) *(24,80,81)*. Despite this, it is this researcher's view that Hcy levels are more likely to be indicative of rather than causal for NTD, with some other folate locus in addition to C677T MTHFR contributing to the increase in Hcy (*24*, *91a*). There is certainly still much to discover about this devastating condition. As discussed earlier, one can consider the level of cellular $5,10CH_2$-H_4folate as being a critical factor in many of these disorders; one only has to study Fig. 2 to realize that this coenzyme sits at a metabolic branch point for three important pathways *(134)*. Although

5,10CH_2-H_4folate is used by 5,10MTHFR to produce 5CH_3-H_4folate in a committed step that then requires B_{12}-dependent MetSyn to cycle Hcy into methionine, it is also required for both thymidylate synthase and MTHFD in the synthesis of DNA thymine and purine, respectively.

Given the critical nexus that 5,10CH_2-H_4folate sits at and taking into account the known NTD–SNP risk factors (and the as-yet major unidentified NTD lesion site), it is quite possible that a metabolic imbalance could easily arise. For instance, if all of these factors unite to alter the concentration of folate substrates for enzymes that have K_m values higher than the cellular concentration of their natural substrates, then one-carbon transfers may be restricted. In particular, the enzymes of nucleotide biosynthesis (thymidylate synthase, dihydrofolate reductase, MTHFD/methenyltetrahydrofolate cyclohydrolase, aminoimidazolecarboxamide ribonucleotide transformylase and glycinamide ribonucleotide transformylase) belong in this category *(134)*. Because of their high K_m values, catalytic activity is critically dependent on the concentration of specific folate substrates. In NTD, lowering of folate substrates for such enzymes of purine and pyrimidine pathways may, in fact, turn out to be crucial in the occurrence of these birth defects. It is quite possible that at the end of the day, a number of folate-related factors will end up being discovered and that they conspire to produce a threshold effect on NTD occurrence.

FOLATE SUPPLEMENTATION

Given the importance of folate nutrition and the high frequency of SNPs in genes coding for folate-dependent enzymes, a compelling argument can be made for mandatory fortification of foods with pteroylmonoglutamate, a simple measure that could lead to an enormous improvement in public health. In the context of NTD, the neural tube closes during postovulation d 22–28 *(135)*, unfortunately, at this point in time, many women are not aware of their pregnancy and that they should have taken folate supplements prior to conception. Therefore, perhaps it is not so much whether we should fortify, but by how much. Current recommendations are that all women planning a pregnancy should take an additional 400 μg folate per day. Pharmacological pteroylmonoglutamate as occurs in supplements and fortified foods does not occur in nature. However, it is both inexpensive and extremely stable, unlike many native forms of the vitamin such as 5CH_3-H_4folate. The body metabolizes pteroylmonoglutamate into 5CH_3-H_4folate, the normal transport form of the vitamin found in plasma. However, research shows that this absorption and metabolic transformation step is saturated at doses in the region of 266–400 μg pteroylmonoglutamate *(136,137)*. This

raises the issue of extended exposure to unmetabolized pteroylmonoglutamate, were mandatory fortification to go ahead above a certain critical level. Although such exposure may present no health risk at all, decisions need to be made that take into account both biochemical and epidemiological viewpoints on the issue of mandatory fortification of the population.

CONCLUSIONS

The role of folate embraces the production of several important biological metabolites, particularly methionine and nucleotides. The availability of folate from the diet, the cellular reserve of folate, and SNP status of genes that code for folate-dependent enzymes all conspire to influence health. The specific folate-related molecular mechanisms that influence disease formation are numerous. Recognized and putative mechanisms include Hcy toxicity, impaired methylation reactions, and reduced nucleotide formation, including misincorporation of uracil into DNA. Despite significant advances in our understanding of folate metabolism and a clear role for folate in preventing NTD, these birth defects still have no coherent biochemical explanation and remain the major folate-related health enigma.

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14

Vascular Disease in Women

Folate and Homocysteine

Tom K. A. B. Eskes

CARDIOVASCULAR DISEASE

Gender Difference in Cardiovascular Disease

Within the preventive aspects of health care in women, cardiovascular disease should have more attention. The main argument for this statement is the fact that about 40% of women will die from cardiovascular disease and around 20% from malignant disease. In other words, risk factors for cardiovascular disease and blood pressure are more important than a Pap smear.

In ischemic heart disease, there is a very marked gender difference: Women die 10–15 yr, later than men and the death rate in women increases exponentially after the age of 50. In case of artificial menopause, the risk for atherosclerosis is two to three times higher than a menopausal age of 50 yr. The mortality figures for coronary heart disease vary from 50 to 200 per 100,000 inhabitants (male more than female) and for cerebrovascular disease, 100 per 100,000 inhabitants (female more than male).

Among the risk factors known for artery disease, such as smoking, cholesterol, body weight, and familial occurrence, homocysteine is an independent risk factor *(1)*. Hyperhomocysteinemia is attributed to commonly occurring genetic and acquired factors, including low values of folate and vitamin B_{12} *(2)*.

Homocysteine: A Small and Crucial Sulfurated Amino Acid

Homocysteine is the demethylated derivate of the essential amino acid methionine. Homocysteine and methionine are small sulfur-containing aminoacids.

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In one of the pathways, homocysteine is transsulfurated, requiring the enzyme cystathionine synthase and vitamin B_6 as a cofactor. In the other pathway, homocysteine is remethylated to methionine, requiring methionine synthase with vitamin B_{12} as a cofactor, and 5,10-methylenetetrahydrofolate reductase (MTHFR) with folate as a cofactor. Because of the critical position of MTHFR in the methionine–homocysteine cycle, homocysteine has become a very sensitive marker for the folate status.

Homocysteine is methylated to methionine by the transfer of the methyl group of methyltetrahydrofolate *(3)*. The so-called methylation cycle is therefore a methyl-donor cycle and serves as "one-carbon metabolism." Methyl groups are important for various biochemical systems like DNA and tRNA synthesis, proteins (myelin), and lipids. The transfer of a methyl group is of vital importance for cell functioning. Phospholipid methylation, for instance, is necessary for the proper function of cell membranes. Methyl groups are necessary for the synthesis and metabolism of neurotransmitters. The synthesis of creatine is important for high-energy buffering systems.

In the interpretation of homocysteine values, one has to take into account a number of variables that can explain low or high values. Factors like age, gender, race, renal insufficiency, folate status, vitamin B_6 and B_{12} concentrations, genetic mutations, and antifolate medication have to be known for the clinical interpretation of homocysteine values.

For more than 30 yr, moderately raised homocysteine concentrations have been associated with an increased risk of atherothrombotic events *(4,5)*.

Homocysteine is known as an independent risk factor for vascular disease *(1,6)* and birth defects like neural tube defects *(7,8)*, schisis *(9)*, and congenital heart defects *(10)*.

Homocysteine Metabolism

The only source of homocysteine in man is demethylation of methionine, an essential amino acid. Homocysteine may either be transsulfurated or remethylated. The former pathway includes the conversion of homocysteine to cystathionine. This step is catalyzed by the enzyme cystathionine β-synthase with vitamin B_6 (pyridoxal 5'-phosphate) as a cofactor. In the remethylation pathway, homocysteine is converted into methionine by the enzyme methionine synthase and vitamin B_{12} as a cofactor and 5-methyltetrahydrofolate (methyl-THF) as a substrate. Methyl-THF is formed from 5,10-methylenetetrahydrofolate; this reaction is catalyzed by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). Thus, the enzymes involved in the metabolism of homocysteine are dependent on the B vitamins B_6, B_{11} (folic acid), and B_{12}. The crucial role of the methionine–

homocysteine cycle is to donate methyl groups to a variety of substances, among them DNA and mRNA. Folates do play a crucial role in this so-called "one-carbon metabolism."

In short, homocysteine derived from dietary methionine is converted to methionine in a folate- and vitamin B_{12}-dependent reaction (remethylation) or to cystin in a vitamin B_6-dependent reaction *(3)*. Causes of hyperhomocysteinemia are genetic and lifestyle factors, vitamin deficiency (nutrition), systemic disorders, and drugs (antifolates).

The methionine–homocysteine metabolism can be stressed by oral loading with L-methionine (0.1 g/kg body weight).

The Methionine Loading Test

The methionine–homocysteine cycle can be challenged by methionine loading (0.1 g/kg body weight) taking the 6 h homocysteine value into account *(11)*. Depending on the cutoffs used, about 50% of patients with a normal fasting level of homocysteine have hyperhomocysteinemia after methionine loading (12).

In the European Concerted Action Project, approx 27% of patients with hyperhomocysteinemia could be identified only after methionine loading. These patients had an increased incidence of arteriosclerotic disease, with a relative risk of 1.8 compared with a relative risk for fasting hyperhomocysteinemia of 1.9. The authors concluded that hyperhomocysteinemia conferred an independent risk of vascular disease similar to that of smoking and hypertension *(13)*.

Fasting homocysteine levels are lower in women than in men. Levels of homocysteine show a positive association with age for both sexes. In the postmenopausal age category, female post-methionine-load homocysteine levels surpass the levels of men.

Elevation of homocysteine (>80th percentile of controls) appeared to be at least as strong a risk factor for vascular disease in women as in men, even before menopause. For post-methionine-load homocysteine, there is a 40% stronger association with vascular disease in women than in men. In both sexes, low vitamin B_6 conferred a twofold to threefold increased risk of vascular disease, independent of homocysteine. Folate levels lower than the 20th percentile were associated with a 50% increased risk of vascular disease *(14)*.

The intra-individual coefficients of variation varies from 15 to 23%. This variability was significantly related to the variability in serum folate levels *(15)*.

Homocysteine Is an Independent Risk Factor of Vascular Disease

In 1969, McCully made the clinical observation linking elevated plasma homocysteine concentration with vascular disease *(4)*. He reported autopsy evidence of extensive arterial thrombosis and atherosclerosis in two children with elevated plasma homocysteine concentration and homocysteinuria.

There is substantial evidence that homocysteine is a strong and independent risk factor for vascular disease *(1,16,17)*. The evidence can be found in more than 80 epidemiologic studies with more than 10,000 subjects. The risk for cardiovascular disease is dose dependent without a cutoff level. For venous thrombosis, data from case-control studies support hyperhomocysteinemia as a risk factor (*18,19*).

A strong, graded association between plasma homocysteine concentrations and overall mortality has been reported in patients with coronary artery disease *(20)*. This indicates that the low risk of coronary artery disease may be related to reproductive hormones.

In the European Concerted Action Project, approximately one-third of patients with hyperhomocysteinemia could be identified only after methionine loading. These patients had an increased incidence of arteriosclerotic disease (relative risk 1.8) compared with the risk for fasting hyperhomocysteinemia *(21)*.

Premenopausal women have significantly lower plasma homocysteine values than premenopausal women. This holds for both the fasting as well as the post-methionine-loading values. Homocysteine levels are negatively correlated with estrogen levels *(22)*. In women, one has to recognize hyperhomocysteinemia when thromboembolic episodes occur during the use of oral contraceptives, the postmenopause, in a postoperative period, or during pregnancy and the postpartum period.

A randomized controlled trial of the effect of vitamins (including folic acid) on the risk of vascular disease has still to be undertaken.

Methylenetetrahydrofolate Reductase (MTHFR)

Homocysteine derived from dietary methionine is converted to methionine in a folate- and vitamin B_{12}-dependent reaction (remethylation) or to cystin in a vitamin B_6-dependent reaction (transsulfuration) *(3)*. Homocysteine is methylated to methionine by the transfer of the methyl group of methyltetrahydrofolate, which is formed by reduction of the methylene group of methylene tetrahydrofolate in a reaction catalyzed by methylene tetrahydrofolate reductase (MTHFR). Kang *(23)* described a thermolabile

variant of MTHFR that is associated with decreased enzyme activity and mildly elevated plasma homocysteine levels. Frosst et al. *(24)* identified the responsible mutation in the MTHFR gene: a C-T substitution at bp 677 leading to an exchange of alanine to valine. This mutation (TT genotype) is present in approx 12% of the population.

The Common MTHFR Gene Mutation (C677T) Does Not Always Lead to Vascular Disease

Brattström et al. *(25)* published an important paper on the clinical effect of the MTHFR mutation. In a meta-analysis of 13 studies, 23 case-control studies and 5869 genotyped cardiovascular patients, it could be clearly established that mild hyperhomocysteinemia was associated with arterial disease. Around 12% of the white population is mutant homozygous for the TT genotype, which is frequently accompanied by mildly elevated circulatory levels of homocysteine. The meta-analysis, however, could not conclude that the mutation as such did increase the cardiovascular risk, namely for coronary disease.

The authors did not find a clear-cut explanation for this finding. One has to realize, however, that the expression of this mutation is dependent on the folate status *(26)*. In this regard it is striking that analysis of the European studies in this meta-analysis does show positive odds ratios, whereas the American studies, except one, demonstrate odds ratios around 1.0. Probably the American study patients, although "well nourished" like the Europeans, do take many more vitamins and eat much more and folaterich or folate-enriched food.

One also has to realize that a mutation can have beneficial, effects like the protection for colon carcinoma *(27)*.

Homocysteine, the Menstrual Cycle, and Reproductive Steroids

The Menstrual Cycle

The menstrual cycle is characterized by an increasing ovarian production of estrogenic hormones during the first (follicular) phase of the cycle followed by the addition of progesterone in the second (luteal) phase of the cycle. Fasting homocysteine levels were lower in women than in men.

In premenopausal women, fasting and post-methionine-load homocysteine serum levels are significantly lower than in postmenopausal women *(28)*. Levels of homocysteine showed a positive association with age. Silverberg et al. *(29)* stressed the need for specific reference ranges for homocysteine values also after methionine loading.

De Cree et al. *(30)* found lower homocysteine levels in the follicular than in the luteal phase of the cycle. These differences were attenuated by training exercise. These observations suggests an inverse correlation between reproductive steroid hormones and homocysteine as demonstrated in cortisol- and estradiol-treated rats *(31)*.

The interdependency of homocysteine, folate, and vitamin B_{12} was nicely demonstrated in serum and follicular fluid *(32)*.

During human pregnancy, when steroid hormones increase dramatically, low homocysteine values are found *(33–35)*. The lower homocysteine values in premenopausal women can partially be explained by body mass, by activity of the transsulfuration pathway of homocysteine, and, most likely, by estrogen hormones. lt seems quite plausible that reproductive steroids like estrogens and progesterone are responsible for the unique difference in efficiency of homocysteine–methionine metabolism.

It is interesting to note that the variance of the menstrual cycle is lower during the use of multivitamins including folic acid *(36)*.

Oral Contraceptives Produce Cyclically Recurrent Periods of Hyperhomocysteinemia

Estrogens and progesterone are also present in oral contraceptives in the form of synthetic steroids like ethinylestradiol and various progestational agents. As in the ovulatory menstrual cycle, the blood levels of reproductive steroids are the highest in the second phase of the cycle (i.e., around d 23) and the lowest during menstruation or during the period of withdrawal bleeding.

Early studies on oral contraceptives containing high levels of estrogens suggested an adverse effect on folate status. Later surveys reported no influence on folate metabolism.

Methionine metabolism, as judged by urinary excretion, was reported not to be influenced by the use of (high dose) oral contraceptives *(37)*.

Steegers-Theunissen et al. *(38)* demonstrated that homocysteine levels in serum were significantly higher in the low-hormonal phase of the contraceptive cycle than in the high-hormonal phase. The levels were comparable to those of heterozygotes for homocystinuria. Brattström et al. *(39)* found no difference in fasting plasma homocysteine concentrations in women on oral estrogen-containing contraceptives. The day of blood sampling, however, was not reported.

In women who used oral contraceptives and had documented vascular occlusion, higher levels of homocysteine were reported compared to controls *(40)*.

An interesting observation has been made by Hladovec et al. *(41)* demonstrating a decrease in methionine tolerance and an increase of detached endothelial cells in the blood in young women using oral contraceptives. This phenomenon could be prevented by using pyridoxine.

Several authors suggest that women using combined oral contraceptives have raised plasma concentrations of vitamin A and reduced concentrations of vitamin B_{12} and folic acid. This could be confirmed, except for folic acid, in a case-control study with sub-50 oral contraceptives.

Multivitamin supplementation (Gravitamon®) increased the levels of the vitamins administered, except vitamin A (intake 1000 IU) and 1 µg/d of vitamin B_{12} *(42)*.

Oral contraceptives containing less than 50 µg of estrogens alter folate kinetics. After oral folate loading, significantly lower serum folate and vitamin B_{12} concentrations were found *(43)*.

Homocysteine, the Menopausal Transition and Reproductive Steroids

The menopause is defined as the moment of the last vaginal bleeding. Because of the loss of production of ovarian estrogens and progesterone, the levels of gonadotrophins (FSH and LH) rise markedly. When the gonadotrophins levels are above 10 IU/L or when vaginal bleeding does not recur in 1 yr the period of the postmenopause has begun. The menopausal transition affects 10–20% of women with climacteric complaints in which the estrogen-dependent flushes are predominant.

In the post-menopausal age category, post-methionine-load homocysteine levels surpass levels of men.

Because vascular disease sharply increases in postmenopausal women and estrogen loss because of ovarian failure occurs, it is tentative to correlate both events *(44)*.

Estrogens and agonists lower plasma homocysteine levels in postmenopausal women (Table 1). Women with the highest pretreatment homocysteine levels showed the largest absolute decrease in plasma homocysteine concentrations. When estrogens are combined with progestational agents, homocysteine decreased more than estrogens alone *(51)*.

Tamoxifen is a nonsteroidal estrogen agonist and antagonist used for the treatment of advanced breast cancer. Tested in postmenopausal women with breast cancer, tamoxifen lowered plasma homocysteine values by 25–30% *(45)*.

Raloxifene, a novel nonstreroidal compound, binds to a unique area of DNA and produces tissue-specific estrogen agonistic and antagonistic effects. This drug is now under investigation for the treatment of climacteric

Table 1
Studies on the Homocysteine Lowering Effect of Estrogens and Agonists in Postmenopausal Women

Population	Exposure	Methods	Results	Remarks	Ref.
n = 31 (Norway)	Tamoxifen (30 mg/d)	Observational	–29.8% after 9–12 mo; –24.5% after 13–18 mo	Median age 65 yr	*45*
n = 21 healthy (Netherlands)	17β Estradiol (2 mg/d); dydrogesterone (10 mg/d); 14 d/28 d cycle; 2 yr	Prospective; observational	–10.9% lasting decrease after 6 mo	FSH > 40 IU/L, no previous folate suppl.	*46*
n = 39 healthy (Netherlands)	Conjugated estradiol (0.625 mg/d); medrogestone (10 mg/d); 14 d/28 d cycle; 1 yr treatment	Prospective; longitudinal; open folate suppl.	–12.3% lasting decrease after 6 mo	FSH > 36 IU/L, no previous folate suppl.	*47*
n = 27 healthy (Netherlands)	Estradiol (1–2 mg/d); dydrogesterone (10 mg/d) 14 d/28 d cycle; controls; 15 mo	Randomly assigned; controlled	–12.6%	FSH > 21 IU/L, no previous hormone or folates	*48*

Table 1 (*Continued*)
Studies on the Homocysteine Lowering Effect of Estrogens and Agonists in Postmenopausal Women

Population	Exposure	Methods	Results	Remarks	Ref.
n = 135 healthy (Netherlands)	Micronized 17β estradiol (2 mg/d); dydrogesterone (2.5, 5.0, 10, and 15 mg/d); 6 mo	Uncontrolled	–13.5% lasting decrease after 3 mo	FSH > 35 IU/L, no previous hormones or folates	*49*
n = 52 Hysterectomized	Raloxifene (60, 150 mg/d); conjugated estrogen (0.625 mg/d)	Randomized double-blind placebo-controlled	–16.0% for raloxifene after 12 mo; –13.0% for raloxifene and –10% for estrogen after 24 mo	FSH > 40 IU/L, estradiol < 73 pmol/L, no previous folate suppl.	*50*
n = 59	Estradiol (2 mg/d) + trimegestone (0.5 mg/d) or dydrogesterone (10 mg/d) or 2 mg estradiol/d only	Prospective; randomly assigned	9.4%; – 5.1% estradiol only	FSH > 20 IU/L, estradiol < 150 pmol/L	*51*

complaints and the treatment of osteoporosis. Raloxifene significantly reduces plasma homocysteine concentrations in postmenopausal women *(50)*.

The mechanisms underlying the estrogen-lowering effect of homocysteine are currently under investigation.

HOMOCYSTEINE AND EARLY MISCARRIAGE

Clinical Background of Early Miscarriage

The term *miscarriage* is used to emphasize the spontaneous interruption of pregnancy. For clinical and scientific reasons, it is important to avoid the international confusion on the duration of pregnancy when the term *abortion* is used because the range of pregnancy duration can be as wide as from 0 to 28 wk. Another reason for this distinction is that the causes for embryonic loss are quite different from those of fetal loss. We therefore tried to unravel literature reports in embryonic loss (up to 10 wk of menstrual age, or 8 wk postconceptional age) and fetal loss (from 10–8 wk up to the 16–24-wk period).

The prevalence of neural tube defects in miscarriages is 10-fold higher compared to the prevalence at birth *(52,53)*.

Despite all research efforts, not much can be offered to a couple that experiences repeated recurrent early pregnancy loss. In only 5% of cases are factors associated with miscarriage found. Among the maternal factors, uterine malformations and positive coagulation factors are the most frequent. Even when these associated factors are found, there is, up to now, no evidence-based proposal for treatment of such disorders.

When early embryonic loss is found, chromosomal abnormalities are reported in more than 50% of cases. This does not include that one always has to accept "the filter of nature" as an unavoidable consequence of genetic deviations residing in the germ cells and/or the processes that govern meiotic divisions and the fertilization process.

Women Who Experience Repeated Unexplained Early Pregnancy Loss Have a Twofold to Threefold Increased Risk for Recurrence as a Result Low Folate Status, Hyperhomocysteinemia, and a Higher Prevalence of the C677T Mutation

With the start of the homocysteine research in relation to neural tube defects and the genetic mutations found, it was logical to also explore this fascinating field of early embryonic development.

Hibbard et al. *(54)* was the first to suggest a possible relationship between miscarriage and *folate* deficiency. An increased FIGLU excretion was found

after histidine loading in 32% of women with an isolated unexplained "abortion" and in 60.5% of women with two or more recurrent events.

Sutterlin et al. *(55)* did not find significant differences in serum concentrations of folate and cobalamin in 29 patients with a history of 3 or more consecutive early losses. Patients with at least four previous events showed a significant negative correlation with the number of miscarriages and serum folate concentration.

Mild hyperhomocysteinemia was suggested in patients with recurrent early pregnancy loss *(56)*.

In later studies, mild homocysteinemia was confirmed *(57–59)*. The pooled odds ratio (OR) of these studies was 2.7 (95% confidence interval [CI]: 1.4–5.2) for fasting homocysteine and 4.2 (95% CI: 2.0–8.6) for homocysteine after methionine loading.

The common mutation C677T was found *(60)* in 16% of 185 Dutch women with unexplained recurrent early loss and in 5% of 113 case controls (OR: 3.3; 95% CI: 1.3–10.1) and 1250 population controls (OR: 2.0; 95% CI: 1.2–3.2). This was confirmed in a small French retrospective study *(61)*.

Ray and Laskin *(62)* calculated a pooled odds ratio of 3.4 (95% CI: 1.2–9.9) for folate deficiency, 3.7 (CI: 0.96–16.5) for hyperhomocysteinemia following methionine loading, and 3.3 (CI: 1.2–9.2) for the MTHFR mutation.

One of the factors recently found for early miscarriage is a defect in the vascularization of the chorionic villi. Nelen et al. *(63)* found that women with elevated homocysteine concentrations also after methionine loading showed significant smaller vascular areas and perimeters. This suggests that the vascular influence of homocysteine is also apparent in the vessels of the early placenta.

The higher prevalence of the C677T mutation in women with recurrent early pregnancy loss was confirmed by Guttormsen et al. *(64)* in the Norwegian Hordaland study.

Homozygotes for the mutant gene of thermolabile MTHFR were sensitive to 0.5 mg of folic acid per day and normalized their plasma homocysteine concentrations *(65)*.

It is important to realize that the possibly preventive effect of folic acid on the recurrence of early pregnancy loss cannot be investigated any longer in a placebo-randomized fashion because of the evidence-based prevention of neural tube defects with folic acid *(66)*, a preventive approach that has to start around conception. Therefore, more in-depth research into the mechanisms that interfere with embryonic development is necessary.

HOMOCYSTEINE AND PREECLAMPSIA

Preeclampsia: A Serious Disease

Preeclampsia is defined as the occurrence of hypertension (diastolic blood pressure greater than 90 mm Hg on two separate occasions 4–6 h apart and proteinuria greater than 300 mg/24 h}.

Preeclampsia occurs mainly in primigravidae without previous hypertensive disease (pregnancy-induced hypertension) or in multigravidae with previous hypertensive disease (pregnancy-aggravated hypertension). The mother is at risk for renal and liver dysfunction (HELLP syndrome), placental abruption, or even eclampsia (convulsions) and death. The fetus is at risk for fetal growth retardation, preterm birth, mortality, or morbidity.

The prevalence of the preeclamptic syndrome is around 10% in the pregnant population, with a recurrence rate of 1–2%. The risk factors are primigravidity, pathologic uterine enlargement (mola, multiple pregnancy) and hypertensive renal disease. Vascular endothelial damage is a central feature of the pathophysiology of preeclampsia (*67*).

Folic acid deficiency was not found in cases of preeclampsia *(68)*. Rajkowic et al. *(69,70)* reported elevated homocysteine levels in pregnant nulliparous American women with preeclampsia and case controls tested at the time of delivery. The hematocrit values were not different between the groups, therefore excluding hemoconcentration. Folic acid concentration did not differ. This finding was confirmed by Powers et al. *(71)*. Furthermore, these authors found evidence for endothelial activation, as demonstrated by an increase of cellular fibronectin, a marker of oxidative stress.

Sorensen et al. *(72)* found elevated levels of serum homocysteine in pregnant women with preeclampsia in the second trimester. The risk of developing preeclampsia was calculated as 3.2 (95% CI: 1.1–9.2). Nulliparous women with elevated homocysteine levels experienced a 9.7-fold increased risk of preeclampsia without homocysteine elevations (95% CI: 2.1–14.1) compared with multiparous women. These risk values are comparable with the study of Raikowic et al. *(70)*.

In patients with severe early-onset preeclampsia, hemostatic and metabolic disturbances, associated with a tendency to vascular thrombosis were found in 79 women tested postpartum: protein S deficiency (24.7%), activated protein C resistance (16.0%), and hyperhomocysteinemia in plasma fasting and after methionine loading (17%) *(73)*.

Pooled data on homocysteine and preeclampsia showed an odds ratio of 20.9 (95% CI: 3.6–121.6) *(62)*.

Folic acid and vitamin B_6 supplementation lowered homocysteine loading values in patients with preeclampsia, fetal growth restriction, and hyperhomocysteinemia *(74)*.

MTHFR Polymorphism Also Plays a Role in Preeclampsia

The C677T mutation of the MTHFR gene was significantly increased in Japanese preeclamptic women (24%) compared with normal pregnant women (11%) and healthy adults (11%). The odds ratio was calculated to be 2.5 (95% CI: 1.3–4.8) for the homozygous genotype *(75)*.

The effect of the MTHFR polymorphism and the genetic susceptibility to preeclampsia can be attenuated by the presence of factor V Leiden *(76)*. In an Italian population, TT homozygotes were found in 29.8% of cases and 18.6% of controls (OR: 1.8; 95% CI: 1.0–3.5). In cases in which factor V Leiden was also present, an illustrative case history demonstrated a high risk for a complicated pregnancy *(76)*.

Kupferminc et al. *(77)* reported a prevalence of the genes of factor V Leiden, MTHFR, and prothrombin of 53% among Jewish women with preeclampsia (OR: 5.4; 95% CI: 2.3–12.4). Van Pampus et al. *(78)* and De Groot et al. *(79)* found no differences in the prevalence of genetic risk factors (factor V Leiden, prothrombin 20210 A allele, protein C and S, and antithrombin deficiency) in women with preeclampsia compared with control subjects.

Three reports *(71,80,81)* could not confirm an increased risk for eclampsia due to the 677 C-T polymorphism.

A meta-analysis of the above-mentioned studies comprised 518 patients and 1100 controls *(81)*. The odds ratio was 2.0 (95% CI: 1.4–2.9). In the interpretation, one has to keep in mind confounding factors like folate status, the population involved, the number of patients, and the type of controls. Nevertheless, the homozygous 677 TT genotype seems to be a moderate risk factor for preeclampsia. This finding is in line with research in the general vascular area.

Homocysteine Promotes Endothelial Dysfunction in Preeclampsia

Rajkowic et al. *(69)* reported elevated homocysteine levels in pregnant nulliparous American women with preeclampsia tested at the time of delivery. The hematocrit values were not different between the groups, therefore excluding hemoconcentration. Folic acid concentration did not differ. This finding was confirmed *(71)*. Furthermore, these authors found evidence for endothelial activation, as demonstrated by an increase of cellular fibronectin, a marker of oxidative stress.

In patients with severe early-onset preeclampsia hemostatic and metabolic disturbances, which are associated with a tendency to vascular thrombosis, were found in 79 women tested postpartum: protein S deficiency (24.7%), activated protein C resistance (16.0%), and hyperhomocysteinemia in plasma fasting and after methionine loading (17.%) (73).

The basic mechanism for preeclampsia is probably a disturbance in the physiologic endothelial replacement mechanism in the maternal spiral arteries supplying the placenta.

PLACENTAL ABRUPTION

Placental Abruption: Great Risk for Mother and Child

In obstetrics, various clinical syndromes are accompanied with disturbances of blood coagulation. Placental abruption is a classic example for such disease. Placental abruption is an obstetric syndrome with great risks for mother and child *(82,83)*. The placenta separates from the uterine wall during pregnancy by retroplacental bleeding and blood clotting. Histology points to a rupture of the spiral artery or arteries. When the placenta is partially ruptured, a retroplacental "delle" with a fitting blood clot can be seen. In the case of total separation, these features are lacking.

Hypovolemic shock can occur in the mother as a result of intrauterine and extrauterine blood loss. This can result in multiorgan failure involving the coagulation and renal system.

Risk factors described for placental abruption are a small-for-gestational-age infant, intrauterine fetal death, premature rupture of the membranes, chronic hypertension, the use of drugs like marijuana and cocaine, preeclampsia, preterm labor, age above 35 yr and smoking *(84,85)*.

The pathology of coagulation can be summarized as a consumption coagulopathy in which too much fibrin is used.

Placental Infarcts Usually Present in Cases of Placental Abruption and Fetal Growth Retardation

In cases of placental abruption, placental infarcts are usually present, as well as fetal growth retardation. When the placenta is totally abrupted fetal death will occur. When the placenta is partially separated from the uterine wall, there is a high risk for fetal brain damage *(83)*.

Remarkably, the incidence of congenital heart defects in the offspring of these mothers is high *(86)* but can be explained by the novel risk factor homocysteine interfering with the developing heart comparable to the closure of the neural tube.

The prevalence of placental abruption is reported as about 3% with a recurrence rate of 6–10%. The risk factors like maternal hypertension, smoking, age, cocaine use and a history of fetal growth retardation point toward a disease of arteries, namely the spiral arteries ramifying from the uterine arteries and supplying blood to the placenta.

Endothelium of Spiral Arteries Replaced by Trophoblast

The endothelium of the spiral arteries is replaced by trophoblast cells in normal early pregnancy. First, the distal tips of the spiral artery are plugged with cytotrophoblast, which is continuous with the proliferating tips of the anchoring villi. This might have a function for providing a low-oxygen milieu in the embryonal period. After invasion of the decidual parts of the spiral artery, there is a new wave of intra-arterial trophoblast, at 15–16 wk of pregnancy, beyond the deciduo–myometrial junction into the true myometrial segmants of the spiral arteries. The trophoblast is incorporated into the arterial wall which has lost its normal histological characteristics. The vessel has now become a tube of fibrinoid material. The described changes are called "the physiologic changes of pregnancy." The histologic appearance suggest that these changes lead to vessels free from vasomotor control and allowing a wide vasodilatation and a low-resistance placental vascular bed *(87,88)*. In cases of placental abruption, this "physiologic change" of the blood vessels does not occur and one or more signs of vasculopathy can be seen, like atherosis, narrowing, necrosis, and thrombosis, as in patients with vascular disease *(89,90)*.

Vasoactive substances from the local circulation may initiate the more widespread maternal vascular endothelial damage, as demonstrated in cases with preeclampsia *(67)*, leading to disturbed coagulation, maternal vasoconstriction, and reduced organ perfusion.

There are numerous markers for endothelial function that can be determined in the peripheral blood and are endothelium-derived proteins like von Willebrand factor, tissue-type plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), cellular fibronectin, and thrombomodulin.

Thrombosis in decidual blood vessels may lead to fetal demise, subsequent abortion or stillbirth *(76,91–93)* especially in women with hereditary thrombophilia.

Endothelial cells can also be studied in vitro. Usually, endothelial cells are taken from the umbilical vein. One has to recognize, however, the fact that there are variations in secretory function and responses to stimuli between large vessels and capillary endothelial cells, as well as between fetal and adult endothelial cells *(94–96)*.

Folic Acid and Placental Abruption

Hibbard and Hibbard *(97)* and Hibbard *(54)* were the first to ask attention for the possible role of folic acid in the pathogenesis of placental abruption. In their studies, the presence of a positive FIGLU test and megaloblastic bone marrow were more common in women with placental disease. Later studies demonstrated that folate deficiency was a prominent risk factor for placental abruption/infarction. Ray and Laskin *(62)* calculated a pooled odds ratio of 25.9 with a 95% CI of 0.9–736.3 in four studies *(97–99)*. The wide range of this odds ratio, however, signifies the fact that these studies had too few numbers.

Van der Molen *(100)* found an odds ratio of 2.5 (95% CI: 1.0–6.1) for serum folate in 165 women with placental abruption/infarcts and 139 matched controls.

To understand the mechanism behind low folate concentrations and placental disease, Steegers-Theunissen et al. *(56)* launched the hypothesis of a derangement of homocysteine metabolism.

Maternal Hyperhomocysteinemia: A Risk Factor for Placental Abruption and Congenital Heart Defects

Steegers-Theunissen et al. *(56)* from the homocysteine research group at Nijmegen (NL) noted that homocysteine could be a risk factor for placental abruption. From the same group, Goddijn-Wessel et al. *(101)* found hyperhomocysteinemia in 26 out of 84 Dutch patients (31.0%) who experienced placental abruption, placental infarcts, and fetal growth retardation—all in a combination summarized as placental vasculopathy—and in 9% of 46 controls. For the fasting hyperhomocysteinemia (above the 97.5th percentile), the odds ratio was 4.8 (95% CI: 1.2–21.5). For the post-methionine-loading, the odds ratio was 4.8 (95% CI: 1.0–31.9). Serum and red cell folate and serum vitamins B_{12} and B_6 were significantly lower in the study group as compared with the controls. This finding was confirmed in a small study from South Africa *(102)* without differences in vitamin profiles (odds ratio: 7.2; 95% CI: 0.7–177.4) for fasting hyperhomocysteinemia being more than the 95th percentile and an odds ratio post-methionine-loading of 3.0 (95% CI: 0.24–82.7).

Also, in the Hordaland homocysteine study in Norway comprising 18,000 subjects, mild hyperhomocysteinemia was found in cases of placental abruption with an odds ratio of 3.0 (Guttormsen, personal communication, 1998).

In extended studies on placental abruption in the Dutch population, van der Molen et al. (100) found a significantly higher plasma homocysteine in

165 women with placental vasculopathy compared with 139 matched controls. The odds ratio (OR) above the 97.5th percentile was 4.7 (95% CI: 1.6–14.0).

The association of hyperhomocysteinemia with birth defects *(103)* was observed in an epidemiologic study for placental abruption and congenital heart disease (*86*) and confirmed for the association with hyperhomocysteinemia *(10)*.

The MTHFR Gene Mutation (C677T) Is a Major Determinant of Hyperhomocysteinemia

Another defect associated with hyperhomocysteinemia is the thermolabile variant of methylene tetrahydrofolate reductase (MTHFR). The cDNA for humans has been isolated and it has been shown that the thermolability is caused by a point mutation 677 C to T transition, resulting in a valine substitution for alanine *(24,104)*. The C677T mutation of the MTHFR gene was found in 19/165 (12.0%) in Dutch women with placental vasculopathy, in 7/139 (5%) of matched controls (OR: 2.5; 95% CI: 1.0–6.0) and in 106/1250 (8.5%) of the population-based controls (OR: 1.8; 95% CI: 0.8–3.9) in the study of Van der Molen et al. *(105)*.

The involvement of the C677T mutation was also found in a much smaller population of 34 women with placental abruption and 110 healthy pregnant women (*77*).

Coagulation, Fibrinolysis, and Homocysteine

The blood coagulation cascade has the abilty to amplify a small initiating stimulus into a large fibrin clot. Upon vascular injury, the extrinsic and intrinsic pathway of blood coagulation is initiated when blood is exposed to tissue factor in the subendothelial space. Tissue factor binds to activated factor VII and this complex activates factors IX and X of the intrinsic coagulation pathway. Factor IX activates factor X, a reaction accelerated by a cofactor, factor VIII. Activated factor X converts prothrombin to thrombin (factor IIa), a reaction that needs phospholipid surfaces and is accelerated by factor V. Thrombin then cleaves fibrinogen to generate fibrin monomeres, which polymerize and crosslink to form a stable clot. The coagulation mechanism is further amplified by the feedback loop of the activation of factors VIII and V by thrombin.

Fibrine has a temporary role and needs to be resolved in order to resume normal tissue architecture and function. The fibrinolytic system takes care of the degradation of fibrin, plasminogen is converted into plasmin, which cleaves fibrin. The conversion of plasminogen is mediated by plasmino-

genactivators such as tissue-type plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA). The fibrinolysis is further regulated at the level of plasminogen-activator inhibitors (PAI), of which PAI-1 is the most prominent inhibitor of t-PA and u-PA.

Some plasma concentrations of factors in coagulation and fibrinolysis are markers of endothelial function. Related to the coagulation status are the von Willebrand factor (vWF) and thrombomoduli (TM). Related to the fibrinolytic status are plasminogen activator (tPA), plasminogen activator inhibitor (PAI-1) and the tPA/PAI ratio.

General activation of the coagulation or fibrinolysis is refelected in the plasma concentration of prothrombin fragment 1 and 2 (F1 + 2) and the fibrinogen product D-dimer. Finally plasma nitric oxide (NO) has vasodilating properties *(106)*.

Elevated concentrations of vWF, tPA, PAI-1, and tPA/PAI are markers of endothelial dysfunction and associated with arteriosclerosis and thrombosis.

In 120 healthy volunteers, Van der Molen *(100)* found that the high homocysteine subgroup (more than 16 µmol/L; $n = 29$) had a higher tPA/PAI ratio compared with the low-homocysteine group, suggesting endothelial dysfunction.

In 101 nonpregnant women with a history of placenta vasculopathy (study group) vs 92 controls, a significant higher vWF, TM, and F1 + 2 was found. Only TM was correlated significantly with homocysteine. In this population, a randomized placebo-controlled study demonstrated that multivitamin therapy (folic acid, hydroxycobalamin, pyridoxine) significantly lowered the tPA/PAI ratio but did not affect vWF concentrations. In 101 nonpregnant women with a history of placental vasculopathy and hyperhomocysteinemia, 5 mg folic acid for 8 wk lowered the vWF *(100)*.

The combination of thrombotic risk factors (homocysteine, MTHFR mutation, activated protein C resistance, protein C) raised the odds ratio for two risk factors to 3.40 (95% CI: 1.80–6.42) and for three risk factors to 6.83 (95% CI: 1.52–30.7), respectively *(105)*.

Endothelial Cells

Endothelial Cells In Vitro May Reflect the Natural In Vivo Feature

Homocysteine metabolism in endothelial cells is dependent on folic acid. When endothelial cells from the human umbilical vein are studied in vitro, homocysteine concentrations increase by constant amounts under standard culture conditions. Folic acid supplementation lowers the homocysteine ex-

port in a dose-dependent manner. Methyltetrahydrofolate and folinic acid, its precursor, are 10 times more active than folic acid. Additions of vitamin B_6 or B_{12} did not show any effect on the in vitro homocysteine cellular export *(107)*.

Gallery et al. *(108)* measured thromboxane generation by cultured decidual cells using the Dynabead technique, with which magnetically loaded monoclonal antibodies can isolate endothelial cells *(108)*. The in vitro thromboxane secretion by decidual endothelial cells was lower than that of endothelial cells of the human umbilical vein, and the responsiveness to specific stimuli was different.

Among the many hypotheses for the atherosclerotic action of homocysteine, the demonstrated dual action of homocysteine of promoting growth of smooth-muscle cells of blood vessels while inhibiting endothelial cell growth is very attractive *(109,110)*. Furthermore, homocysteine has an impact on vascular endothelial cell surface anticoagulant mechanisms leading to thrombosis *(111)*.

The Pathogenesis of Atherothrombotic Disease Resulting from Homocysteine

The endothelium is an important locus of control of vascular functions. It actively regulates vascular tone and permeability, the balance between coagulation and fibrinolysis, the composition of the subendothelial matrix, the extravasation of leukocytes, and the proliferation of vascular smooth-muscle cells. The endothelium produces regulatory mediators for these functions such as nitric oxide, prostanoids, endothelin, angiotensin II, tissue-type plasminogen activator, plasminogen activator inhibitor-1, von Willebrand factor, adhesion molecules, and cytokines *(112)*.

Among the many hypotheses for the atherosclerotic action of homocysteine, the demonstrated dual action of homocysteine of promoting growth of smooth-muscle cells of blood vessels while inhibiting endothelial cell growth is very attractive *(109,110)*.

Moderate folate depletion increases plasma homocysteine. Folate depletion results in decreased lymphocyte DNA methylation as demonstrated in postmenopausal women *(113)*. Hypomethylation of DNA can lead to misincorporation of uracil into DNA and increased DNA repair. The clinical consequences of this very interesting finding could be an increased vascular risk, chromosomal damage, and preneoplastic lesions. The DNA hypomethylation could be reversed by folate repletion.

TREATMENT OF HYPERHOMOCYSTEINEMIA

Folic acid administration (5 mg/d) reduced homocysteine concentrations before and after methionine loading in postmenopausal women *(114)*.

Recent data on vitamin B administration, as potent homocysteine lowering drugs, are promising *(115)*. Moderate doses of folate and vitamin B_{12} have been shown to lower plasma homocysteine levels considerably, especially in the presence of the C677T mutation *(6,116)*. Serum folate was the lowest in homozygotes *(117)*.

Reduction of fasting and post-methionine-load plasma homocysteine values could be lowered by the administration of 250 mg vitamin B_6 and 5.0 mg folic acid per day for 6 wk *(118)*. The activity on this mutation is strongly dependent on the folate status of the body, being worst when the folate status is minimal *(26)*. This opens perspectives for the prevention of the recurrence of placental abruption to be validated in proper clinical placebo randomized trial. In such a trial, all women must be given folic acid in the periconceptional period because of the strong protective effect of folic acid on the prevention of neural tube defects *(66)*. Thereafter, it is possible to mount a placebo-randomized multicenter study.

The plasma homocysteine concentration is determined by genetic factors and nutritional deficiencies of vitamins B_6 and B_{12} and folic acid.

At any age, the absolute risk of a coronary event in women is lower than in men, and, on average, this event happens 10–15 yr later in life.

Fasting homocysteine and postmethionine levels of homocysteine are lower in women than in men. Post-methionine-load homocysteine predicts vascular disease more strongly in women than in men; the reverse is true for the fasting plasma concentrations of homocysteine *(14)*.

The genetic mutation of MTHFR an important enzyme for the remethylation of homocysteine, doubles the risk of neural tube defects *(119–121)* but seems to be of minor importance for the risk of vascular disease *(25)*.

When the end point of treatment is to lower plasma homocysteine values, the preference will be to use simple means devoided of side effects. Although steroids do lower homocysteine values by 17% *(46)*, estrogens and progestins do have unwanted side effects, such as a slightly elevated risk of mammary carcinoma and thromboembolism.

Fasting hyperhomocysteinemia is usually sensitive to folate treatment, with a reduction of approx 25% *(6)*. Vitamin B_{12} reduces fasting homocysteine by an additional 7% *(122)*. Vitamin B_6 has no effect on fasting homocysteine levels, but it decreases postmethionine homocysteine levels by 20–30% *(123)*. By using folates occurring in natural food or enrichment of cereals with folic acid, plasma homocysteine can decrease by 4–11% *(124,125)*.

Moderate folate depletion increases plasma homocysteine. Folate depletion results in increased DNA methylation, as demonstrated in postmeno-

pausal women *(113)*. Hypomethylation of DNA can lead to misincorporation of uracil in to DNA and increased DNA repair. The clinical consequences of this very interesting finding could be an increased vascular risk, chromosomal damage, and preneoplastic lesions. The DNA hypomethylation could be reversed by folate repletion.

Furthermore, folate and vitamin B_6 reduce the risk of coronary heart disease among women *(126)* and decrease the risk of atherosclerotic coronary events, as demonstrated in a randomized placebo controlled trial *(127)*.

In conclusion, one can state that B vitamins and folic acid are an innocuous means to reduce plasma homocysteine with clinical effects.

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Folate, Homocysteine, and Cardiovascular Disease

Jayne V. Woodside and Ian S. Young

INTRODUCTION

Homocysteine is a sulphur-containing amino acid that is an intermediary product in methionine metabolism *(1)*. The metabolism of homocysteine is closely linked to certain B-group vitamins, including folate, cobalamin, pyridoxine, and riboflavin. In 1969, McCully proposed that homocysteine may promote the development of vascular lesions *(2)*, based on studies of postmortem findings in patients with very high homocysteine levels resulting from rare genetic defects. Subsequent investigations have focused on the possibility that more moderate elevations may also be associated with increased risk of vascular disease *(3)*. There has also been recent interest in the potential links between total homocysteine (tHcy), folate, and cancer, in particular colorectal and lung cancer *(4–6)*, and between tHcy and cognitive function, especially Alzheimer's disease *(7,8)*. This chapter will focus on recent advances in the understanding of factors, both genetic and environmental, that influence homocysteine levels, including folate status. The intervention trials currently underway to assess the effect of lowering homocysteine on cardiovascular end points will be described. Finally, possible mechanisms that contribute to the vascular toxicity of homocysteine will be discussed.

HOMOCYSTEINE AND VASCULAR DISEASE

Retrospective case-control studies have consistently suggested that total homocysteine is an independent risk factor for cardiovascular disease, with an increase in plasma tHcy of 5 μmol/L being associated with an increase in risk of approx 1.5–1.6 *(9)*. Prospective studies have been less positive, but a

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recently updated meta-analysis confirmed that, overall, they were also supportive, suggesting an increment in risk of 1.20 (1.14, 1.25) for a 5-μmol/L increase in tHcy *(10)*. However, Christen et al. *(11)* reviewed 43 studies and found that prospective studies showed a smaller or no association between tHcy and cardiovascular disease (CVD). Cleophas et al. *(12)* produced a pooled odds ratio (OR) of 1.49 (1.33–1.67) (where OR is the odds of coronary artery disease (CAD) in patients with elevated tHcy levels/odds of CAD in patients with normal tHcy levels) in prospective cohort studies. However, they stated that the relevance of these results was minimized by significant heterogeneity in study results. Five of the 11 cohort studies (45%) could not demonstrate a significant association between elevated tHcy levels and CAD. Indeed, in a critical review of the epidemiological evidence, Eikelboom et al. *(9)* did not perform a formal meta-analysis because of the heterogeneity observed in study design and method.

These discrepancies, even in meta-analyses, have led to the suggestion that (1) elevations in tHcy are a consequence of cardiovascular disease rather than a cause *(13)* or (2) tHcy has a greater effect on short-term outcome than long-term outcome.

If tHcy is a consequence of CVD, then one possible mechanism whereby tHcy becomes elevated would be via atherosclerotic renovascular disease. Patients with renal impairment have long been recognized to have elevated tHcy, which is associated with increased cardiovascular risk *(14)*, reflecting the importance of the kidney in homocysteine metabolism. Minor degrees of renal dysfunction may also increase tHcy. Bostom et al. *(15)* investigated the relationship between renal function and tHcy in 164 patients with stable coronary disease and normal renal function as defined by serum creatinine <1.4 mg/dL. They used serum cystatin C as a sensitive indicator of renal function and showed that subtle variation in renal function was strongly related to tHcy. Minor degrees of renal dysfunction are common in patients with vascular disease and this might, therefore, partly explain why retrospective case control studies demonstrate a stronger relationship between tHcy and CVD than prospective studies.

Total homocysteine may also be associated with other risk factors for CVD. In the European COMAC project on tHcy and vascular disease *(16)*, hyperhomocysteinemia had a more than multiplicative effect on risk in smokers and hypertensive subjects and also enhanced the risk conferred by elevated cholesterol. The heterogeneous results of the various cohort studies also suggest complex interactions between tHcy and other risk factors. A prospective study by Rimm et al. *(17)*, which found a protective effect of folate and vitamin B_6 on CVD in women, supports the concept that tHcy is a

confounding variable rather than a causal factor. Lifestyles of women in the lowest quintile were unhealthy in terms of the presence of hypertension, the intake of fat, fiber, and alcohol, and the amount of exercise. In contrast, women in the highest quintiles had healthy lifestyles, including taking vitamin supplements. A strong effect modification of the tHcy–CVD association by conventional risk factors may also explain the recent observation that plasma tHcy is not related to CHD in patients with a low-cardiovascular-risk profile *(18)*. Because the mechanisms of CVD are multifactorial, it will be important to evaluate the significance of tHcy as a risk factor in populations with different conditions and different risk factors and to control for other established risk factors within these study analyses.

Several studies have now demonstrated a clear relationship between tHcy and adverse outcome over a relatively short time scale *(19–21)*. For instance, Morita et al. report that elevated tHcy is associated with restenosis following coronary angioplasty, particularly in diabetes mellitus *(22)*, and Omland et al. report a positive relationship between 2-yr all-cause mortality and tHcy in patients presenting with an acute coronary syndrome *(23)*. Nygard and coworkers *(19)* reported a strong graded relationship between tHcy and overall mortality in persons with angiographically demonstrated CHD; the mortality ratio was 4.5 (confidence interval [CI]: 1.22–16.6) for persons with the highest levels of tHcy compared to those with the lowest levels over a mean follow-up period of 4.6 yr. This suggests that the mechanisms by which homocysteine causes vascular toxicity may operate over a shorter time-period than the follow-up duration of prospective studies. The relationship between tHcy and outcome may therefore be diluted by long-term follow-up.

METABOLISM OF HOMOCYSTEINE

Homocysteine, a sulphur-containing amino acid, is produced during catabolism of the essential amino acid methionine. Homocysteine can be metabolized by two major pathways. When methionine is in excess, homocysteine follows the transsulfuration pathway, where it is irreversibly conjugated to serine by cystathionine β-synthase in a process which requires vitamin B_6 as a cofactor. Under conditions of low methionine, homocysteine is primarily metabolized through the methionine-conserving remethylation pathway. In most tissues, homocysteine is remethylated in a process requiring methionine synthase, vitamin B_{12} as a cofactor, and methyltetrahydrofolate as a cosubstrate. This pathway requires an adequate supply of folic acid and the enzyme methylenetetrahydrofolate reductase (MTHFR). Genetic and acquired abnormalities in the function of these

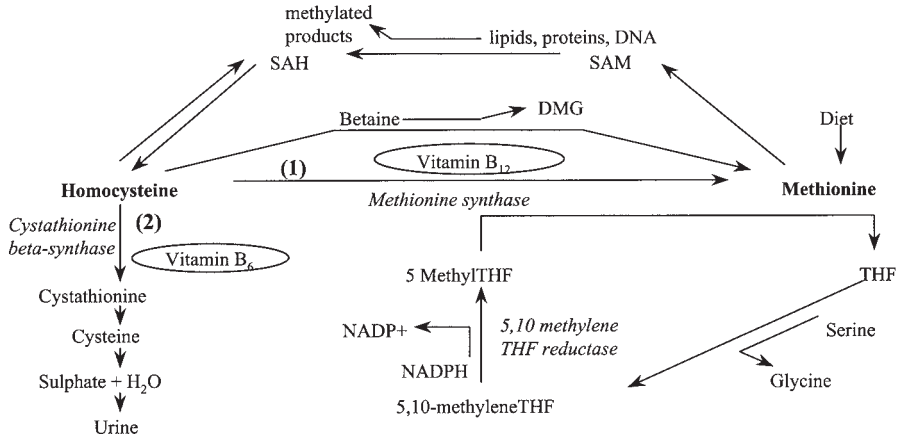

Fig. 1. Methionine cycle: metabolic cycle of homocysteine metabolism. THF = tetrahydrofolate; DMG = dimethylglycine; SAH = *S*-adenosyl homocysteine; SAM = *S*-adenosylmethionine; NADP+ = nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH = nicotinamide adenine dinucleotide phosphate (reduced form).

enzymes or deficiencies in folic acid, vitamin B_6, or vitamin B_{12} cofactors can lead to elevated tHcy levels *(24)* (*see* Fig. 1).

THE RELATIONSHIP BETWEEN HOMOCYSTEINE AND FOLATE

Plasma total homocysteine is strongly influenced by B-group vitamins, with folate status being the most important determinant of tHcy in the general population *(3)*. Epidemiological studies cannot fully exclude the possibility that folate and vitamins B_6 and B_{12} may have an association with cardiovascular risk and atherogenesis that is independent of tHcy levels. In fact, two recent studies have shown an association between low serum folate and CVD *(25,26)*, whereas vitamin B_6 has previously been shown to have an independent effect on CVD incidence *(27)*. Folate supplementation, however, has been consistently shown to reduce tHcy, whether given as supplements or fortified foodstuffs *(28,29)*, and more recently increased fruit and vegetable consumption has also been shown to modestly reduce tHcy *(30)*.

As yet, there are no intervention studies reporting a reduction in cardiovascular morbidity or mortality with folate supplementation. Vermeulen et al. recently reported a randomized, placebo-controlled trial among 158 healthy siblings of 167 patients with premature atherothrombotic disease *(31)*. Eighty were assigned placebo and 78 were assigned 5 mg folic acid

and 250 mg vitamin B_6 daily for 2 yr. The primary end point was the development or progression of subclinical atherosclerosis as estimated from exercise electrocardiography, the ankle-brachial pressure index, and carotid and femoral ultrasonography. Vitamin supplementation reduced tHcy and was associated with a significant reduction in abnormalities on exercise electrocardiography, although there was no effect on other end points. An editorial accompanying ref. *31* queries the use of these surrogate end points, pointing to a lack of sensitivity and specificity *(32)*. This work adds to an earlier study in which a combination of folate and vitamins B_{12} and B_6 reduced the rate of progression of carotid artery plaques in patients with elevated tHcy *(33)*. In another study, 70 patients with post-methionine-load hyperhomocysteinaemia were given a combination of folate and vitamin B_6 and had the same incidence of new cardiovascular events as did 162 patients with normal tHcy levels *(34)*. However, although these studies are encouraging, it remains possible that the benefits observed were a direct effect of B-group vitamins independent of any tHcy lowering effect *(27)*.

A recent study in monkeys failed to show a beneficial effect of lowering tHcy on development of atherosclerosis *(35)*. The authors tested the hypothesis that dietary supplementation with B vitamins (folic acid, vitamin B_6, and vitamin B_{12}) would prevent hyperhomocysteinaemia, vascular dysfunction, and atherosclerotic lesions in monkeys. After 17 mo, tHcy increased from 3.6 ± 0.3 to 11.8 ± 1.7 μmol/L in monkeys fed an unsupplemented atherogenic diet, but it did not increase in monkeys fed an atherogenic diet supplemented with B-group vitamins (3.8 ± 0.3 μmol/L). Responses to endothelium-dependent vasodilators, both in resistance vessels in vivo and in the carotid artery ex vivo, were impaired to a similar extent in both groups. Anticoagulant responses to the infusion of thrombin were also impaired to a similar extent in both groups. Vitamin supplementation also failed to prevent intimal thickening in the carotid or iliac arteries. The diet given also produced hypercholesterolemia, so the authors suggested that interventions to lower tHcy may have limited clinical benefit unless other risk factors are also controlled *(35)*. However, this does not fit well with the previous suggestion that elevated tHcy enhances the risk associated with elevated cholesterol *(16)*.

As stated earlier, to date, no published randomized clinical trials have evaluated the effect of decreasing tHcy on major cardiovascular events in humans. The ability of randomized clinical trials to detect a treatment effect, if one exists, depends on the choice of population, the specific treatment regimen, and the duration of therapy. Targeting persons at high risk for fatal and nonfatal cardiovascular events (i.e., those with previous vascular dis-

ease), enrolling a sufficiently large sample, optimizing folic acid, vitamin B_6, and vitamin B_{12} doses, and allowing for a sufficiently long duration represent the best strategies in the design of randomized clinical trials evaluating the effects of decreasing tHcy *(9)*.

At least nine large randomized clinical trials of decreasing tHcy are currently in progress *(9)*. These studies have sample sizes ranging from 2000 to 12,000 and are assessing a variety of end points including stroke, myocardial infarction, and unstable angina *(9)*. In total, these trials are expected to included approx 50,000 persons and will be able to detect even a 10% relative risk (RR) reduction in the incidence of major vascular events, such as death or myocardial infarction *(9)*.

GENETIC FACTORS INFLUENCING HOMOCYSTEINE

The relationship between folate and tHcy is strongly influenced by genetic factors, particularly the *C677T* polymorphism of the methylene tetrahydrofolate reductase (*MTHFR*) gene *(37)*. The activity of *MTHFR* is crucial in determining the distribution between folate species used for homocysteine remethylation and DNA/RNA synthesis. Individuals homozygous for the thermolabile variant of the gene (TT) comprise 10–15% of Caucasian populations and have reduced *MTHFR* activity and a significant elevation in tHcy if folate intake is low *(36)*. This is associated with hypomethylation of DNA *(37)*. However, they also have an increased intracellular methylenetetrahydrofolate pool and, hence, improved purine and pyrimidine syntheses, which may contribute to a protection against cancer in certain contexts *(5)*. Epidemiological studies have failed to show a consistent relationship between the *C677T* polymorphism and CVD, although because many of these studies have failed to take folate status into account, this is probably not surprising, and a modest effect of the polymorphism cannot be excluded *(10)*. In fact, in several articles showing no association between *C677T MTHFR* and CVD risk, the authors state that their population was probably well nourished *(38)*. In contrast, two recent studies in Turkish men, who, in general, have a high prevalence of CVD, low cholesterol, and low folate, the TT genotype was a significant predictor of the extent of coronary artery disease *(39,40)*. Another explanation for the lack of association, put forward by Refsum et al. *(10)*, concentrates on the low power of the studies carried out so far. From the calculated effects of *MTHFR* genotype on tHcy levels and the consequent effect of tHcy on RR as assessed by meta-analysis, the authors deduced that the sample size required to show such an association exceed the sample size in any published study or meta-analysis of CVD and *MTHFR*.

Chen et al. *(41)* have created *MTHFR* knockout mice to study the effects on the vascular system. Heterozygous mice had decreased enzyme activity similar to that seen in humans homozygous for the thermolabile *MTHFR* allele (about 35–40% of normal levels). No vascular abnormalities were observed in the young, but abnormal lipids deposition in the aorta was observed in older heterozygotes, but not in wild-type animals, suggesting an atherogenic effect of hyperhomocysteinemia in these mice. This finding also points to a possible inadequacy in the epidemiological studies carried out in humans to date.

Although most research has concentrated on the interaction between *C677T* and folate, recent evidence also suggests that the *C677T* polymorphism influences the relationship between riboflavin and tHcys. Riboflavin plays an essential role in the remethylation pathway of homocysteine metabolism, and because pyridoxyl 5'-phosphate (the active form of vitamin B_6) is formed through pyridoxine oxidation by an flavin mononucleotide (FMN)-dependent enzyme (pyrodoxine phosphate oxidase), riboflavin is also required for the transsulfuration pathway. *MTHFR* is a flavin-dependent enzyme, and the thermolabile variant is more likely than the wild-type enzyme to dissociate from the flavine adenine dinucleotide (FAD) cofactor *(42)*. In vitro, folates stabilize the binding of *MTHFR* to FAD in both thermolabile and wild-type enzymes, suggesting a mechanism by which folate supplementation can reduce tHcy. Hustad et al. determined plasma tHcy, serum folate, serum cobalamin, serum creatinine, and *MTHFR C677T* genotype in 423 healthy blood donors, ages 19–69 yr *(43)*. In addition, they measured riboflavin and its two coenzyme forms, flavin mononucleotide and flavin adenine dinucleotide. Riboflavin determined tHcy independently in a multiple linear regression model with adjustment for sex, age, folate, cobalamin, creatinine, and *MTHFR* genotype ($p = 0.008$), although the effect was modest, with tHcy being 1.4 μmol/L higher in the lowest compared with the highest riboflavin quartile. The riboflavin–tHcy relationship was modified by genotype ($p = 0.004$) and was essentially confined to subjects with the *C677T* transition of the *MTHFR* gene. To date, little is known about the effect of riboflavin supplementation on tHcy, but it may provide additional tHcy lowering, particularly in subjects homozygous for the thermolabile variant.

More recently, other common polymorphisms have been identified in *MTHFR* and other genes that may influence tHcy. A multiplex heteroduplexing method allows several of these to be assessed in a single procedure *(44)*. In general, the effects of these additional polymorphisms seem modest when compared with *C677T*. For instance, Harmon et al. described

the effects of one relatively common polymorphism in the methionine synthase gene (*D919G*), an A to G transition at 2756 bp *(45)*. In a male population (n = 607), the polymorphism was significantly associated with homocysteine concentration (p = 0.03), with the DD genotype contributing to a moderate increase in homocysteine levels, independent of folate concentration.

However, the DD genotype had a larger homocysteine-elevating effect in individuals with low vitamin B_6 levels. This polymorphism has not been linked with the risk of myocardial infarction in the Physicians' Health Study *(46)*. Other common functional variants in enzymes involved in homocysteine metabolism include *MTHFR E429A (47)*, *cystathione β-synthase 844ins68 (48)*, and *methionine synthase reductase I22M (49)*. These common variants may interact with each other and with B-group vitamins to influence tHcy and CVD risk. However, given the larger number of genetic and environmental variables involved in the regulation of tHcy, very large studies will be required to disentangle their relative contributions.

MECHANISMS OF VASCULAR TOXICITY

A wealth of mechanisms have been proposed to contribute to the vascular toxicity of homocysteine, including platelet aggregation, increased coagulation or reduced thrombolysis, endothelial dysfunction, and effects on the blood vessel wall *(50)*.

Modest elevations of tHcy have been associated with endothelial dysfunction *(51)*. Endothelial function is impaired after a methionine-loading test or even as a result of a high-protein meal *(52)*. Administration of either folate or ascorbate or a combination of ascorbate and vitamin E can ameliorate this endothelial dysfunction *(53,54)*. In the former case, this is presumably the result of a reduction in tHcy, whereas in the latter two cases, it has been suggested that ascorbate functions as an antioxidant to scavenge superoxide or other reactive oxygen species and, hence, increase nitric oxide availability. Elevated tHcy is also associated with increased levels of a variety of biochemical markers of endothelial damage that are reduced following treatment to normalise tHcy *(55)*. The situation is obviously complex, as a methionine load has been shown to simultaneously cause endothelial dysfunction and also increase both coagulation parameters (fibrinopeptide A, plasminogen activator inhibitor 1, and tissue plasminogen activator) and adhesion molecules (serum ICAM-1 and VCAM-1) *(56)*.

One mechanism that may contribute to homocysteine-induced endothelial dysfunction is increased synthesis of asymmetrical dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor *(57)*. The biosyn-

thesis and metabolism of dimethylarginines are not completely understood; however, they are probably formed from the degradation of methylated proteins *(58)*. Endothelial cells are capable of synthesizing small amounts of ADMA *(59)*, and this synthesis is upregulated in the presence of methionine or homocysteine *(60)*. The production of ADMA by human endothelial cells is regulated by *S*-adenosylmethionine-dependent methyltransferases, and production is upregulated by native or oxidized low-density lipoprotein. This provides a mechanism by which homocysteine can impair endothelial function, particularly in the presence of hypercholesterolemia. The hypothesis that ADMA is an important mediator of homocysteine-induced dysfunction is further supported by data showing that, in monkeys, diet-induced hyperhomocysteinaemia produced twofold to threefold increases in both tHcy and ADMA. However, supplementation with B-group vitamins decreased the plasma level of tHcy but did not affect the plasma level of ADMA or endothelial function *(61)*. The authors suggested that decreasing level of tHcy may not decrease protein methylation or the generation of ADMA because intracellular levels of *S*-adenosylmethionine may actually increase as a consequence of increased turnover of the tHcy–methionine pathway (*see* Fig. 1).

Alternative mechanisms are also likely to contribute to the vascular toxicity of homocysteine. Several authors have suggested that homocysteine may exert pro-oxidant effects leading to oxidative damage to endothelial cells. However, many of the in vitro studies on which this concept is based have used supraphysiological concentrations (1–10 mmol/L) of homocysteine. There are strong reasons to believe that pro-oxidant effects of homocysteine are unlikely to be important in vivo *(62)*, although Wilcken et al. reported an association between tHcy and extracellular superoxide dismutase (EC-SOD) in homocystinuria *(63)*, suggesting that increased EC-SOD expression may represent a protective response to homocysteine-induced oxidative stress.

Arterial stiffness is an independent determinant of cardiovascular risk *(64)*. Arterial stiffening may result from both structural and functional changes in the large arteries *(65)*. Pulse-wave velocity (pwv) is may be used as an indirect index of the stiffness of the arterial wall, and tHcy has been shown to be associated with pwv in end-stage renal disease patients (lower-limb pwv) *(66)* and in hypertensive patients (aortic pwv) *(67)*. More recently, Vermeulen et al. *(68)* have compared the morphology of the muscular femoral artery in patients with atherosclerosis and hyperhomocysteinemia with those with atherosclerosis and normal tHcy levels. Hyperhomocysteinemia was associated with a significant decrease of the smooth-muscle cell/extra-

cellular matrix ratio of the media of muscular femoral arteries without a significant change in medial thickness, an observation that clearly requires further study.

Another promising area of further study, which highlights the seemingly far-reaching biochemical and molecular consequences of hyperhomocysteinemia, is the pro-inflammatory effect of homocysteine. Hofmann et al. *(69)* showed that apoE-null mice fed a normal chow diet became hypercholesterolemic, developing foam cell accumulation after 10 wk and advanced fibrous plaques after 15 wk. However, when these animals were placed for 8 wk on a diet enriched with methionine and deficient in folic acid and vitamins B_6 and B_{12}, they developed atherosclerotic lesions of increased size and complexity. Moreover, unlike apoE-null controls, mice that were both apoE deficient and hyperhomocysteinemic showed nuclear translocation of NF-κB (necrosis factor-κB), a redox-activated inflammatory transcription factor, high plasma levels of the inflammatory cytokine TNF-α (tumor necrosis factor-α). There was also increased expression of the receptor for advanced glycation end products (RAGE) and its signal-transducing ligand (ENRAGE), newly identified signaling partners in chronic inflammation. Several downstream gene products, VCAM-1, tissue factor, and matrix metalloproteinase-9 were also activated *(69,70)*. None of these effects were observed in mice on a diet replete in B vitamins.

Many other potentially toxic effects of homocysteine continue to be reported. However, these are often demonstrated at very high tHcy concentrations. It is also important to demonstrate that toxic effects are specific to homocysteine and are not shared by other thiol-containing compounds. For instance, Woo et al. reported that homocysteine stimulates MAP kinase in aortic smooth-muscle cells *(71)*, which might be expected to stimulate cell proliferation. A positive effect was observed, but only in the presence of 2 m*M* homocysteine, a condition that would not be encountered in vivo. Homocysteine has been reported to stimulate vascular smooth-muscle cell proliferation at 25 μmol/L or above *(72)*, suggesting that mechanisms other than mitogen-activated protein (MAP) kinase activation must be important. A similar caveat must be applied to the report of Midorikawa et al. *(73)*, who reported enhanced expression of plasminogen activator inhibitor-1 (*PAI-1*) gene expression and secretion from vascular endothelial and smooth-muscle cells, but only at concentrations of homocysteine greater than 500 μmol/L, although a correlation has been reported between tHcy and *PAI-1* in vivo by several groups *(74,75)*.

CONCLUSION

Plasma tHcy is influenced by both genetic and environmental factors, particularly B-group vitamins, which interact in a complex manner. The epidemiological association between tHcy and CVD is strong and consistent in retrospective case-control studies, although weaker in prospective studies, suggesting that it is possible that tHcy may simply be a risk marker for CVD. tHcy is strongly influenced by renal function, and it is important to control carefully for this, and other cardiovascular risk factors that may interact with tHcy, in future studies. The vascular toxicity of homocysteine may operate over a relatively short time scale, and prothrombotic effects and impairment of endothelial function are likely to be particularly important. Inflammatory effects and structural changes to the arterial wall are areas that deserve further study in relation to tHcy. tHcy can be effectively lowered by folate, which can be given as pharmacological supplements or in the form of fortified foods. Other B-group vitamins, including cobalamin, pyridoxin, and riboflavin, have a smaller effect. The response to vitamin supplementation is likely to be influenced by polymorphisms in the genes that regulate homocysteine metabolism. On balance, it remains likely that homocysteine contributes to cardiovascular morbidity and mortality, although the ultimate test of this will be provided by the large-scale B-vitamin supplementation studies with clinical end points that are currently underway.

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