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Folates and the fetus

Tom K.A.B. Eskesa,b,*

aInstitute for the Prevention of Birth Defects, University Hospital Nijmegen Sint Radboud, Nijmegen, Netherlands
bThe Department of Obstetrics and Gynaecology, University Hospital Nijmegen Sint Radboud, Nijmegen, Netherlands

Abstract

It is proven that folic acid supplied in the periconceptional period can lower the recurrence and occurrence rate of neural tube defects (NTDs). Our research team on prevention of birth defects could demonstrate that folic acid preventable NTDs are partly based on hyperhomocystinemia and a genetic predisposition (mutation of the methylenetetrahydrofolate-reductase gene (MTHFR)). Reduced activity of the folate methylation cycle seems to be an attractive working hypothesis in the aetiology of NTDs. This genetic metabolic defect can be overcome by treatment with folic acid and/or vitamin B12. © 1997 Elsevier Science Ireland Ltd.

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1. Folates are not only important for the treatment of megaloblastic anemia

Obstetricians are familiar with folates (folic acid) because of their central role together with iron in the treatment of anaemia especially during pregnancy. But folates are also necessary for numerous cellular functions. There is now ample evidence that folic acid is of importance for the prevention of birth defects, particularly neural tube defects (NTDs).

It has long been suspected that nutrition played a role in the cause of NTDs with emphasis on folic acid [1,2].

Observational reports indeed demonstrate folate deficiency judged by the FIGLU test and later serum and red cell folate in women with NTD offspring. These 'deficiencies' led to intervention with multivitamins demonstrating a ten-fold reduction of the recurrence of NTD [3]. Our research group at the University of Nijmegen started in 1983 on the topic of primary prevention of birth defects, in particular NTDs.

* Tel.: +31 24 3614725; fax: +31 24 3541194; e-mail: G.Theunissen@obgyn.azn.nl

2. Folic acid reduces the recurrence and occurrence rates of NTDs

These observations called for a randomised trial to prove or disprove the benefit of folic acid.

A randomised double-blind prevention trial was carried out in 33 centres [4]. Hungary contributed 769 women to the total group of 1817 women (42%). These women were at risk of having a pregnancy with NTD, because of a previous affected pregnancy. They were allocated at random before pregnancy to one of three groups: (i) folic acid (4 mg); (ii) other vitamins; (iii) both folic acid and other vitamins and (iv) neither folic acid or other vitamins. Each group used their pills from at least 1 month before conception until the 12th week of pregnancy. A completed pregnancy was achieved in 1195 women; six NTDs occurred in the folic acid groups and 12 in the two other groups. A 72% protective effect was calculated (relative risk, 0.28; 95% confidence interval (CI95% 0.12–0.71)). Compliance of tablets was studied by determining folic acid in blood samples.

Czeizel and Dudás (1992) [5] studied the extent to which vitamin supplementation could reduce the first occurrence of NTD. Women planning in most cases the first pregnancy were randomly assigned to receive a
single tablet of a vitamin supplement (containing 12 vitamins including 0.8 mg of folic acid, four minerals and three trace elements) or a trace-element supplement (containing copper, manganese, zinc and a very low dose of vitamin C) daily for at least 1 month before conception and until the date of the second missed menstrual period or later. Compliance was not determined. Pregnancy was confirmed in 4753 women. Compliant women were not different from women who did not take any supplements at all.

There were six cases of NTDs in the group receiving the trace elements, as compared with none in the vitamin supplement group (P = 0.02).

The interest in folic acid has now been raised by the startling finding that folic acid c.q. multivitamins can prevent the recurrence and occurrence of NTDs.

3. The neural tube of the human is closed 4 weeks post-conception

The neural groove and folds are first seen during Carnegie stage 8, i.e. about 18 post-ovulatory days. The three main divisions of the brain appear at stage 9. The neural folds begin to fuse at stage 10. The rostral (anterior) neuropore closes within a few hours during stage 11 (about 24 post-ovulatory days). The caudal neuropore closes during stage 12 (about 26 days). At stage 13 (4 weeks) the neural tube is completely closed [6].

Clinically seen this means that the development and closure of the neural tube takes place very early in human pregnancy.

Recently, genes have been identified whose expression marks the formation and regionalization of the neural plate and which encode cell adhesion molecules or putative transcription factors [7].

The major classes of NTD are anencephaly, encephalocele and spina bifida. The yearly number of affected babies is about 400 000 worldwide, with a wide range (0.5–12/1000) among different countries [8].

Most cases (92%) of isolated NTD do have a multifactorial origin. The multiple or syndromic NTD group include chromosomal aberrations (trisomia 18), gene mutations (Meckel syndrome) and teratogenic medication (valproic acid).

4. Folic acid plays a keyrole in one carbon metabolism

Folic acid or vitamin B₁₂ was first recognized in 1930 as a factor present in the yeast preparation marmite, which was able to cure megaloblastic anemia. The term 'folic acid' is derived from the latin word folium which means 'leaf'. The substance was successfully synthesized in the form of pteroylmonoglutamate.

The major dietary sources of folates are fresh and green leafy vegetables, liver, kidney, asparagus, citrus fruits, juice whole wheat bread and legumes (wheat and dry beans).

The absorption process of dietary folate includes the conversion of polyglutamates to monoglutamates and a full reduction to tetrahydrofolate (THF), the parent compound for all biological active folates. The predominant folate in serum and in tissues is 5-methyl-THF.

Folate is involved in two cycles, one involving DNA biosynthesis (guanine, adenine and thymine), essential for cell division, the other being the methylation cycle (or one carbon metabolism), essential for the provision of methylgroups for a wide range of cellular methyltransferases (Fig. 1) [9].

5. Folate is an important substrate in the metabolism of homocysteine

Homocysteine is a sulfur-containing amino-acid, formed after demethylation of the essential amino acid methionine (Fig. 2) [10]. Intracellular homocysteine is bound with serine to form cystathionine. This reaction is catalyzed by the enzyme cystathionine β synthase (CBS). Cystathionine is then split to cysteine through the transsulphuration pathway. Homocysteine can also be remethylated.

Three B-vitamins are involved in methionine-homocysteine metabolism: B₆ (pyridoxal 5′ phosphate), B₁₂ (folate) and B₃. The enzyme CBS requires vitamin B₆ as a co-factor. Methionine synthase (MS) requires vitamin B₁₂ as a cofactor and 5-methyl-THF as a substrate. Methyl-THF is formed upon the reduction of 5,10-methylenetetrahydrofolate reductase (MTHFR).

Thus, vitamin B₆ is important in homocysteine transsulphuration, while folate and B₁₂ play a significant role in homocysteine remethylation.

Defective trans-sulphuration as well as remethylation disorders will result in increased blood (hyperhomocysteinaemia) and urine levels of homocysteine.

Classic homocystinuria is the homozygous form of the autosomal recessively inherited cystathionine synthase deficiency. The clinical characteristics are premature atherosclerosis and thrombosis, ectopic lentis, scoliosis, marfanoid features and mental retardation [11].

As folate is an important substrate in the metabolism of homocysteine, it was suggested that a subset of NTD might be due to a maternal disturbance in this metabolism [12]. Furthermore, it was thought that ho-
mocysteine levels could give a rather accurate picture of vitamin B status.

Hyperhomocysteinaemia could be the result of inadequate nutrition or of an inborn error of metabolism.

In our studies total homocysteine was determined in plasma using high pressure liquid chromatography (HPLC) [13].

Plasma concentrations were measured in serum and red blood cells, and vitamin \( B_{12} \) levels in serum, by using the Dualcount Solid Phase Boil Radioassay (Diagnostic Products, Los Angeles, CA). The assay of vitamin \( B_{6} \) as pyridoxal phosphate in whole blood was performed with HPLC.

Steegers-Theunissen et al (1994) [14] studied 41 non-pregnant women who had given birth to infants with an NTD, as well as 50 control women who previously had normal offspring. Fasting plasma homocysteine concentrations were significantly higher in the NTD group than in controls. In nine (22%) patients from the NTD group, post load plasma homocysteine concentrations exceeded the mean plus twice the standard deviation of controls. All nine of these women had fibroblast cystathionine synthase activities within the reference range. The authors suggested that not the trans-sulfuration but the remethylation pathway could be impaired in these women.

Supportive evidence linking impaired homocysteine remethylation to increased risk for NTD was published by Mills et al. (1995) [15]. Mothers with NTD pregnancies in the lower-normal serum vitamin \( B_{12} \) range had less effective homocysteine metabolism (remethylation) compared with controls matched for vitamin status.

6. Thermolabile 5,10-MTHFR has been cloned, a mutation identified and recognised as an important genetic risk for NTD

The gene for MTHFR has been cloned [16] and the DNA mutation responsible for the heat-labile variant
THE METABOLISM OF METHIONINE IN MAN

Fig. 2. The metabolism of methionine in man (after Boers 1985) [10]. Methionine is demethylated in steps to homocysteine. Homocysteine can then enter the transsulphuration pathway leading to cysteine or be remethylated to methionine. For this remethylation folates and vitamin B₁₂ are essential.

has been identified [17]. The frequency of homozygotes for this polymorphism was 12% among French Canadians [18] and 5.4% in the Dutch population [17].

Fasting homocysteine levels were almost twice as high in homozygotes for this variant compared to those who did not have the polymorphic allele [18].

Van der Put et al. (1995) [19] studied the frequency of the 677 C–T mutation in the 5,10-MTHF-R gene in 55 patients with spina bifida and parents of such patients (70 mothers, 60 fathers). Five percent of 207 controls were homozygous for the mutation compared with 16, 10 and 13% of mothers, fathers and patients, respectively.

The odds ratios for the homozygous mutation were 3.7 (CI₉₅% = 1.5–9.1) for the mothers, 2.2 (CI₉₅% = 0.8–6.3) for the fathers and 2.9 (CI₉₅% = 1.0–7.9) for the patients versus the controls.

The mutation was associated with decreased MTHF-R activity, low plasma folate and high plasma homocysteine and red cell folate concentrations. These results
were confirmed by the study of Whitehead et al. (1995) [20].

The abnormal thermolabile allele of MTHFR can explain the association between elevated homocysteine and NTDs because of the importance of the MTHF role in remethylating homocysteine to methionine. It could in part also explain the efficacy of folic acid in preventing NTDs by overcoming a partial enzymatic block.

These studies provide evidence of a genetic explanation for NTDs and suggest a mechanism whereby a genetic factor (the abnormal enzyme) and an environmental factor (folic acid) interact.

Posey et al. (1996) [21] calculated (based upon the available literature) that 13% of NTD cases can be attributed to the homozygous 677 C-T mutation. If heterozygosity is a causal risk factor for NTD an additional 11% of NTD cases may be attributed to the mutation. These fractions fall well below the 70% reduction in NTD rates [4]. Therefore the protective effect of folic acid against NTDs has to involve other biological and/or gene interactions as well.

7. Is homocysteine toxic for the embryo?

Fetal loss has been observed in 25 of 52 pregnancies of untreated homozygous CBS deficient women. However, these data were sampled in retrospect and strongly influenced by the high number of stillbirth and spontaneous abortions in a few individuals [22].

The teratogenic effects of an impaired methionine-homocysteine cycle could be due to cytogenetic damage of homocysteine or insufficient methionine levels leading to insufficient methyldonorship, altered DNA synthesis or abnormalities of DNA methylation.

A possible role of homocysteine in the aetiology of NTDs was tested in vitro by culturing day 10 post coitum post-implantation rat embryos [23]. In high doses (3.6–7.2 mM/h) the mitotic index of the neural epithelium of the rhombencephalon as well as the total morphological score was reduced.

The embryotoxicity of L-homocysteine was stereospecific since D-homocysteine caused no embryotoxic effects.

In lower concentrations (1 and 2 mM) however, the addition of L-homocysteine was not toxic and promoted normal development of rat embryos. At even lower concentrations L-methionine had the same beneficial effect. Methionine is the precursor of S-adenosyl methionine (SAM) which is the universal methyldonor in transmethylation reactions and thus essential to normal embryonic growth and development. As a low ratio of SAM and S-adenosylhomocysteine (SAH) will inhibit many transmethylation reactions [24], it is hypothesized that increased SAH formation resulting from high homocysteine concentrations is the first step in the embryotoxic mechanism of L-homocysteine [25].

This view is in line with the observation that L-methionine deficiency induced NTD in cultured rat embryos [26,27].

8. Hyperhomocystinaemia is associated with recurrent spontaneous miscarriage

In the products of conception, which can be studied after the spontaneous abortion process, the prevalence of NTD is ten-fold increased as compared to the prevalence at birth [28,29].

It was therefore, after the finding of mild hyperhomocystinaemia in NTD, not illogic to study a possible association between hyperhomocystinaemia and spontaneous miscarriage.

The association between hyperhomocystinaemia and recurrent early pregnancy loss could be confirmed [30,31].

In an extended study [32] 180 women with recurrent miscarriage and 46 controls were selected. Hyperhomocystinaemia was defined as a fasting and/or postmethionine load plasma homocysteine concentration above the 97.5 percentile level of the controls. Hyperhomocystinaemia was diagnosed in 22% of women with recurrent miscarriage and 9% in controls. In women with recurrent miscarriage plasma homocysteine concentrations were significantly and negatively correlated to blood concentrations of folate and vitamin B12.

In the same study, enzymes were determined in a subset of women, CBS and thermolabile 5,10-MTHF-R. CBS activity in fibroblasts was within the range of obligate heterozygotes and thermolabile MTHFR in isolated lymphocytes was observed in one out of seven and three out of 17 hyperhomocysteinemic women with recurrent miscarriage, respectively.

Hyperhomocystinaemia could be corrected by daily oral administration of 1 mg folic acid.

The rate of live births in a subsequent pregnancy was 83% in hyperhomocysteinemic women who were treated with B-vitamins. In 17 normohomocysteinemic women using B-vitamins as well the rate was 65%. In 55 normohomocysteinemic women who had not used any vitamins the rate was 53%.

A randomized controlled prevention trial should provide the answer to the intriguing question if vitamin B supplementation prevents spontaneous abortion (at least in a subset of women). Such a trial however is hampered by the fact that folic acid (multivitamins) prevents the occurrence and recurrence of NTD.
9. Multivitamins (folic acid) reduce also the rate of congenital anomalies other than NTDs

Periconceptional multivitamin supplementation can reduce not only the rate of NTDs but also the rate of other major non-genetic syndromatic congenital abnormalities [33].

Maternal periconceptional use of multivitamins reduces the risk for conotruncal heart defects and limb deficiencies [34], schisis [35] and urinary tract anomalies [36].

10. Consumption of extra natural food folate is less effective for folate status than folic acid supplementation

The findings of the MRC vitamin study group [4] led the UK Department of Health to recommend that women of child-bearing age should consume 400 μg of folic acid or folate per day to prevent NTD [37]. These recommendations were also made in the USA [38], the Netherlands [39] and in Australia [40].

These recommendations make the assumption that all routes of intervention like supplements, folic acid fortification natural food folates and dietary advice have similar potential to optimise folate metabolism.

The effect of increasing dietary folate on red-cell folate was studied by Cuskelly et al. [41]. They assessed the effectiveness of folic acid supplement (400 μg per day), folic-acid-fortified foods (400 μg/day added), dietary folate (an additional 400 μg per day), dietary advice and controls. Red cell folate increased significantly over a 3 month period only in the groups taking folic acid supplements or food fortified with folic acid. Dietary folate or dietary advice did not change the folate status.

Synthetic folates (folic acid) have the advantage of a greater stability and better absorption than dietary folates. Folic acid is not just more stable than natural folates (polyglutamates) but it has twice the bioavailability [42]. Also, prolonged cooking destroys some of the natural food folates.

It therefore seems that folic acid supplementation as a tablet or enrichment of food is the most effective way of ameliorating folate status. The USA plans to add folic acid to food products [43].

The fortification of food supply with folic acid is challenged by Gaull et al. (1996) [44]. These authors discuss the uncertainties like the fact that NTDs seem to be a multifactorial polygenic group of disorders so that folate will not help in all cases. In various countries the incidence of NTDs is decreasing. For those not at risk folate may pose safety concerns. There is not yet established a dose-response relationship between folate and NTDs. Finally, pharmacological doses of a nutrient may be required in case of a metabolic abnormality.

It seems to us that the described MTHFR deficiency group fulfils the criteria of these authors.

In conclusion, we can state that the periconceptional use of folic acid reduces the occurrence and recurrence of NTDs substantially. A possible defect of folate metabolism can be diagnosed by determining homocysteine in serum, or more directly by demonstrating thermolabile 5,10-MTHFR. A subgroup of women who experienced NTD do have hyperhomocysteinaemia and a mutation of this MTHFR gene.

Homocysteine seems to be involved in the pathogenesis of NTD. The reduction of homocysteine by stimulation of residual enzyme activity by folates (and/or Vitamin B₁₂) could be an explanation for the effect of folate on NTD occurrence and recurrence.

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