1 General introduction

The impact of the water-soluble B-vitamin folate in the development of neural tube defects (NTDs), one of the most common severe congenital malformations, was first described in 1964 (Hibbard 1964). Since then, many epidemiologic and intervention studies were undertaken to investigate the association of risk markers related to folate metabolism to NTD incidence and the potential of folate to prevent NTDs. The pathobiochemical link between folate and NTDs is discussed to be through the role of folate in deoxyribonucleic acid (DNA) and cell synthesis and in remethylation of homocysteine to methionine which, as S-adenosyl-methionine (SAM), is involved in the synthesis of neurotransmitters and in DNA methylation.

FOLATE METABOLISM

Folate derivatives are involved in pyrimidine and purine synthesis and, together with vitamin B_{12} (cobalamin), in the remethylation of homocysteine to methionine. The folate metabolism is illustrated in a simplified version in **Figure 1.1**. Vitamin B_2 (riboflavin) is cofactor for the enzyme 5,10-methylenetetrahydrofolate reductase (*MTHFR*) and for methionine synthase that catalyzes the remethylation of homocysteine. The polymorphisms 677C>T and 1298A>C of the gene encoding for the enzyme *MTHFR* may influence folate and homocysteine concentrations.

Folate

The term folate summarizes the biologically active metabolites of folate in biological systems. The chemical structure of folate is a pteridine ring attached to a *p*-aminobenzoate and a polyglutamyl chain. The active form of folate is tetrahydrofolate (THF) to which different one-carbon units are bound forming 10-formyl-THF, 5-formyl-THF, 5,10-methenyl-THF, 5,10-methylene-THF, and 5-methyl-THF (5-MTHF). Some of these folate derivatives function as donors of one-carbon units. 10-formyl-THF and 5,10-methylene-THF are involved in nucleic acid metabolism. 10-formyl-THF is involved in purine synthesis and inserts the one-carbon units C_2 and C_8 into the purine ring. 5,10-methylene-THF methylates uridylate to thymidylate. 5-MTHF, the predominant folate form in human metabolism and the transport form in plasma, functions as coenzyme in the remethylation reaction of

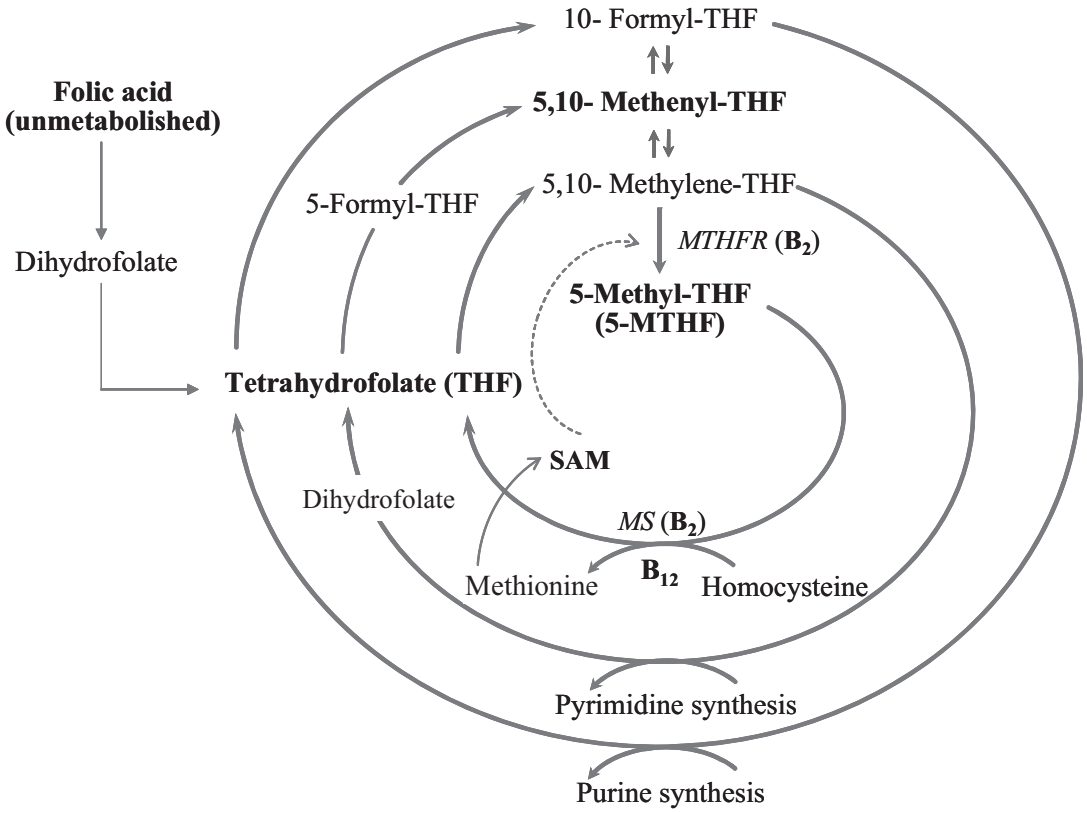

Figure 1.1: Simplified folate metabolism (modified from Bässler et al. (2002))

MTHFR, 5,10-methylenetetrahydrofolate reductase; SAM, S-adenosyl-methionine; *MS*, methionine synthase; B_2, vitamin B_2; B_{12}, vitamin B_{12}

homocysteine to methionine (Bässler et al. 2002). The regeneration of methionine is essential for the formation of SAM, the primary methylating agent involved in the synthesis of neurotransmitters and in DNA methylation. SAM regulates the formation of 5-MTHF through inhibition of the enzyme *MTHFR*. Abnormal one-carbon metabolism can lead to hyperhomocysteinemia, DNA hypomethylation, and neurological disorders (Bailey and Gregory 1999).

Folic acid is the term for a synthetic form of folate that does not occur naturally. The chemical structure of folic acid is the pteroylmonoglutamic acid. Due to its high stability, it is widely used in vitamin supplements and for food fortification.

The absorption of folate in the intestine is a complex process. Food folate consists of mono- and polyglutamyl folates. The polyglutamyl folates are deconjugated by the brush border conjugase and their extent of absorption is 60% to 80% that of monoglutamyl folates. Folate as monoglutamate is transported through the jejunal brush border membrane by a saturable, pH-dependent process which involves a folate binding protein and partly by an unsaturable absorption with passive diffusion (Gregory 1989). Methylated and reduced folates are

transferred faster from intestine to circulation than folic acid (Herbert 1999). Folic acid is absorbed by passive diffusion and converted in the liver via dihydrofolate and THF to 5-MTHF (Whitehead and Cooper 1967; Melikian et al. 1971). At amounts above 200µg/d, folic acid is not totally converted to biologically active folate derivatives in the liver and appears in unmetabolised form in plasma (Leeming et al. 1972; Kelly et al. 1997). In the first passage, 10-20% of the folates are taken up by the liver while the remainders are distributed to other tissues (Gregory 1989).

Biochemical parameters for folate status in human metabolism are red cell and plasma folate concentration. Because red cells only incorporate and accumulate folate during their development, the erythropoiesis, the red cell folate concentration underlies slow changes and is an indicator for long-term folate status. Plasma folate changes immediately after the intake of folate or folic acid and is therefore often used as parameter for bioavailability studies (Shane 1995).

Folate deficiency is defined as plasma folate concentrations below 7nmol/L and red cell folate levels below 360nmol/L (Herbert 1999). Negative folate balance leads to a reduction in DNA cycle and methylation reactions. This results in reduced DNA and cell synthesis and increased plasma tHcy concentrations. Because the effect on DNA synthesis will first be observed in rapidly dividing cells, folate deficiency is diagnosed as reduction in the number of red cells and thus as anaemia (Scott 1999). In case of severe folate deficiency, the diminished cell division leads to megaloblastic anaemia which is morphologically identical to the anaemia caused by vitamin B_{12} deficiency (Bailey and Gregory 1999).

Vitamin B_{12}

In human metabolism, vitamin B_{12} functions as cofactor in two enzymes, the methionine synthase and the methylmalonyl-CoA mutase. Methionine synthase catalyzes the remethylation of homocysteine to methionine; methylmalonyl-CoA mutase converts methylmalonyl-CoA to succinyl-CoA, as part of the fatty acid degradation. Increased concentrations of methylmalonic acid are thus used as diagnostic parameter for vitamin B_{12} deficiency. Further indicators of vitamin B_{12} deficiency are elevated plasma tHcy, low plasma vitamin B_{12}, and low holo-transcobalamin II (holo-TC II) concentrations. Holo-TC II is the transport form of vitamin B_{12} in plasma and an early marker for changes in vitamin B_{12} metabolism (Herbert 1994; Nexo et al. 2002).

Vitamin B_{12} deficiency is manifested in two major clinical conditions, the megaloblastic anaemia and the cobalamin deficiency associated neuropathy. The neuropathy affects the

cerebral cortex, spinal cord and peripheral nerves (Weir and Scott 1999). The pathogenesis of megaloblastic anaemia in vitamin B_{12} deficiency that is morphologically identical to the anaemia in folate deficiency can be explained by the methyl trap hypothesis. Lacking vitamin B_{12} in the remethylation reaction of homocysteine to methionine induces reduced methionine and SAM levels. The low SAM concentration evokes activation of the *MTHFR* leading to increased synthesis of 5-MTHF. However, because 5-MTHF can not be converted to THF firstly due to lacking vitamin B_{12} for the remethylation reaction and secondly due to the irreversible synthesis of 5-MTHF from 5,10-methylene-THF, 5-MTHF accumulates. Thus, the concentration of the folate derivatives THF, 10-formyl-THF and 5,10-methylene-THF decreases leading to a reduced DNA synthesis. The reduced DNA cycle as induced in folate deficiency evokes reduced cell synthesis and thus anaemia (Scott and Weir 1981).

Vitamin B_2

Riboflavin functions as coenzyme in a diversity of redox reactions. The biologically active forms are flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). In folate metabolism, FAD is a cofactor for the enzyme *MTHFR*. Both flavin coenzymes are involved in the vitamin B_{12} metabolism and function as cofactors for the enzyme methionine synthase. Due to its role in folate and homocysteine metabolism, riboflavin was observed to be an independent determinant of plasma total homocysteine (tHcy) (Hustad et al. 2000; Moat et al. 2003). Others found that riboflavin only affects plasma tHcy in subjects homozygous for the 677C>T *MTHFR* polymorphism combined with low riboflavin concentrations (McNulty et al. 2002) or low plasma folate levels (Jacques et al. 2002).

Homocysteine

The sulphur-containing amino acid homocysteine is remethylated to methionine in all cells by the enzyme methionine synthase with 5-MTHF acting as methyl group donor and vitamin B_{12} transferring the methyl group. A second remethylation pathway exists only in the liver and kidney (Craig 2004): betaine, a methylated derivative of glycine, functions as methyl group donor and betaine homocysteine methyltransferase serves as catalyzing enzyme. This reaction is vitamin B_{12} independent. Besides remethylation, homocysteine is degraded through the transsulfuration pathway (see **Figure 1.2**). In the first step of transsulfuration, homocysteine condenses with serine to cystathionine. This reaction is catalyzed by the enzyme cystathionine β-synthase containing vitamin B_6 in form of pyridoxal-5'-phosphate (PLP) as cofactor. Cystathionine is then hydrolyzed to form cysteine and α-ketobutyrate by the second

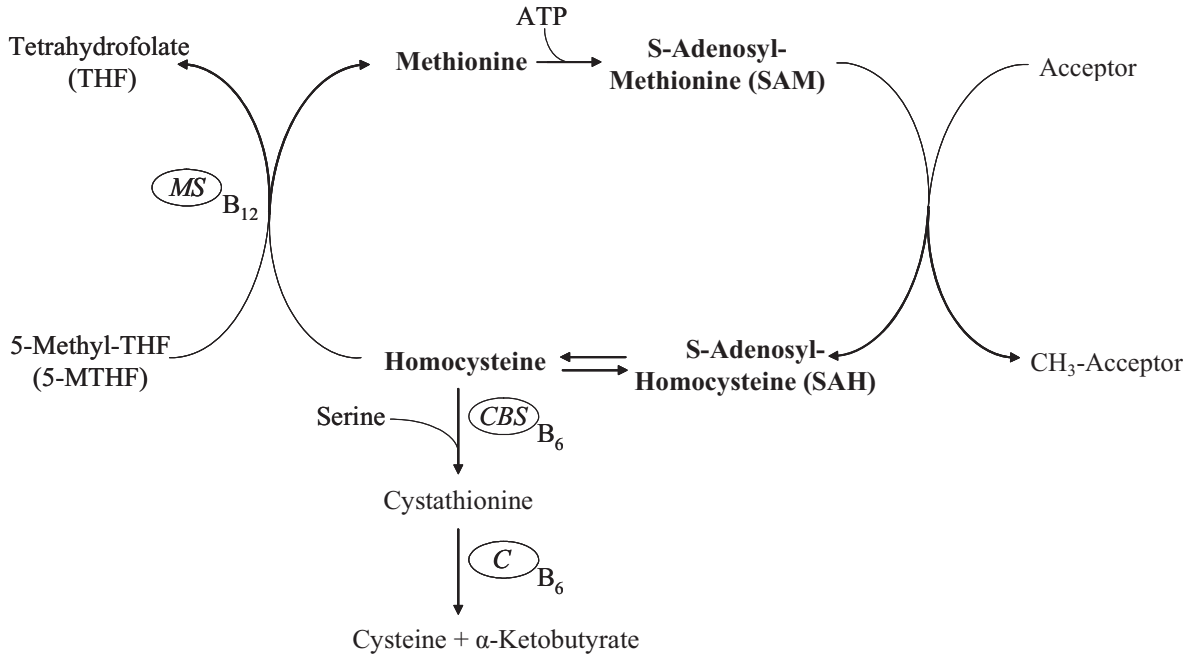

Figure 1.2: Simplified homocysteine-methionine metabolism

MS, methionine synthase; *CBS*, cystathionine β-synthase; *C*, γ-cystathionase; B_{12}, vitamin B_{12}; B_6, vitamin B_6

PLP-containing enzyme γ-cystathionase. Whether homocysteine is degraded through the remethylation or transsulfuration pathway is coordinated by SAM. SAM acts as activator of the enzyme cystathionine β-synthase and, as above mentioned, as allosteric inhibitor of *MTHFR* that synthesizes 5-MTHF. In case of disequilibrium in homocysteine degradation, the increasing intracellular homocysteine concentration activates the homocysteine export mechanism. This mechanism prevents the cells of the potentially toxic homocysteine, however leads to increased homocysteine levels in plasma (hyperhomo-cysteinemia) and in urine (homocystinuria) (Selhub and Miller 1992).

In plasma, homocysteine occurs mainly bound to protein (albumin), but also in free disulfide forms or in the free thiol form. All forms are measured analytically and are referred to as plasma total homocysteine (tHcy).

Homocysteine concentrations are associated with plasma folate, vitamin B_{12} and vitamin B_6 concentrations in men and women (Jacques et al. 2001). Further determinants of plasma tHcy concentration are age, sex, lifestyle and genetic factors. Homocysteine increases with age and is higher in men than in women (Nygard et al. 1995; Jacques et al. 2001). Plasma total homocysteine concentration is inversely related to physical activity (Nygard et al. 1995; Rasmussen et al. 2000; Mennen et al. 2002), and correlates with smoking status (Nygard et al.

1995; Nygard et al. 1998; Jacques et al. 2001), and coffee consumption (Nygard et al. 1998; Jacques et al. 2001; Verhoef et al. 2002). With respect to genetic factors, the common 677C>T and 1298A>C *MTHFR* polymorphisms are linked with higher plasma tHcy concentrations, mainly in subjects homozygous for the 677C>T *MTHFR* (Kluijtmans et al. 2003) and in heterozygotes for both polymorphisms (Lievers et al. 2001). A further determinant of plasma tHcy concentration is the renal function for which serum creatinine serves as marker (Jacques et al. 2001). The intake of drugs interfering with folate metabolism may affect plasma tHcy concentrations (Schneede et al. 2000). Examples for such medical treatments are cholestyramine, methotrexate, sulfasalazin, salicylic acid, antacid and antiepileptic drugs.

***MTHFR* polymorphisms**

The enzyme *MTHFR* catalyzes the reduction of the methyldonor 5-MTHF for homocysteine remethylation (see **Figure 1.1**). The most common genetic polymorphism of the *MTHFR* is a C to T substitution at base pair 677, which, in the homozygous variant (677TT), causes a reduced stability and decreased residual activity of the enzyme and is therefore named thermolabile variant of *MTHFR* (Frosst et al. 1995). The distribution of genotypes was observed to be 43% for the wild-type (677CC), 45% for the heterozygous genotype (677CT), and 12% of homozygotes (677TT) (Brattstrom et al. 1998). A second mutation, the A to C substitution at base pair 1298, also results in decreased enzyme activity (van der Put et al. 1998); however, this variant does not affect the thermostability of the enzyme (Lievers et al. 2001). Studies investigating both genotypes have shown that the 677C>T *MTHFR* polymorphism has an impact on plasma tHcy, red cell folate and plasma folate concentrations in subjects of different populations (van der Put et al. 1998; Friedman et al. 1999; Dekou et al. 2001; Kluijtmans et al. 2003). Subjects homozygous for the 677C>T *MTHFR* polymorphism showed elevated plasma tHcy and low folate concentrations. 5-MTHF is the main folate derivative in plasma, what explains the decreased plasma folate concentrations in homozygotes for the 677C>T *MTHFR* polymorphism. An impact of the 1298A>C *MTHFR* polymorphism in homocysteine metabolism was only shown in subjects with combined heterozygosity (677CT/1298AC) resulting in elevated plasma tHcy and reduced plasma folate levels (van der Put et al. 1998; Lievers et al. 2001).

NEURAL TUBE DEFECTS

Incidence

Neural tube defects are one of the most common severe congenital malformations. Each year estimated 400,000 NTD cases occur worldwide. The prevalence differs greatly between the countries. The lowest incidence was observed in Europe with 0.1-0.6 cases per 1,000 births and in the United States of America and Canada with 0.5-1.0 cases per 1,000 births. In Europe, more than 4,500 pregnancies per year are affected by NTD (Busby et al. 2005). The highest prevalence was found in Ireland and Wales with 6.4-10.9 cases per 1,000 births. Rates of NTD cases per 1,000 births for other countries were: Northern India 3.9-20.0, Southern India 0.5-2.6, Pakistan 7.9, South Africa 4.5, North China 7.3, and Guatemala 20.0.

Pathogenesis

The development and closure of the neural tube are normally completed within 28 days after conception, thus at a point in time when most women are not aware of being pregnant. NTDs are mainly caused by the failure of the neural tube to close. The most common forms of NTDs are spina bifida and anencephaly, two important factors in foetal and infant mortality (Botto et al. 1999).

Spina bifida, the most prevalent NTD form in humans (Groenen et al. 2004), occurs in different forms: spina bifida occulta, meningocele and the most common form is meningomyelocele. Spina bifida occulta is “a bony defect of the spine, usually covered with normal skin”. Meningocele is “a saccular herniation of meninges and cerebrospinal fluid through a bony defect of the spine usually covered by normal skin”. A meningomyelocele is defined as “a herniation of the spinal cord, nerves, or both through a bony defect of the spine” and occurs as an open NTD with either exposed meninges or neural tissue. Infants surviving with spina bifida have severe life-long disability. Anencephaly is characterized by a partial or total absence of the brain and calvaria. All infants with anencephaly are stillborn or die shortly after birth (Botto et al. 1999).

Risk markers

Environmental and genetic factors have a joint role in causing NTDs (Botto et al. 1999). The prevalence of NTDs differs between ethnic groups: the NTD rate is higher among Hispanic and non-Hispanic whites than among blacks in the United States of America. The changing NTD rates observed after migration emphasise the interaction between environmental factors

and ethnicity. Environmental causes for NTDs are maternal diabetes, use of certain antiepileptic drugs, fever and hyperthermia in early pregnancy, and obesity. NTD rates are higher in lower socioeconomic classes. One reason for this is the poor nutrition (Botto et al. 1999). A low status of micronutrients, including folate, vitamin C, and vitamin B_2, were observed in mothers who had a NTD affected pregnancy (Smithells et al. 1976).

The present thesis focuses on the impact of parameters related to folate metabolism, such as folate and vitamin B_{12} status, homocysteine level, and genetic disorders on a women's risk of having a NTD affected pregnancy.

Folate

The association between folate and the development of NTDs was first described by Hibbard in 1964. Smithells et al. (1976) determined low plasma and red cell folate levels in mothers who gave birth to a child with NTD. Kirke et al. (1993) showed that low maternal folate status is an independent risk factor for NTDs. A protective level of folate status for the lowest risk of a NTD affected pregnancy was determined by Daly et al. (1995). The results of their case-control study revealed a continuous dose-response relationship between maternal red cell folate concentration and the risk of having a NTD affected pregnancy. Women with red cell folate concentrations above 906nmol/L had the lowest NTD risk. Based on this risk estimation, a women's risk of NTD would reduce by 88% if her red cell folate concentration increases from 340nmol/L to 906nmol/L (Daly et al. 1995).

Folate is not only related to NTDs, but also to other adverse pregnancy outcomes: An inverse relation was found between plasma folate concentration of the mother and her risk of having early spontaneous abortion (George et al. 2002).

Vitamin B_{12}

Several studies have shown that low maternal vitamin B_{12} status is associated with an increased risk of having a NTD affected pregnancy (Kirke et al. 1993; Afman et al. 2001; Ray and Blom 2003; Groenen et al. 2004). Low levels of holo-TC II were associated with a 3-fold higher risk of a NTD affected pregnancy (Afman et al. 2001). Women with plasma vitamin B_{12} concentrations $\leq$ 185pmol/L had a 3.5-fold higher risk of having an offspring with spina bifida (Groenen et al. 2004). The first genetic link between vitamin B_{12} deficiency and NTDs was described by Wilson et al. (1999): In combination with the homozygous form of the A66G polymorphism in methionine synthase, the risk of having a NTD affected pregnancy increased five times for women with low vitamin B_{12} concentrations (Wilson et al. 1999).