Homocysteine metabolism and risk of schizophrenia

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ISBN-10: 90-9021133-0
ISBN-13: 978-90-9021133-6

Print: PrintPartners Ipskamp, Enschede, The Netherlands.
Cover: Harrie Eijkemans.

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Homocysteine metabolisme en het risico op schizofrenie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. W.H. Gispen,
ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op
woensdag 22 november 2006 des middags te 2.30 uur
door

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geboren op 1 januari 1961 te Elburg

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Dit proefschrift werd mede mogelijk gemaakt met financiële steun van de afdeling Psychiatrie van het UMC Utrecht, de Stichting GGz Nijmegen, AstraZeneca bv, Bristol-Myers Squibb, Eli Lilly bv, Janssen-Cilag bv, Organon bv en Pfizer bv.

Aan Philou

Elke, Stijn en Veerle

Table of contents

List of abbreviations

AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
BHMT	betaine-homocysteine methyltransferase
CASH	comprehensive assessment of symptoms and history
CAT	computer assisted tomography
CBS	cystathionine β-synthase
CI	confidence interval
COMT	catechol-O-methyltransferase
DNA	deoxyribonucleic acid
DSM	diagnostic and statistical manual of mental disorders
EDTA	ethylenediaminotetra-acetate
FAD	flavin adenine dinucleotide
GCPII	glutamate carboxypeptidase II
HPLC	high-performance liquid chromatography
ICD	international classification of diseases
LD / D'	linkage disequilibrium / coefficient of LD
MAT	methionine-adenosyltransferase
Met	methionine
5-MeTHF	5-methyltetrahydrofolate
MRC	Medical Research Council
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
5,10-MTHF	5, 10-methylenetetrahydrofolate
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
NMDA	N-methyl-D-aspartate
NTD	neural tube defect
OMIM	online mendelian inheritance in man
OR	odds ratio
P	probability value
PCR	polymerase chain reaction
PEMT	phosphatidylethanolamine
PLP	pyridoxal-5'-phosphate
RBC	red blood cell
RNA	ribonucleic acid
SD	standard deviation
SNP	single-nucleotide polymorphism
SPSS	statistical package for the social sciences
TDT	transmission disequilibrium test
tHcy	plasma total homocysteine
Val	valine

Chapter 1

General Introduction

Introduction

Schizophrenia is a disabling mental disorder that causes disruptions in thought processes, perceptions, emotions, and cognitive function, resulting in a clinical phenotype with symptoms such as delusions, hallucinations, negativism, or intellectual and social deterioration (American Psychiatric Association, 1994). At the turn of the 20th century Kraepelin first described the clinical phenotype of schizophrenia in detail (Decker, 2004; Engstrom and Weber, 2005), whereas Bleuler introduced its current name (Ban, 2004). Since then it has become clear from numerous epidemiological studies that schizophrenia is a common disorder with a world-wide life-time prevalence of approximately 1.4-4.6 per 1000 population (Jablensky, 2000). Despite the fact that many factors, both genetic and environmental, have been proposed to influence the susceptibility to schizophrenia (Tsuang, 2000), its aetiology is still unknown. Nowadays, schizophrenia is thought to be a complex and chronic brain disorder caused by multiple interacting genes influenced by environmental factors resulting in aberrant neurodevelopment and/or neurodegeneration (Wong and Van Tol, 2003; Pantelis et al., 2005; Rapoport et al., 2005). Interestingly, around 1900 both Kraepelin and Bleuler believed schizophrenia had an organic aetiology and postulated the hereditary nature of a vulnerability to develop schizophrenia. Although several putative susceptibility genes for schizophrenia have been postulated (Harrison and Weinberger, 2005), the pathogenic mechanisms remain to be resolved. Evidence of an association between schizophrenia risk and an aberrant metabolism could reveal additional candidate genes and might generate new insights into the complex aetiology of schizophrenia.

Approximately 50 years ago it was suggested that certain aberrant methylated compounds may affect mental state and behaviour (Osmond and Smythies, 1952) leading to the hypothesis that amino acids associated with the methylation pathway play a role in the pathogenesis of schizophrenia (Kety, 1959a; Kety 1959b). Preliminary evidence for the involvement of the methylation pathway in the pathogenesis of schizophrenia stems from the observation that treatment with high daily doses of L-methionine, the precursor of homocysteine, resulted in exacerbation of schizophrenic symptoms (Pollin et al., 1961). In addition, a higher incidence of schizophrenia patients among homocystinurics, subjects with very high homocysteine levels due to an inborn error of metabolism, was detected compared to the general population (Spiro et al., 1965; Schimke et al., 1965). These observations initiated further investigations about the relationship between aberrant homocysteine metabolism and schizophrenia risk. Nowadays, mild hyperhomocysteinemia is a risk factor for various disorders, such as cardiovascular disease (Boushey et al., 1995; Wald et al., 2002), neural tube defects (NTDs) (Van der Put et al., 1995; Rader 2002), obstetrical pathology (Nelen et al., 2000; De la Calle et al., 2003), and Alzheimer's disease (Clarke et

al., 1998; Seshadri et al., 2002) stressing the pivotal role of homocysteine metabolism in humans. Whether aberrant homocysteine metabolism plays a significant role in the aetiology of schizophrenia remains to be established, and is the aim of this thesis.

Homocysteine

Metabolism

Homocysteine is a sulphur-containing amino acid that cannot be obtained from dietary sources, but is solely the product of the methylation cycle. In human cells, homocysteine is formed by the demethylation of the essential amino acid methionine. Methionine is converted by methionine-adenosyltransferase (MAT) into S-adenosylmethionine (AdoMet) (Figure 1.1) (Mudd et al., 2001). AdoMet is the most important methyl donor in humans, and donates its methyl group in numerous transmethylation reactions, such as methylation of DNA, RNA, proteins and lipids, hormones and neurotransmitters (Scott and Weir, 1998). These transmethylation reactions, catalysed by methyltransferases, form S-adenosylhomocysteine (AdoHcy), which is an inhibitor of many methyltransferases, such as catechol-O-methyltransferase (COMT) or phosphatidylethanolamine methyltransferase (PEMT). In its turn, AdoHcy rapidly hydrolyses to adenosine and homocysteine by a reversible reaction involving S-adenosylhomocysteine hydrolase (AHCY). Homocysteine can then be metabolised via three metabolic pathways that function to maintain low intracellular concentrations of homocysteine. Firstly, homocysteine can be remethylated to methionine via the enzyme methionine synthase (MTR), which uses vitamin B12 as co-factor. In this remethylation reaction 5-methyltetrahydrofolate (5-MeTHF) donates its methyl group to vitamin B12, which, in its turn, transfers it to homocysteine to form methionine and tetrahydrofolate (THF). Folate is solely obtained via the diet and is reduced via several steps involving enzymes such as serine-hydroxymethyltransferase (SHMT) and methylenetetrahydrofolate reductase (MTHFR) into 5-MeTHF, the predominant form of folate in plasma. 5-MeTHF is formed from 5,10-methylenetetrahydrofolate (5,10-MTHF) by MTHFR, which is a key enzyme in the folate-dependent remethylation of homocysteine. MTHFR uses flavin adenine dinucleotide (FAD), a biological active form of vitamin B2 as co-factor. Apart from the remethylation of homocysteine folate is also involved in thymidylate and purine synthesis necessary for DNA synthesis. Secondly, in the liver and kidney homocysteine can also be remethylated into dimethylglycine by the enzyme betaine-homocysteine methyltransferase (BHMT) with betaine as methyl donor. Thirdly, intracellular homocysteine can be irreversibly degraded to cysteine through the transsulphuration pathway that is mainly limited to cells of the liver and kidneys. In this pathway vitamin B6 dependent cystathionine β-synthase (CBS) is the regulating enzyme. As BHMT and CBS are

absent in central nervous tissue (Sunden et al., 1997), 5-MeTHF is the only methyl donor involved in the methylation of homocysteine in central nervous tissue.

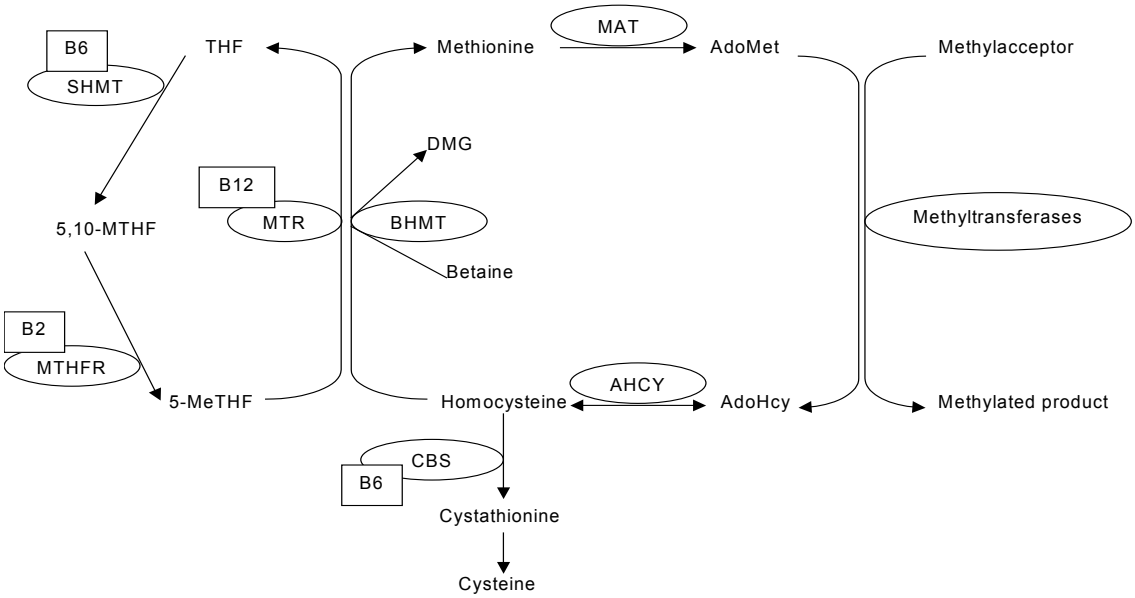

Figure 1.1. Simplified scheme of folate-dependent homocysteine metabolism.

Main regulating enzymes with corresponding cofactors are depicted. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; AHCY, S-adenosylhomocysteine hydrolase; B2, vitamin B2; B6, vitamin B6, B12, vitamin B12; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine beta-synthase; MAT, methionine-adenosyltransferase; MeTHF, methyltetrahydrofolate; MTHF, methylenetetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; SHMT, serine-hydroxylmethyltransferase; THF, tetrahydrofolate.

Hyperhomocysteinemia

Disturbances in intracellular homocysteine metabolism lead in most cases to elevated plasma total homocysteine (tHcy) levels. The intracellular concentration is precisely regulated, and in case of higher intracellular production of homocysteine than the elimination by remethylation or transsulphuration, homocysteine will be exported from the tissues to the plasma (Mudd et al., 2001; Blom and De Vriese, 2002). The term plasma total homocysteine refers to all forms of homocysteine in plasma. THcy levels are measured after an overnight fast or after a methionine loading test. In the loading test the metabolism is stressed by oral supplementation of methionine, and homocysteine levels are determined at peak levels, 4-8 hours after ingestion. Methionine loading is known to increase tHcy concentrations. In fasting state, hyperhomocysteinemia is supposed to reflect in particular abnormalities in homocysteine remethylation, whereas an abnormal tHcy increase upon methionine

provocation is considered to reflect defects in homocysteine transsulphuration. Moderate elevations in the tHcy concentration refers to fasting plasma concentrations >15-30 µmol/L, while severe hyperhomocysteinemia refers to concentrations >100 µmol/L (Kang et al., 1992).

Environmental and genetic determinants of homocysteine

Many environmental and genetic factors influence the tHcy concentration (Table 1.1) (Refsum et al., 2004). Low intake of one of the B-vitamins that serve as a co-factor, or a genetically determined reduction in activities of enzymes of homocysteine or folate metabolism may lead to elevated tHcy concentrations. On the other hand, supplementation of folic acid, the synthetic form of folate, is effective in lowering tHcy concentrations (Homocysteine Lowering Trialists' Collaboration, 1998). Furthermore, tHcy concentrations are found to be higher in men than in women, and are associated with increasing age, renal failure, coffee consumption and smoking (Refsum et al., 2004). THcy levels are also often elevated due to various diseases and drugs (Dierkes and Westphal, 2005). In contrast to the rare inborn errors that lead to very high tHcy levels, common variants in genes (polymorphisms) coding for enzymes involved in the homocysteine metabolism are associated with moderate enzyme deficiency and moderate hyperhomocysteinemia. A common variant in the MTHFR gene has been clearly associated with elevated tHcy levels. The MTHFR gene, localized on chromosome 1.p36.3 (Goyette et al., 1994), harbours the most common genetic determinant of hyperhomocysteinemia, the 677C>T single nucleotide polymorphism (SNP). In 1995, Frosst et al. (1995) identified the single base pair substitution of C to T at nucleotide 677 to be responsible for a thermolabile MTHFR and decreased enzyme activity. The prevalence of the homozygous 677C>T MTHFR (677TT) genotype is between 0 and 32% of the population world-wide (Wilcken et al., 2003), ranging from 5 to 15% in Caucasian populations (Brattstrom et al., 1998). Higher tHcy levels were especially seen in 677TT subjects with low plasma folate status compared to subjects with the normal (677CC) genotype (Jacques et al., 1996).

Clinical relevance of hyperhomocysteinemia

The clinical relevance of homocysteine was first described in 1962 by Carson and Neill (1962) and Gerritsen et al. (1962), when they found very high levels of homocystine, a disulfide of homocysteine, in the urine of children with mental retardation. Severe hyperhomocysteinemia or homocystinuria is characterized by accumulation of homocysteine in blood with homocystine excretion in the urine. Since then several inborn errors such as severe CBS or MTHFR deficiency have been reported to cause homocystinuria, showing premature vascular disease, mental retardation, or psychiatric symptoms as the most

common clinical manifestations (Rosenblatt, 1995). However, on the population level these inborn errors are not important causes of elevations in tHcy concentrations, because they are extremely rare. For example, the estimated incidence of severe MTHFR deficiency is 1:3,000,000 (Rosenblatt, 1995), and severe CBS deficiency 1:335,000 (Mudd et al., 2001).

Table 1.1. Major determinants of elevated plasma total homocysteine concentration*

Genetic factors	*Lifestyle factors*
Variants in genes involved in homocysteine metabolism	Smoking
Gene-nutrient interaction	Coffee consumption
	Alcohol consumption
Physiological determinants	Lack of physical activity
Increasing age	
Postmenopausal state	*Drugs*
Male sex	Antagonists of B-vitamins
	L-Dopa
Clinical conditions	Diuretics
Dietary folate deficiency	Oral conceptives
Dietary vitamin B6 deficiency	Oral antidiabetic drugs
Dietary vitamin B12 deficiency	Lipid lowering drugs
Renal failure	Methotrexate
Hyperproliferative disorders	Nitrous oxide
Endocrinological disorders	
(Diabetes, Hypothyroidism)	
Atrophic gastritis	

* The data are based on the systematic review of Refsum et al. (2004)

First evidence for clinical relevance of mild hyperhomocysteinemia stems from the intervention trials which showed that periconceptional folic acid supplementation reduces the risk of having offspring with a NTD (Medical Research Council [MRC] Vitamin Study Research Group, 1991; Czeizel and Dudas, 1992). In addition, homozygosity for the MTHFR 677C>T polymorphism present in mother, child, or both has been associated with an increased risk of neural tube defects (Van der Put et al., 1995). It was postulated that folate administration may protect pregnant women from having a child with a NTD, possibly by overcoming the folate-dependent genetic defect present in mother or child (Van der Put et al., 1995; Whitehead et al., 1995).

At the time this thesis was initiated an association was shown between hyperhomocysteinemia and a variety of disorders, including cardiovascular disease (Boushey et al., 1995), venous thrombosis (den Heijer et al., 1998), NTD (Botto et al., 1999), Alzheimer's disease (Clarke et al., 1998), cancer (Duthie, 1999), depressive disorder (Fava et al., 1997), and obstetric complications such as recurrent early pregnancy loss (Nelen et al., 1998) stressing the pivotal role of the homocysteine mechanism.

Schizophrenia and homocysteine

The hypothesis linking transmethylation reactions with neuropsychiatric disorders was put forward in 1952 by Osmond and Smythies (Osmond and Smythies, 1952). They proposed that schizophrenia might be related to aberrant methylation of neurotransmitters, such as catecholamines, subsequently leading to toxic compounds like mescaline. The hypothesis was based on the fact that the psychotomimetic drug mescaline is an O-methylated derivative of dopamine. Evolving from this original transmethylation hypothesis it was postulated that disturbances in the one-carbon metabolism, in which homocysteine, methionine, AdoMet and folate are involved, itself might be causative (Smythies, 1963; Levi and Waxman, 1975). At the time this thesis was initiated, several lines of evidence supported the theory of an association between methyl carbon metabolism, in which homocysteine is an intermediate metabolite, and schizophrenia.

Initial clinical evidence for an impaired methyl-carbon pathway in schizophrenic patients was provided by methionine loading studies. It was found that 40% of chronic schizophrenia patients developed an exacerbation of their psychotic symptoms after oral administration of 20 g/day of L-methionine, the precursor of homocysteine (Pollin et al., 1961). Remarkably, this observation showed to be a consistent finding as it has been replicated in ten different studies (Cohen et al., 1974). In addition, as compared to controls the rate of whole body methylation was found to be decreased in patients with schizophrenia after methionine loading (Antun and Kurkjian, 1982; Sargent et al., 1992). It was suggested that these findings might reflect an enzymatic defect or a failure of a control mechanism of the single-carbon cycle in schizophrenic patients (Smythies et al., 1997).

Subjects with homocystinuria due to CBS deficiency have been found to be predisposed to schizophrenia in several case reports (Spiro et al., 1965; Beals, 1969; Rahman,1971; Freeman et al., 1975; Bracken and Coll, 1985), and one open-study (Schimke et al., 1965). Unfortunately, most of these studies were hampered by the fact that included subjects were not diagnosed according to standard diagnostic criteria for schizophrenia such as described in the Diagnostic and Statistical Manual of Mental Disorders (DSM) (American Psychiatric Association, 1994) or International Classification of Diseases (ICD) (World Health Organization, 1977). Following studies suggested that psychiatric disorders are common (approximately 50%) among homocystinurics, but psychotic symptoms are not (Abbott et al., 1987). Because of the rarity of severe homocystinuria it is not a likely underlying cause of general psychiatric disorders, such as schizophrenia.

Several case reports observed severe MTHFR dysfunction with homocystinuria (Freeman et al., 1975) or mildly MTHFR dysfunction with concomitant elevated tHcy levels in patients with “schizophrenia-like” symptoms (Pasquier et al., 1994; Regland et al., 1994; Regland et al., 1997). Interestingly, these patients improved clinically on supplementation with folate

(Freeman et al., 1975), cobalamin (Regland et al., 1994), or a combination of B-vitamins (Pasquier et al., 1994). Confusingly, the finding of decreased MTHFR activity in schizophrenia patients could not be replicated in two case-control studies examining MTHFR enzyme activity in platelets of 18 patients and 31 control subjects (Berger et al., 1977) or tissue samples of six brain regions of 10 schizophrenia patients and 9 control subjects (Elliott et al., 1978).

The first case-control study examining the relationship between tHcy and schizophrenia reported a high frequency of hyperhomocysteinemic subjects in 9 out of 20 patients compared to 10% of the controls (Regland et al. 1995). Susser et al. (1998) observed increased tHcy levels among schizophrenia patients (n=6) compared to controls (n=8) only when serum folate levels were low. These findings of an association between elevated tHcy levels and schizophrenia could not be replicated in a much larger sample of 210 schizophrenia patients and 218 controls (Virgos et al., 1999).

The MTHFR 677C>T polymorphism was detected in 7 out of 11 (64%) hyperhomocysteinemic schizophrenia patients (Regland et al., 1997) suggesting a strong and causal association between this genetic variant, and tHcy levels in these patients. However, at the start of this thesis only three case-control studies had examined the MTHFR 677C>T polymorphism in relation to schizophrenia risk. The results of these studies were inconclusive, showing an increased risk of 1.9 (95%CI: 1.3-2.9) in the initial study comprising 297 patients and 419 controls (Arinami et al., 1997), but no increased frequency of the 677TT genotype in schizophrenia patients compared to controls in the following studies by Kunugi et al. (1998) (343 patients and 258 controls), and Virgos et al. (1999) (210 patients and 218 controls). Only one family-based study was published when we initiated our studies of this thesis. Wei and Hemmings (1999) genotyped MTHFR in 56 Caucasian families consisting of parents, and their affected offspring and found that the 677C allele was more frequently distributed among affected offspring.

A double-blind, placebo-controlled add-on trial with supplements of methylfolate improved clinical status in schizophrenia patients (Godfrey et al., 1990). All patients included had low red blood cell (RBC) folate levels at the entry of the trial. However, the number of schizophrenia patients included was very low comprising only 8 patients with placebo and 9 patients with methylfolate supplementation.

More indirect evidence for an association between aberrant homocysteine metabolism and schizophrenia stems from the findings reported in two epidemiological studies. Individuals who were exposed to severe famine during the periconceptional period in the Dutch Hunger Winter of 1944-1945 had a twofold increased risk of adult schizophrenia (Susser and Lin, 1992; Susser et al., 1996). Interestingly, an increased risk of NTD was also observed in individuals who were exposed to this famine period during pregnancy. A lack of

folate was considered to explain these findings, given that several studies demonstrated that NTDs are related to folate-sensitive genetic defects in homocysteine metabolism (Van der Put et a., 1995; Whitehead et al., 1995).

The metabolic pathway of MTHFR is interconnected with the pathway of the numerous methyltransferases, including COMT (Figure 1.1). COMT is involved in the S-adenosylmethionine-dependent methylation of catecholamines, such as dopamine or noradrenaline, and in this way contributes to homocysteine formation. The COMT gene has been assigned to chromosome 22q11.2. The low enzyme activity Met allele of the common COMT 324G>A polymorphism has been implicated as risk factor for schizophrenia (Kotler et al., 1999; Ohmori et al., 1998). Aberrant methylation of neurotransmitters, such as dopamine or noradrenaline might be due to COMT dysfunction. The genes coding for MTHFR and COMT each show a common variant associated with a reduced enzyme function, and have been implicated in schizophrenia. However, at the time the studies of this thesis were conducted, no data were available with respect to the interplay of COMT, MTHFR, homocysteine and schizophrenia risk.

Objectives

At the time this study was initiated, only a few studies were published about the contribution of homocysteine-methylation metabolism in relation to schizophrenia risk, showing contradicting results. Therefore, the main objective of this thesis was to examine the role of homocysteine metabolism in schizophrenia.

Specific objectives were:

- To elucidate whether a reduced folate level plays a role in the aetiology of schizophrenia.
- To study whether hyperhomocysteinemia is a risk factor in developing schizophrenia.
- To study the contribution of the MTHFR 677C>T polymorphism to the risk of developing schizophrenia.
- To examine the effect of two common and functional genetic variants of enzymes of the methylation pathway, the COMT 324 G>A and MTHFR 677C>T polymorphisms, on schizophrenia risk.
- To examine whether COMT variants themselves may contribute to hyperhomocysteinemia.

Outline of this thesis

In chapter 2 the association between homocysteine, B-vitamins, MTHFR 677C>T polymorphism and schizophrenia risk was explored. Given the strong correlation between tHcy concentration and folate status, we considered folate an important homocysteine determinant. In addition, low folate itself may reflect inadequate diet or genetic predisposition to reduced folate status. Therefore, an overview of the studies examining folate levels in schizophrenia patients is provided in chapter 3. In chapter 4 the investigation of the preliminary study (chapter 2) was extended with regard to the association between tHcy concentration, MTHFR polymorphism, and schizophrenia risk. When the studies of this thesis were initiated several other studies have been published on the relationship between aberrant homocysteine metabolism and schizophrenia. In chapter 5 a meta-analysis of these studies is presented on the association between schizophrenia risk and tHcy levels and MTHFR 677C>T polymorphism, respectively. Chapter 6 deals with the association between MTHFR 677C>T polymorphism and schizophrenia in a family-based study design. The metabolic pathway of MTHFR is interconnected with the pathway of the numerous methyltransferases, such as COMT. A common COMT 324G>A polymorphism has been implicated as a risk factor for schizophrenia. In chapter 7 the contribution of the COMT 324G>A polymorphism alone and when in combination with the MTHFR 677C>T polymorphism, to the risk of schizophrenia is reported. In chapter 8 the effect of several single nucleotide polymorphisms (SNPs) of the COMT gene on tHcy levels was examined. This thesis concludes with a general discussion of our findings, followed by suggestions for future research in chapter 9.

Chapter 2

Homocysteine metabolism and B-vitamins in schizophrenic patients: low plasma folate as a possible independent risk factor for schizophrenia

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Psychiatry Research 2003; 121: 1-9

Abstract

Objective: Two apparently unrelated disorders, neural tube defects (NTDs) and schizophrenia showed increased risks in birth cohorts exposed to famine during early gestation. NTD is associated with impaired folate metabolism. We investigated whether schizophrenia is also linked with a dysfunctional folate metabolism.

Methods: In addition to the prevalence of the 677C>T mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, we compared plasma and red blood cell (RBC) folate, vitamin B6, vitamin B12, and plasma total homocysteine (tHcy) concentrations of 35 schizophrenic patients with those of 104 unrelated controls.

Results: Schizophrenic patients had significantly lower plasma folate concentrations after adjustment for tHcy levels, and elevated RBC folate levels compared to controls. Vitamin B6, vitamin B12, and tHcy levels did not differ from control values. Plasma folate levels below the 10^{th} percentile of controls were associated with an approximate 4 to 7-fold (before and after adjustment of folate levels for tHcy respectively) risk of having schizophrenia. In addition, a significant dose-respons relation between plasma folate concentrations and risk for schizophrenia suggested a protective effect by high plasma folate concentrations. Elevated tHcy levels and, in line with this finding, homozygosity for the 677C>T mutation in the MTHFR gene were not associated with an increased risk for schizophrenia.

Conclusion: Evidence is presented suggesting that the folate metabolism is disturbed in schizophrenic patients, independently of tHcy.

Introduction

There is a growing body of evidence suggesting that there may be abnormal development of the brain, arising possibly *in utero* or in early life, in some cases of schizophrenic patients (Weinberger, 1987). Genetic and environmental factors have been frequently discussed as the agents disrupting neurodevelopment (Waddington, 1993). The findings of an increased risk of both schizophrenia and neural tube defects (NTDs), as observed in a birth cohort exposed to maternal nutritional deficiency during early gestation (Susser et al., 1996), is therefore of great interest. The authors suggested that NTD-like defects could play a role in the aetiology of schizophrenia. Low folate levels (Smithells et al., 1976), mild hyperhomocysteinemia (Steegers-Theunissen et al., 1994) and homozygosity for the 677C>T mutation (677TT) of the 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme (Van der Put et al., 1995) are reported to be associated with NTDs. Periconceptional folate administration reduces occurrence (Czeizel and Dudás, 1992) and recurrence (MRC Vitamin Study, 1991) risk of NTD. Probably by overcoming a mild folate-related defect in the homocysteine metabolism, the developing child is protected against NTD. Folate (vitamin B11), together with pyridoxal-5'-phosphate (an active form of vitamin B6) and cobalamin (vitamin B12), is essential for an adequate metabolism of homocysteine.

In contrast to NTD, controversy and a lack of evidence remain regarding the association between lowered plasma folate, (mild) hyperhomocysteinemia, MTHFR dysfunction and schizophrenia. Low levels of plasma folate have been reported to be related with psychiatric illness in a consistent manner, in particular depression. The association of folate deficiency to any form of psychosis is less clear (Levi and Waxman, 1975; Hutto, 1997; Smythies et al., 1997; Herrán et al., 1999). Supportive evidence linking impeded homocysteine remethylation to schizophrenic patients has been reported in three controlled studies (Regland et al., 1995; Susser et al., 1998; Levine et al., 2002). Findings of a recent study failed to demonstrate a significant difference in plasma total homocysteine (tHcy) levels between cases and controls (Virgos et al., 1999). MTHFR deficiency, which results in (mild) hyperhomocysteinemia in plasma, has been associated with schizophrenia-like psychosis in several case-reports (Freeman et al., 1975; Pasquier et al., 1994; Regland et al., 1994). This positive association is inconsistent with the previous finding that platelet MTHFR activity of schizophrenic patients and normal subjects showed no significant difference (Berger et al., 1977). Homozygosity for the thermolabile variant of MTHFR (677TT), a polymorphism which results in hyperhomocysteinemia, has not been consistently linked with schizophrenia. An increased frequency of 677TT genotype among cases as compared to controls was recently found (Arinami et al., 1997; Joober et al., 2000), whereas others could not replicate these findings (Kunugi et al., 1998; Virgos et al., 1999).

Evidence for a folate-sensitive genetic defect in homocysteine metabolism in schizophrenic patients is still meagre, especially because of some non-reproducible findings. We examined the relationship between a disturbed folate and homocysteine metabolism, and schizophrenia by exploring plasma and red blood cell (RBC) folate levels, vitamin B6, vitamin B12 and tHcy levels in schizophrenic patients and comparing them with levels in healthy controls. The relative risk for having schizophrenia was estimated at different cut-off levels of tHcy and folate. In addition, we investigated the presence of the 677C>T mutation. This is the first time such potential risk factors, such as tHcy, vitamins B6 and B12, plasma and RBC folate levels, and the 677C>T mutation in the MTHFR gene have been examined in the same study in schizophrenic patients.

Methods

Subjects

Thirty-five unrelated Dutch schizophrenic patients (27 men and 8 women; mean age [±SD]: 39.4±9.0 years) took part in the study, which was conducted at an outpatient clinic and several inpatient clinics of the GGz Nijmegen, Institute of Mental Health in Nijmegen, the Netherlands. All enrolled cases fulfilled the DSM-IV (American Psychiatric Association, 1994) criteria for a diagnose of schizophrenia, and most of them had chronic or refractory illness. All patients were receiving regular antipsychotic treatment. The exclusion criteria were hormonal therapy, the use of vitamins or folate antagonists, overt liver and renal dysfunction, cleft lip and NTD, which were verified by medical records. After complete explanation of the study to the subjects, written informed consent was obtained.

Comparison data were derived from a reference population consisting of 104 healthy and unrelated Dutch hospital employees (30 men and 74 women; mean age [±SD]: 35.5±8.4 years), who had participated in a health survey of risk factors for NTD, as described elsewhere (Heil et al., 2001). The protocol of this study was approved by the medical ethics committee of the University Medical Centre St Radboud in Nijmegen and local ethics committee.

Homocysteine and vitamin analysis

Venous blood samples were collected after an overnight fast. THcy, plasma and RBC folate, vitamin B6 and vitamin B12 levels were determined in all patients. Blood samples for measurement of tHcy concentrations in plasma were drawn in ethylenediaminetetra-acetate (EDTA) vacutainer tubes of 4 ml and centrifuged within 30 minutes at 3,000 x g for 10 minutes. The plasma was separated and immediatedly stored at -20ºC. THcy concentrations were measured by high-performance liquid chromatography (HPLC) with fluorimetric

detection (Te Poele-Pothoff et al., 1995). Folate and vitamine B12 levels were determined in EDTA plasma using ion capture IMx (Abbott Laboratories, Abott Park, IL, USA). For RBC folate hemolysates were prepared by diluting a 50 µl whole blood sample with 1 ml of IMx folate RBC Lysis reagent. Determination, using ion capture IMx as well, was followed by calculation of the RBC folate using % hematocrit and plasma folate concentration. The determination of vitamin B6 as pyridoxal-5'-phosphate (PLP) in EDTA-whole-blood was performed with an HPLC technique (Schrijver et al., 1981), using precolumn derivatisation with semicarbazide to obtain PLP-semicarbazone (Ubbink et al., 1985)

For the control group, the blood sampling and determination procedures of tHcy were the same as the methods used for patients. Vitamin levels were determined by using the Dualcount Solid Phase Boil Radioassay (Diagnostic Product Corporation, Los Angeles, CA, USA).

Mutation analysis

The investigated mutation in the MTHFR gene is a C to T substitution at basepair 677, altering an alanine into a valine residue. The prevalence of this mutation was investigated by polymerase chain reaction and HinfI digestion, as reported before (Frosst et al., 1995).

Statistics

In order to correct for differences in determination methods between patients and controls, concerning plasma and RBC folate, vitamin B6 and vitamin B12, regression formula were used. Data are presented as medians (range) because of their skewed distribution. Prior to the statistical analyses, variables were logarithmically-transformed to achieve a normal distribution. P values for differences in tHcy and vitamin levels between patients and controls were calculated using linear regression analysis, and were adjusted for age and sex because of marked differences between both groups. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated as estimates of the relative risk of schizophrenia for different tHcy and vitamins concentrations using logistic regression analysis, and were also adjusted for age and sex. To examine possible trends in ORs for schizophrenia, linear trend tests using logistic regression were performed on the consecutive 10^{th} percentiles of the different tHcy and vitamins concentrations. Correlational analyses were done with Spearman rank correlation tests. The OR and 95% CI were calculated to estimate the relative risk of homozygosity for the 677C>T mutation in relation to schizophrenia. No analysis of genotype could be performed in one patient and seven control subjects. Probability values of 0.05 or less in two-tailed tests were considered significant. All statistical analyses were performed with the SPSS-software package 9.0.

Results

Homocysteine and vitamins

Table 2.1 documents the median values of vitamins and tHcy levels in the schizophrenic patients and controls. Age- and sex-adjusted ORs at several cut-off points of plasma and RBC folate, and tHcy concentrations are shown in Table 2.2.

Plasma folate levels of the schizophrenic patients were non-significantly lower when compared to control levels when the P value was adjusted for age and sex only. After additional adjustment for tHcy levels, a significant difference was found (P=0.031). We observed that 13 out of 35 patients (37%) had plasma folate levels below the 10th percentile of plasma folate levels in controls, resulting in an age- and sex-adjusted OR of 4.4 (95% CI: 1.5-12.6) of having schizophrenia. To examine whether the observed risk of low plasma folate levels is related to tHcy levels or not, we also adjusted the OR for tHcy concentrations (Table 2.2). This resulted in an OR of plasma folate levels below the 10th percentile for schizophrenia of 6.9 (95% CI: 2.1-23.1), indicating that low folate is an independent risk factor for having schizophrenia. In line with this finding, an increase in ORs, adjusted for age, sex and tHcy levels, with declining plasma folate concentrations and an overall significant trend (P=0.0281) was observed, which suggested that low plasma folate is a risk factor and high plasma folate a protective factor for schizophrenia. RBC folate levels of patients were significantly higher compared to levels in controls and in addition high RBC folate concentrations tended toward an increased risk for schizophrenia (Table 2.2). We also observed an increase in ORs with increasing RBC folate levels and an overall significant trend (P=0.0005).

Table 2.1. Fasting homocysteine, vitamin B6, vitamin B12, red blood cell and plasma folate concentrations in the patient versus control group[a]

	Patients (n=35)	Controls (n=104)	P value
Homocysteine (µmol/L)	13.4 (8.9-39.5)	12.8 (7.3-31.9)	0.54
Vitamin B6 (nmol/L)	48 (28-123)	46 (22-160)	0.27
Vitamin B12 (pmol/L)	235 (141-598)	215 (89-600)	0.09
Red blood cell folate (nmol/L)	771 (466-1980)	580 (120-1200)	0.0001*
Plasma folate (nmol/L)	11.1 (6.4-36.5)	14.0 (3.2-80.0)	0.11

[a]Results are expressed as medians with minimum and maximum value in parentheses.
*Significant difference (P<0.05), P values are adjusted for age and sex.

The vitamin B6 and vitamin B12 levels of schizophrenic patients were similar to those of controls. In line with those findings, low vitamin B12 and low vitamin B6 concentrations

(below the tenth, 20th or 30th percentile), were not associated with an increased risk for schizophrenia (data not shown).

THcy levels of the schizophrenic patients did not differ significantly from control values. When hyperhomocysteinemia was defined as the concentration exceeding the 90th percentile of the control group (>19.2 µmol/L), no increased risk could be detected. Fasting hyperhomocysteinemia was observed in 4 out of 35 (11%) patients only, compared with 10 out of 104 control subjects (10% by definition), resulting in an age- and sex-adjusted OR of 0.5 (95%CI: 0.1-2.0). THcy concentrations over the 70th and 80th percentiles showed a slightly elevated risk for schizophrenia, albeit not statistically significant. Accordingly no significant overall trend was observed. Additional adjustment of the tHcy levels for plasma folate did not change the outcome in a substantial way (Table 2.2).

Table 2.2. Odds ratios (OR) and 95% confidence intervals (95% CI) for different cut-off points of plasma and red blood cell folate and homocysteine levels of schizophrenic patients versus controls

	Patients (n=35)	Controls (n=104)	OR (95% CI)*	OR (95% CI)§ tHcy-adjusted	OR (95% CI)# folate-adjusted
Folate (nmol/L)					
Plasma (percentile)					
< 11.9 (30th)	18	43	2.9 (1.2- 7.4)[1]	4.1 (1.4-11.8)[2]	
< 11.0 (20th)	16	30	2.1 (0.8- 5.2)	2.6 (1.0- 7.4)[1]	
< 10.0 (10th)	13	16	4.4 (1.5-12.6)[2]	6.9 (2.1-23.1)[2]	
< 9.6 (5th)	10	5	5.6 (1.5-20.3)[2]	9.0 (2.1-37.9)[2]	
Red blood cell (percentile)					
> 675 (70th)	22	31	3.0 (1.2- 7.5)[1]	3.1 (1.2- 8.1)[1]	
> 740 (80th)	19	19	4.2 (1.7-11.2)[2]	4.8 (1.7-12.9)[2]	
> 840 (90th)	13	9	4.0 (1.4-11.9)[2]	4.2 (1.4-12.7)[2]	
> 958 (95th)	13	5	10.5 (2.8-39.2)[3]	10.8 (2.8-41.3)[3]	
Homocysteine (µmol/L)					
Plasma (percentile)					
> 14.4 (70th)	15	29	1.2 (0.5-3.0)		1.0 (0.4-2.6)
> 15.5 (80th)	12	19	1.4 (0.5-3.8)		1.2 (0.4-3.3)
> 19.2 (90th)	4	10	0.5 (0.1-2.0)		0.3 (0.1-1.4)
> 24.8 (95th)	1	5	0.2 (0.0-2.1)		0.1 (0.0-1.4)

*Adjusted for age, sex. §Adjusted for age, sex and plasma homocysteine. #Adjusted for age, sex and plasma folate. [1]P<0.05: [2]P<0.01: [3]P<0.001.

To score for associations between the different vitamin and tHcy levels of schizophrenic patients and controls, we calculated the Spearman correlation coefficients (Table 2.3). Plasma folate correlated with RBC folate and vitamin B6, and showed an inverse correlation

with fasting tHcy. RBC folate levels showed an inverse correlation with tHcy levels in patients and correlated positively with vitamin B6 and vitamin B12 in controls. A low but significant inverse correlation between vitamin B12 and tHcy levels was present in controls only. No other significant correlations were observed.

Table 2.3. Relationship between plasma homocysteine, vitamin B6, vitamin B12, and red blood cell (RBC) folate and plasma folate levels of schizophrenic patients and controls[a]

	Vitamin B6	Vitamin B12	RBC folate	Plasma folate
Patients				
Homocysteine	0.32	0.00	-0.46*	-0.64*
Vitamin B6		0.09	0.16	0.52*
Vitamin B12			0.04	0.08
RBC folate				0.51*
Controls				
Homocysteine	0.01	-0.25*	-0.18	-0.42*
Vitamin B6		0.06	0.37*	0.26*
Vitamin B12			0.31*	0.14
RBC folate				0.43*

[a]Values given in Spearman correlation coefficients.
*Correlation is significant at the 0.01 level (two-tailed).

677C>T mutation

We obtained no higher prevalence of homozygosity for the 677C>T mutation in patients when compared to the control group. The 677TT genotype was observed in 3 out of 34 patients (9%) only and in 17 of 97 control subjects (18%), resulting in an age- and sex-adjusted OR of 0.4 (95% CI: 0.1-1.8) for patients versus controls. Heterozygosity for the 677C>T mutation was present in 16 (47%) patients and 35 (36%) controls. The wild type (677CC) was present in 15 (44%) patients and 45 (46%) controls. The observed frequency of the T allele of the 677C>T mutation was 0.36 for controls and 0.32 for the group of patients.

Discussion

Our data provide evidence that decreased plasma folate levels may act as a risk factor for schizophrenia, indicating a disturbance in folate metabolism in schizophrenic patients, independently of tHcy. We found no evidence for a major role of homozygosity of the 677C>T mutation in the MTHFR gene in schizophrenia. In line with this finding, no conclusive evidence for the involvement of homocysteine metabolism as a major risk factor for schizophrenia could be established. However, other polymorphisms of the folate metabolism

could be involved.

In our study, schizophrenic patients showed a redistribution of folates, namely lowered plasma and elevated RBC folate levels compared to controls after adjustment for age, sex and tHcy levels. These findings can be explained as a result of decreased MTHFR activity due to the 677C>T mutation as previously described by Van der Put et al. (1995). They reported a redistribution of folates due to the 677C>T mutation in the MTHFR gene, resulting in decreased plasma folate and elevated RBC folate. Although a higher frequency for the 677C>T mutation was not observed among our schizophrenic patients, other polymorphisms in the MTHFR gene may account for this result. RBC folate is an indicator for the stored derivatives in the red blood cell and is lowered when folate uptake is deficient or in case of malnutrition, whereas plasma folate may reflect recent dietary intake (Bailey, 1995). In line with previous studies, plasma and RBC folate showed a positive correlation (Table 2.3). Schizophrenic patients are in general, expected to be an undernourished group and, as a consequence, may show low vitamin levels. Therefore, differences in lifestyle between the study groups may account for differences in folate levels found. Nevertheless, we have no indication that a nutritional shortage or a defect in folate uptake explained the lowered plasma folate levels in the group of schizophrenic patients in our study. The different B-vitamins are frequently present in the same types of foods; thus the intake of one vitamin can be a marker for the intake of another vitamin (De Bree et al., 2001). Folate is especially highly correlated with vitamin B6 intake. We did not found a deficiency of vitamin B6 in our patients group, indicating that they had a normal nutritional intake regarding these B-vitamins. The additional importance of our study involve the findings of a high prevalence of low plasma folate concentrations in schizophrenic patients and the increase in risk with decreasing plasma folate independent of tHcy levels. The findings are suggestive of an increased metabolic need for folate and a protective effect of high plasma folate levels in schizophrenic patients.

We observed, as did Virgos et al. (1999), that fasting tHcy levels and the prevalence of hyperhomocysteinemia in the group of patients were comparable to those of controls. In contrast, Regland et al. (1995) observed high tHcy levels in 45% of schizophrenic patients. One explanation for those conflicting findings may be the related high frequency of the 677TT genotype in our reference group of 18%, resulting in a high tHcy concentration as cut-off level for hyperhomocysteinemia. The study of Regland et. al. (1995) may have been biased by the inclusion of patients from a broader diagnostic spectrum, including schizoaffective disorder. In general, the conflicting results in previous studies may be due to the heterogeneity of the schizophrenia syndrome. Differences in several confounding factors as age, gender, coffee consumption and cigarette smoking, which have been associated with increases in tHcy levels (Nygård et al., 1998), might explain the differences in reported tHcy

levels also.

Genetic defects that impair the metabolism of vitamin B6 or vitamin B12 or malnutrition of those vitamins could disturb folate and homocysteine metabolism. We observed no decreased levels of vitamin B6 or vitamin B12 in patients compared to controls. Therefore, we have no indication for an impaired metabolism or malnutrition of vitamin B6 or vitamin B12.

If homozygosity for the 677C>T mutation of the MTHFR gene were a strong determinant for having schizophrenia, then the observed frequencies of the 677TT genotype should have been higher in the group of schizophrenic patients compared to controls. In accordance with the findings of Kunugi et al. (1998) and Virgos et al. (1999), we failed to find an increased frequency of homozygosity for the 677T allele of the MTHFR gene in schizophrenic patients. Some caution is warranted when interpreting our result because of the relatively small number of schizophrenic patients in the present study. However, the lack of a positive association between the 677TT genotype and schizophrenia is inconsistent with the findings of the studies by Arinami et al. (1997) and Joober et al. (2000). The latter study found that the 677TT genotype was only more frequent in a subgroup of schizophrenic patients, i.e. excellent responders to neuroleptic medications, compared to controls. In order to circumvent a false-positive rate due to population stratification in a population-based association study, Wei and Hemmings (1999) investigated 56 Caucasian families consisting of parents and affected offspring with schizophrenia. They concluded that the 677C>T mutation itself may not play a role in determining the susceptibility to schizophrenia but serves as a genetic marker indicating a locus for schizophrenia. In a previous study of a Dutch population of healthy individuals, the 677TT genotype occurred in 8.4% of the population (Van der Put et al., 1997). We obtained a much higher frequency of 18% in the group of healthy controls, which is likely attributable to chance.

It cannot be ruled out that, despite correction for age and gender, our study was susceptible to other confounding factors, including medication and nutrition status and the duration of schizophrenia, which we did not assess. Psychotropic drugs were used by patients only, and these drugs have not been related to tHcy (Regland et al., 1995) or folate (Herrán et al., 1999) levels thus far. Although food questionnaires can provide data on dietary habits and therefore vitamin intake, it is well known that many schizophrenic patients have cognitive deficits, including memory problems. Therefore, the reliability of taking dietary histories must be questioned. We preferred to asses blood vitamin status instead of dietary intake.

In conclusion, our findings suggest that decreased plasma folate is a risk factor for schizophrenia. Low folate status could affect the cell's ability to methylate important compounds such as DNA, proteins, lipids and myelin, impairing cellular functions and thus

the functioning of the brain. Possibly a disturbed folate metabolism may hamper the development of the brain *in utero*. Understanding of the pathogenetic processes that are responsible for the appearance of schizophrenia could lead to the development of therapeutic strategies for the prevention of schizophrenia or to the reduction of its symptoms. Further investigation should focus on the identification of the molecular basis of decreased plasma folate levels in schizophrenic patients, particularly in relation to MTHFR dysfunction and possible genetic markers.

Acknowledgements

The authors thank Nelleke J. Hamel-Van Bruggen for her assistance in data collection, and Lydia A. Afman, PhD, for statistical evaluation, and Dorine W. Swinkels, PhD, for her critical comments. Henk J. Blom, PhD is an Established Investigator of the Netherlands Heart Foundation (Grant No. D97.021).

Chapter 3

Aberrant folate status in schizophrenic patients: what is the evidence?

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Progress in Neuro-Psychopharmacology and Biological Psychiatry
2005; 29: 1133-1139

Abstract

Objective: A vast amount of case reports, open-studies, and to a lesser extent, case-control studies have been published on the topic of psychopathology and folate deficiency. These studies reported a high incidence of serum folate deficiency in patients with various psychiatric disorders. Folate deficiency seems to be a particular consistent finding in depressive patients. The evidence for an association between aberrant folate status and schizophrenia seems less convincing. The lack of stringent methodology such as inclusion of age and sex matched controls was thought to be the main reason for the inconclusive results.

Methods: The purpose of this article is to review the published case-control studies that provide data on folate levels in the population of patients with schizophrenia. Data extracted from these studies comprised methodological design, clinical characteristics and folate measurements.

Results: We found that none of the seven case-control studies included in this review (in total 325 cases and 560 control subjects) explicitly reported on all critical factors in the assessment of folate. In addition, only three studies found lower plasma folate levels more frequently in patients with schizophrenia compared to controls.

Conclusion: Further research on this topic is required to clarify the relationship between folate status and schizophrenia and should avoid the methodological pitfalls mentioned in this review. In addition, research should also focus on polymorphisms of genes related to folate metabolism.

Introduction

The name folate is derived from the Latin word folium or “leaf”. This water soluble B vitamin was first isolated from spinach leaves in 1941 and synthesized in 1946. The generic term folate includes both naturally occurring polyglutamates as the synthetic form folic acid (Lucock, 2000). Humans cannot synthesize folate, and thus bioavailability depends on dietary intake and absorption of the vitamin. In mammalian tissues, folate acts as donor and acceptor of one-carbon units required for vital cellular processes like nucleic acid biosynthesis, protein biosynthesis, amino acid metabolism, methyl-group biogenesis, and vitamin metabolism. Since the discovery of folate a variety of clinical conditions such as cardiovascular disease (Wald et al., 2002), adverse birth outcomes including neural tube defects (Scholl and Johnson, 2000), cancers (Duthie, 1999), Parkinson’s disease (Allain et al., 1995), and Alzheimer’s disease (Clarke et al., 1998) have been associated with aberrant folate metabolism, stressing the pivotal role of folate. The report on the occurrence of mental effects such as sleeplessness, forgetfulness, and irritability related to experimentally induced folate deficiency in a healthy male, disappearing after folate supplementation (Herbert, 1962) resulted in extensive research on the topic of psychopathology and folate status. It was hypothesized that an aberrant folate metabolism could also contribute to the pathogenesis of schizophrenia (Levi and Waxman, 1975). A vast amount of case reports, open-studies and, to a lesser extent, case-control studies have been published on the topic of psychopathology and folate deficiency discussed in several reviews. In the extensive review by Young and Ghadirian (1989) a high incidence of serum folate deficiency was reported varying between 8 and 50% in patients with various psychiatric disorders including depression and schizophrenia. In addition, many studies investigated the prevalence of psychiatric symptoms in epileptic patients on anticonvulsants providing evidence for an association of folate deficiency and psychiatric symptomatology (Reynolds, 1967; Young and Ghadirian, 1989). Anticonvulsants decrease folate absorption from the gastrointestinal tract and might induce folate deficiency, subsequently resulting in mental effects. In line with these findings several clinical studies demonstrated therapeutic effects of folate administration on psychiatric symptoms especially in those subjects with folate deficiency (Carney and Sheffield, 1970; Godfrey et al., 1990). Folate deficiency seems to be a particular consistent finding in depressive patients (Crellin et al., 1993), with a correlation of symptom severity and effect of folate as an adjunct therapy (Fava et al., 1997). The evidence for an association between aberrant folate status and schizophrenia seems less convincing. The lack of stringent methodology such as well-defined exclusion criteria and inclusion of age and sex matched controls was thought to be the main reason for the inconclusive results (Skeritt, 1998). An overview of the factors contributing to the contrasting findings is shown in Table 3.1.

Table 3.1. Methodological shortcomings of studies on folate levels in schizophrenic patients

Small sample size
Lack of case-control design
No matching for age and sex
Differences in diagnostic instrument used
Inclusion of subjects with clinical conditions associated with folate deficiency
Inclusion of subjects using folate level interfering drugs
Lack of data on red blood cell levels as determinant of folate storage reservoir
Arbitrary cut-off values for folate deficiency
Different assay techniques
Different geographical locations

We reviewed the published case-control studies with data on the association of folate levels and schizophrenia. Positive results in this matter might be a rationale for the utility of assessing folate status in schizophrenic patients and, in addition, treating these patients with folate supplements. Implications for future research are being discussed.

Methods

Study identification

Eligible studies were identified by searching the electronic NLM MEDLINE database for relevant reports published up to and including September 2004. Subject headings were folate, folic acid, vitamin B and schizophrenia. Additionally, the reference list of original articles on this topic were scanned to identify articles missed by the computerized search.

Study selection

Studies examining serum, plasma or red blood cell (RBC) folate levels were included in the review. The following additional criteria were used to select studies for data extraction: (a) a diagnosis of schizophrenia for cases, (b) use of a case-control design, (c) publication in an English-language, peer-reviewed, indexed scientific journal. Control groups composed of medical ill subjects or psychiatric patients were not allowed. Studies on subjects with neurological disorders such as epilepsy or (psycho-)geriatric disorders were excluded.

Data extraction

Information was extracted from each study on the outcome of statistical comparison of the mean folate levels in each study group and whether this analysis revealed significant differences between cases and controls. In addition, the number of cases and controls above

and below a particular folate concentration as cut-off point either from the control distribution in each study or from a laboratory reference value were extracted from the studies.

The selected studies were also screened on whether specific data were reported on exclusion criteria or determinants of folate levels in order to minimize confounding or systematic differences. Based on the knowledge of the most relevant determinants screening was performed on: physiological conditions such as age, sex, and pregnancy, lifestyle factors such as alcohol consumption and smoking, supplementary vitamin intake and vitamin status. Screening was also conducted for drugs and diseases known to modulate folate levels. The presence of an uniform blood sampling procedure such as food intake requirements before blood sampling for subjects were also registered for each study. All abstracted information was tabulated and discussed by making a summary.

Results

Overall results

A total of ten eligible articles contained data of studies on folate measurements in schizophrenic patients versus healthy control subjects. Two articles reported the same study group (Carney, 1967; Carney and Sheffield, 1970) and therefore only the first published study was included. Two studies were excluded because the results of schizophrenic patients were unrecognisably mixed up with the total group of psychiatric patients in the studies reported (Reynolds et al., 1971; Skeritt, 1998). This review contains data on seven eligible case-control studies with a total number of 325 cases and 560 control subjects (Carney, 1967; Gundersen, 1969; Hermesh et al., 1988; Susser et al., 1998; Herrán et al., 1999; Muntjewerff et al., 2003; Goff et al., 2004). Study specific characteristics are presented in Table 3.2, and discussed below. RBC levels were determined in only two studies (Herrán et al., 1999; Muntjewerff et al., 2003), therefore only serum or plasma folate levels are shown in Table 3.3. Data on lifestyle factors were presented in only one study (Goff et al., 2004). Requirements for blood sample collection or assay method used were different for cases compared to controls in five out of seven studies (Table 3.3).

Study specific results

Carney (1967) performed an extensive study on 423 psychiatric patients of which 78 patients were diagnosed as schizophrenic. In this study more schizophrenic patients than controls had low serum folate, although mean serum levels did not differ significantly. Analysis between various age groups and sex distribution showed no differences in relation to the folate levels, but these data were only provided for the total group of included psychiatric patients. Low folate psychiatric patients had more haematological abnormalities

including low serum vitamin B12 than the psychiatric patients with normal folate levels. Severe malnutrition, use of folate level depressing drugs, pregnancy and physical illness were reported for several patients in the low folate group. No specific data were provided in this matter for the group of schizophrenic patients.

In the study of Gundersen (1969) data on folate and vitamin B12 levels in serum, and haematological parameters were presented for three groups of female subjects: 21 schizophrenic patients with mean age 70 years, 18 healthy young controls with mean age 25 years, and 19 healthy old controls with mean age 82 years. In Table 3.2 the total number of control subjects is shown, because mean values and distributions of vitamin levels and haematological parameters were similar for all three study groups. However, no detailed data were provided on the statistical analyses used. All patients were under treatment with tricyclic neuroleptics. It was concluded by the author that these drugs did not depress folate levels in serum.

Hermesh et al. (1988) determined vitamin B12 and folate levels in serum of 30 inpatients with obsessive compulsive disorder and of 30 inpatients with schizophrenia. Results of the patient groups were compared with healthy subjects matched for age and sex. Neither mean folate levels nor the frequencies of low folate showed significant differences between groups. The frequency of low levels of serum vitamin B12 observed in the group of obsessive compulsive patients (20%) was significantly higher than the frequency found among controls or schizophrenic patients (approximately 4%).

The study by Susser et al. (1998) included patients with a diagnosis of schizophrenia or schizoaffective disorder admitted to a research unit. Stored blood samples drawn for a prior study on control subjects (not matched for age and sex) were utilized. Serum folate, cobalamin, and plasma total homocysteine (tHcy) were determined. Mean tHcy was significantly higher only in the group of cases with low folate compared to controls with low folate. Cobalamin levels were similar in the low folate groups.

The study by Herrán et al. (1999) was the first case-control study providing data on both serum and RBC folate levels in a distinct group of schizophrenic outpatients. Control subjects were matched for age and sex. Subjects with conditions known to be associated with folate deficiency were excluded. Although serum folate levels were within normal range for both study groups, it was reported that clinical features as measured with Clinical Global Impression (Bech et al., 1993) and the Positive and Negative Syndrome Scale (Kay et al., 1987) correlated negatively with serum folate levels in patients. The higher the degree of severity, the lower serum folate levels. Mean RBC folate concentrations were similar for patients and controls and in only 2 out of 55 patients RBC folate was below the reference value.

Table 3.2. Study characteristics of seven case-control studies on the association of folate levels and schizophrenia

Study	Study area	Exclusion criteria	Age and sex matching or adjustment	Cases			Controls
				N	Diagnostic instrument	Duration illness	N
Carney (1967)	UK	not reported	no	78	not reported	66% of patients with duration >3 years	62
Gundersen (1969)	Denmark	physical illness, like cancer or liver dysfunction, pregnancy, anti-convulsants, barbiturates	female subjects in age subgroups	21	not reported	chronic	37
Hermesh et al. (1988)	Israel	not reported	yes	30	not reported	chronic	30
Susser et al. (1998)	USA	substance abusers	no	17	DSM-III-R	not reported	24
Herrán et al. (1999)	Spain	physical illness, pregnancy, vitamin users, vegetarians, drugs associated with vitamin deficiency	yes	53	DSM-IV	12.7 years (SD: 10.8)	55
Muntjewerff et al. (2003)	NL*	liver or renal dysfunction, vitamin users, folate antagonists	yes	35	DSM-IV	chronic	104
Goff et al. (2004)	USA	drugs known to affect folate or homocysteine levels, renal dysfunction, alcohol abuse	no	91	not reported	chronic	248

* The Netherlands

In the study by Muntjewerff et al. (2003) data from a mixed group of 35 in- and outpatients with schizophrenia were compared with data from a control group utilized in a previous study. Statistical analyses were adjusted for age and sex. In order to correct for differences in determination methods between patients and controls concerning vitamin analyses, regression formula were used. The results on plasma folate levels are presented in Table 3.3, which shows showing a significant association between low plasma folate and schizophrenia. Remarkably, patients had significantly higher RBC folate levels compared to controls. Elevated RBC folate levels were associated with an increased risk for schizophrenia. The distributions of tHcy concentrations, vitamin B6 and vitamin B12 levels and homozygosity of the 677C>T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene were also determined and showed no significant differences between patients and control subjects.

Goff et al. (2004) observed significantly lower serum folate concentrations in a group of 91 schizophrenic outpatients than the published value of a control sample. When analysed by smoking behaviour folate levels of both smoking and non-smoking patients were lower than folate levels of control subjects. Remarkably, serum folate significantly correlated with severity of negative symptoms as recorded by the Scale for Assessment of Negative Symptoms (Andreasen, 1984), a relationship that was significant only in non-smokers. The mean tHcy concentration of the patient group did not differ from the control group value.

Discussion

Summary

As reviewed previously, most epidemiological studies on the topic of folate levels in psychiatric patients suffered from many methodological limitations (Young and Ghadirian 1989; Crellin et al.,1993; Skeritt, 1998). Especially the lack of well-defined cut-off values for folate deficiency with values for the normal range varying between assay techniques and laboratories stresses the importance of the implementation of a case-control design. We showed that none of the case-control studies included in this review on the topic of folate levels in schizophrenic patients explicitly reported on all critical factors in the assessment of this vitamin. Heterogeneity in distribution of confounders was not only observed between studies, but also between cases and controls within studies. For example, the two most recent studies on folate levels in schizophrenic patients used different assay methods for cases and controls (Muntjewerff et al., 2003; Goff et al., 2004), thereby possibly introducing confounding. Although the influence of each determinant and assessment factor on folate concentration is moderate, when accumulating systematically in either the group of patients or controls it could result in false positive or negative findings.

Table 3.3. Data of seven case-control studies on the association of serum or plasma folate levels and schizophrenia

Study	Requirements for blood sampling	Assay method	Cut-off point	% of Subjects with low folate		Statistical analyses of cases versus controls as reported in study
				Cases	Controls	
Carney (1967)	not reported	microbiological assay	10th percentile of control distribution	20	10	similar mean folate levels
Gundersen (1969)	fasting	microbiological assay	laboratory reference	38.1	29.7	similar mean folate levels, similar frequency of low folate subjects in both groups
Hermesh et al. (1988)	fasting	not reported	laboratory reference	6.7	3.3	similar mean folate levels , similar frequency of low folate subjects in both groups
Susser et al. (1998)	not reported	radioassay	bottom tertile for controls	35.3	33.3	no analyses on folate reported
Herrán et al. (1999)	fasting	radioimmunoassay	laboratory reference	0	0	mean folate level lower in cases (P=0.00)
Muntjewerff et al. (2003)	fasting	radioimmunoassay for cases, radioassay for controls	10th percentile of control distribution	37	10	similar mean folate levels, folate levels below cut-off point were associated with a 4-fold risk for schizophrenia [odds ratio=4.4 and 95% confidence interval: 1.5-12.6]
Goff et al. (2004)	non-fasting cases, fasting controls	immunoassay for patients, microbiological assay for controls	laboratory reference	16.4	1.7	mean folate level lower in cases (P=0.001)

Folate levels and nutritional status

In order to gain further insight in the association between folate levels and schizophrenia, it will be of utmost importance to standardize the measurement of folate. One crucial factor in this respect is determination of RBC folate in subjects. RBC folate correlates well with liver stores and reflects folate status in the previous 3 months and is therefore less dependent on the daily food intake. RBC folate has no known metabolic role and is thought to be a storage reservoir and long term buffer for maintaining folate homeostasis. It is used as a measure of folate status, which, unlike plasma levels, is not affected by recent dietary intake. Folate, together with vitamin B6 and vitamin B12, is essential for an adequate metabolism of homocysteine showing a reciprocal relationship between homocysteine levels and blood levels of these B-vitamins. In line with this, determination of plasma folate should also be accompanied with determination of tHcy concentration which is elevated in tissue deficiency of folate or vitamin B12.

Despite the methodological shortcomings three studies included in this review found lower plasma folate levels more frequently in patients with schizophrenia compared to controls (Carney, 1967; Muntjewerff et al., 2003; Goff et al., 2004). Low folate levels could be the resultant of poor nutritional status secondary to the psychiatric disease. Only a few studies reported on the dietary habits of psychiatric patients and folate levels in an attempt to clarify this relationship. Similar food intake patterns as examined by dietary rating scales were observed for patients with or without low serum folate (Thornton and Thornton, 1978), and similar folate levels were observed in patients whose diet was assessed as poor, moderate or good (Reynolds et al., 1970). Schizophrenic patients are in general, expected to be an undernourished group and as a consequence may show low vitamin levels including folate, vitamin B6 and vitamin B12. Although low plasma folate levels were reported for schizophrenic patients in the study by Muntjewerff et al. (2003) no deficiency of vitamin B6 or vitamin B12 was found, which is indicative of a normal nutritional intake regarding these B-vitamins. The different B-vitamins are frequently present in the same types of foods, thus the intake of one vitamin can be a marker for the intake of another vitamin (De Bree et al., 2001). Folate is especially highly correlated with vitamin B6 intake. Koren et al. (2002) observed a significant correlation between serum folate levels and folate intake by schizophrenic patients as determined from food diaries indicating the relevance of the latter.

Genes of folate metabolism

Folate deficiency could be related to the pathogenic process of schizophrenia by reflecting aberrant genes encoding for enzymes, transporters or receptors related to folate metabolism. Folate metabolism is complex, and subsequently more than 30 gene loci have been identified as folate-related (Johnson, 1999). Table 3.4 shows some of the folate-related

genes localized to a chromosomal region. The gene encoding for MTHFR is of special interest, because a common polymorphism in this gene has been identified in which a C to T substitution at nucleotide 677 converts an alanine to valine (Frosst, et al., 1995). Individuals who are homozygous for this mutation show reduced activity of MTHFR leading to a decreased availability of 5-methyltetrahydrofolate required for the remethylation of homocysteine and resulting in hyperhomocysteinemia. Thus far several studies have showed an association between elevated tHcy levels and schizophrenia (Regland et al., 1995; Susser et al., 1998; Levine et al., 2002; Applebaum et al., 2004). Although association between other enzymes such as methionine- adenosine transferase, serine-hydroxymethyltransferase (Smythies, 1997), or glutamate carboxypeptidase II (Goff et al., 2004) and schizophrenia have been proposed, solid evidence is still lacking. It can be hypothesized that genetic variants of folate metabolism in the individual *in utero* are interacting with environmental factors of the mother. Maternal nutritional deficiency such as low folate status during early pregnancy has been postulated as a risk factor for schizophrenia in the offspring. Indirect evidence for this hypothesis has been provided by epidemiological studies (Susser et al., 1996; Smits et al., 2004).

Table 3.4. Enzymes and gene loci related to folate metabolism[a]

Enzym	Location
Methylenetetrahydrofolate reductase	1p36.3
Methionine synthase	1q43
Dihydrofolate reductase	5q11.2-13.2
Folylpolyglutamate synthase	9cen-q34
Methionine-adenosyltransferase	10q22
Glutamate carboxypeptidase II	11p11.2
Folate receptor complex	11q13.3-q14.1
	11q13.3-q13.5
Serine-hydroxymethyltransferase, mitochondrion	12q13
5, 10-Methylenetetrahydrofolate dehydrogenase	14q24
Serine-hydroxymethyltransferase, cytoplasm	17p11.2
Thymidylate synthetase	18p11.31-p11.22
	18p11.32
S-adenosylhomocysteine hydrolase	20cen-q13.1
Phosphoribosylglycinamide formyltransferase	21q22.1
Reduced folate carrier-1	21q22.2-22.3
Cystathionine beta-synthase	21q22.3

[a]Location information available on Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/Omim/

Folate supplementation

Irrespective of the cause of folate deficiency or subnormal folate levels in schizophrenic patients, folate supplementation might contribute to symptom reduction in patients with schizophrenia. Thus far, only one double-blind, placebo-controlled add-on clinical trial with folate supplements in schizophrenic patients has been published (Godfrey et al., 1990). Supplementation of methylfolate improved clinical and social recovery in schizophrenic patients with low RBC folate at the entry to the trial. In a more recent double-blind controlled trial the efficacy was assessed of adjunctive megavitamin treatment in schizophrenic patients (Vaughan and McConaghy, 1999). Patients were treated with vitamin A, C and E and with a variety of B-vitamins including folate. The study provided no evidence supporting a positive relationship between regulation of vitamin levels in serum and clinical outcome in schizophrenic patients. Unfortunately, the study design showed several methodological pitfalls. Preliminary data from a randomized double-blind controlled crossover trial conducted in Israel showed significantly reduction of clinical symptoms of patients with schizophrenia when treated with a combination of B vitamins (folic acid, vitamin B6 and vitamin B12) (Levine et al., 2004). The patients included were all hyperhomocysteinemic showing tHcy concentrations above 15 µmol/L. Data on plasma or RBC folate levels were not provided in this study.

Conclusions

It was stated previously that low folate levels appeared to be common enough in the population of psychiatric patients to justify folate determinations routinely and that supplemental folate should be administered to all patients with low folate (Carney, 1967). Up to now evidence of an association between aberrant folate status and schizophrenia seems too meager to justify routine folate determination in schizophrenic patients. Further research on this topic is required to clarify the relationship between folate and schizophrenia, if any, and should avoid the methodological pitfalls mentioned in this review. In addition, research should also focus on polymorphisms of genes related to folate metabolism, both in patients and in their parents.

Chapter 4

Hyperhomocysteinemia, methylenetetrahydrofolate reductase 677TT genotype, and the risk for schizophrenia. A Dutch population based case-control study

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American Journal of Medical Genetics part B (Neuropsychiatric Genetics)
2005; 135: 69-72

Abstract

Objective: Evidence for an involvement of aberrant homocysteine metabolism in the aetiology of schizophrenia is limited and controversial. A case-control study was performed to quantify the risk of schizophrenia in the presence of elevated homocysteine concentrations or homozygosity for the 677C>T polymorphism (677TT) in the methylenetetrahydrofolate reductase (MTHFR) gene in subjects of Dutch ancestry.

Methods: We determined the 677C>T MTHFR genotype distribution in 254 well-defined patients and 414 healthy controls. Plasma total homocysteine (tHcy) concentrations were measured in 62 patients with schizophrenia and 432 control subjects.

Results: When tHcy concentrations were stratified into quartiles of the control distribution, we calculated an increased risk for schizophrenia in the fourth and third quartile versus the lowest quartile [odds ratio (OR)=3.3; 95% confidence interval (CI): 1.2-9.2, and OR=3.1; 95%CI: 1.2-8.0, respectively]. A significant dose-response relation of increasing tHcy levels and increasing risk for schizophrenia was observed (P=0.036). The 677TT genotype was associated with an OR of 1.6 [95% CI: 0.96-2.8] of having schizophrenia. Heterozygosity for the 677T allele compared to 677CC subjects accounted for an OR of 1.3 [95% CI: 0.91-1.8].

Conclusion: Elevated tHcy levels and the MTHFR 677TT genotype are associated with an increased risk for schizophrenia. These observations support a causal relation between disturbed homocysteine metabolism and schizophrenia.

Introduction

Elevation of plasma total homocysteine (tHcy) is positively associated with various disorders involving the central nervous system, such as neurodevelopmental disorders including neural tube defects (NTDs) (Botto and Yang, 2000), but also with neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Mattson and Shea, 2003). Evidence is accumulating that the aetiology of schizophrenia contains both neurodevelopmental and neurodegenerative components (Wong and Van Tol, 2003). In line with this, schizophrenia, a common and debilitating psychiatric disorder has also been associated with hyperhomocysteinemia (Regland et al., 1995; Susser et al., 1998; Levine et al., 2002; Applebaum et al., 2004), although not consistently (Virgos et al., 1999; Muntjewerff et al., 2003; Goff et al., 2004). One of the enzymes pivotal in homocysteine metabolism is methylenetetrahydrofolate reductase (MTHFR [MIM 236250]), which is involved in the folate-dependent remethylation of homocysteine to methionine. Homozygosity for the common 677C>T variant (677TT) in the MTHFR gene has been shown to be associated with higher levels of tHcy, especially under the conditions of low folate status (Frosst et al., 1995). The 677TT genotype has been reported to be associated with schizophrenia in some studies (Arinami et al., 1997; Joober et al., 2000; Sazci et al., 2003), but not in others (Kunugi et al., 1998; Virgos et al., 1999; Muntjewerff et al., 2003).

Thus far, only a few studies have explored the relation between elevated tHcy levels or the 677C>T polymorphism in the MTHFR gene and schizophrenia, showing contradictory results. We studied the MTHFR 677C>T genotypes in a large group of well-diagnosed patients with schizophrenia and in controls, all of Dutch ancestry. Furthermore, we studied the effect of the biochemical phenotype at the level of tHcy concentrations in a subset of the patients.

Methods

Subjects

A total of 269 patients with schizophrenia according to DSM-IV criteria were recruited from the psychiatric inpatients and outpatients clinics of the University Medical Centre Utrecht, Utrecht and GGz Nijmegen, Mental Health Institute, Nijmegen, The Netherlands. All patients were diagnosed by a well trained rater (M.L.C.H) using the Dutch translation of the Comprehensive Assessment of Symptoms and History (CASH) (Andreasen et al., 1992). Mutation analysis was performed in a group of 254 patients (mean age 41 ± 14 years; 72% male), and in a subgroup of 62 patients recruited in GGz Nijmegen (mean age 42 ± 10 years; 74% male) homocysteine levels were also determined. A small number of patients (n=10)

participating in our previous study (Muntjewerff et al., 2003) were included in the present study. The mean illness duration since the first psychotic episode was 18 years (SD: 13 years) for the total group.

The control group consisted of 432 subjects (mean age 51 ± 14 years; 43% male) who were recruited from a general practice in the Hague, The Netherlands, to take part in a health survey, as described elsewhere (den Heijer et al., 1995). Homocysteine levels were determined in all control subjects and mutation analysis was successfully performed in 414 subjects (mean age 51 ± 14 years; 43% male).

We obtained a short medical history of all patients by interview and screening of medical charts and of controls by questionnaire. Subjects with a physical illness were excluded. Medication and the use of vitamin supplements involved in homocysteine metabolism in the past three months prior to the homocysteine analysis were not allowed. All subjects were unrelated and of Dutch Caucasian descent. A written informed consent was obtained from all subjects, and the protocol of this study was approved by the medical ethics committee of the University Medical Centre Utrecht, and the local ethics committee of GGz Nijmegen.

Homocysteine and 677C>T MTHFR genotype determination

Blood samples for determination of tHcy were collected during the morning and in sitting position, and immediately placed on ice after drawing. Control subjects were fasting and patients had no requirements for fasting. EDTA blood was centrifuged at 3,500 x g for 5 minutes within 2 hours and subsequently plasma was separated and stored at –20ºC until analysis. Plasma total homocysteine concentrations were determined by HPLC with fluorescence detection (Te Poele-Pothoff et al., 1995). Genomic DNA was extracted from peripheral lymphocytes, and subsequently the 677C>T MTHFR genotypes were determined according to Frosst et al. (1995).

Statistical analysis

THcy concentrations were log-transformed prior to statistical analysis, because of a skewed distribution. Differences in tHcy levels between patients and controls were assessed using linear regression analysis and P values were adjusted for age and sex. Odds ratios (ORs) and 95% confidence intervals (95% CIs) as estimates of relative risk were calculated with logistic regression and only adjusted for age and sex for estimates on tHcy. Test for linear trend in the observed distribution of ORs was calculated using logistic regression and adjusted for age and sex. All P values reported are two-tailed, and statistical significance was accepted at $P<0.05$. All statistical analyses were performed with the SPSS 9.0 software package.

Results

Median tHcy value (range) was 11.2 (5.0-23.1) µmol/L for patients and 10.7 (2.0-43.4) µmol/L for controls, resulting in a statistical significant difference (P=0.037). Because homocysteine concentrations are age and sex dependent, subjects were stratified in subgroups according to sex and age groups (20-50 and 51-65 years) showing no significant differences between patients and control subjects (Table 4.1).

Table 4.1. Median plasma homocysteine (tHcy) and range (minimum-maximum) concentrations in µmol/L in schizophrenic patients and control subjects stratified by age and sex

Age (years)	Patients			Controls			P-value*
	N	tHcy	range	N	tHcy	range	
				Men			
20-50	35	11.7	5.0-23.1	93	11.0	2.8-43,4	0.437
51-65	11	12.3	9.5-18.6	93	12.2	4.9-27.3	0.816
All ages	46	11.7	5.0-23.1	186	11.7	2.8-43.4	0.817
				Women			
20-50	12	10.4	6.9-18.1	125	9.6	3.2-31.6	0.179
51-65	4	11.7	9.1-20.2	121	10.7	2.0-36.2	0.362
All ages	16	10.4	6.9-20.2	246	10.2	2.0-36.2	0.221

*P values calculated by linear regression analysis with log-transformed data.

Hyperhomocysteinemia was defined as the concentration which exceeds the 90th percentile of the control group (>15.5 µmol/L). Among patients, 9 out of 62 (14.5%) had tHcy concentrations above the cut-off point, versus 42 out of 432 (9.7%) control subjects. This yielded a crude OR of 1.6 (95% CI: 0.7-3.4), which increased to 1.9 (95% CI: 0.8-4.4) after adjustment for age and sex.

Table 4.2. Risk for schizophrenia for strata of homocysteine concentrations

Homocysteine concentrations	Patients (N=62)	Controls (N=432)	OR (95% CI)^{o}
1st quartile (<8.7 µmol/L)	7	109	1*
2nd quartile (8.7-10.7 µmol/L)	16	104	2.4 (0.9-6.1)
3^{td} quartile (10.7-12.8 µmol/L)	22	113	3.1 (1.2-8.0)$^{\#}$
4th quartile (>12.8 µmol/L)	17	106	3.3 (1.2-9.2)$^{\#}$

^{o}Age and sex adjusted odds ratio (OR) and 95% confidence interval (95%CI)
*Reference category, OR=1, $^{\#}$P<0.05

Table 4.2 shows the homocysteine measurements of both study groups stratified into quartiles as calculated from the control group, and ORs for the three highest levels each

compared with the lowest quartile. The largest increase of risk for schizophrenia was observed for an increase of tHcy concentration from the lowest (first) to the highest (fourth) quartile. The OR increased with increasing tHcy concentration, and reached significance (test for trend, P=0.036).

The effect of the MTHFR 677C>T genotypes on tHcy concentration was analysed in both study groups (Table 4.3). In de group of schizophrenic patients, both the 677TT and the 677CT genotypes were associated with significantly higher homocysteine levels than the 677CC genotype (both P<0.05). In the control group, differences in tHcy levels were found only between 677CC and 677TT genotypes (P<0.05). A significant difference in tHcy level between control subjects and schizophrenic patients after stratification by genotype was observed only for the 677CT genotype (P=0.006).

Table 4.3. Median plasma homocysteine (tHcy) and range (minimum-maximum) concentrations in µmol/L in schizophrenic patients and control subjects stratified by 677C>T MTHFR genotype

Genotypes	Patients			Controls			P-value*
	N	tHcy	range	N	tHcy	range	
677CC	30	10.5	5.0-18.6	212	10.7	3.2-19.4	0.495
677CT[1]	28	12.3	7.2-23.1	166	10.7	2.0-36.2	0.006
677TT[2]	4	12.5	9.1-18.1	36	11.4	3.4-43.4	0.680

*P values calculated by linear regression analysis with log-transformed data and adjusted for age and sex, [1]P<0.05 677CT vs. 677CC for patient group, [2]P<0.05 677TT vs. 677CC for patient and control group

The distribution of the 677C>T MTHFR genotypes among patients and controls, and calculated ORs for risk of schizophrenia are shown in Table 4.4.

Table 4.4. Frequencies of the MTHFR 677C>T genotypes among schizophrenic patients and controls, and odds ratios (ORs) as risk estimate for schizophrenia

Genotypes	Patients		Controls		OR (95% CI)*
	N	%	N	%	
677CC	112	44.1	212	51.2	1[#]
677CT	111	43.7	166	40.1	1.3 (0.91-1.8)
677TT	31	12.2	36	8.7	1.6 (0.96-2.8)

*Crude OR and 95% confidence interval (95% CI)
[#]Reference category, OR=1

When subjects homozygous or heterozygous for the 677T allele where combined, a crude OR of 1.3 (95% CI: 0.97-1.8) was calculated relative to the 677CC variant. When the genotype distribution was explored according to sex, an OR of 2.8 (95% CI: 1.2-6.5) was

calculated for the 677TT genotype compared to the 677CC genotype for female patients. Remarkably, no increased risk was estimated for the group of male patients (OR= 1.1 [95% CI: 0.6-2.3]).

Discussion

In this Dutch case-control study, we obtained a significant association of elevated tHcy levels and a higher risk for schizophrenia. The 677C>T polymorphism of the MTHFR gene is a genetic determinant of elevated tHcy levels. Individuals with the 677TT genotype had a 1.6 (95% CI: 0.96-2.8) higher risk for schizophrenia.

We calculated a significant increased risk for schizophrenia in the two highest quartiles of tHcy concentrations compared to the lowest quartile. This finding implies that there is no sharp cut-off point but rather a grading risk for schizophrenia in relation to increasing tHcy concentrations. The confidence intervals of the calculated ORs for the different tHcy levels were wide, due to the relative small sample size of the patient group. We could not replicate the findings of previous studies who reported markedly elevated tHcy levels in young male schizophrenic patients, but not older males or in females (Levine et al., 2002; Applebaum et al., 2004). Homocysteine levels in plasma are influenced by various factors such as genetic, dietary and lifestyle factors which can interfere with transsulfuration and remethylation or by pre-existing diseases associated with homocysteine metabolism (Refsum et al., 2004). The possibility of unadjusted confounding makes it difficult to be sure that the relation between schizophrenia and homocysteine is causal. Given that the tHcy concentration is a graded risk factor for schizophrenia, the observation that the 677C>T variant is associated with increased tHcy concentrations leads to the prediction that this variant is also associated with increased risk of schizophrenia. The distribution of confounding, such as lifestyle factors, is expected to be similar among the three different genotypes. In our study, tHcy levels were similar for patients and control subjects, when stratified by genotype. In case of serious confounding, tHcy levels should have been significantly higher for patients when compared to controls in all three genotype strata. However, the effect of the polymorphism on tHcy levels may differ according to folate status not only in 677TT but also in 677CT individuals. In accordance with the study performed by Virgos et al. (1999), we found a relationship between 677TT or 677CT genotype and elevated tHcy in patients when compared to tHcy levels of patients with the 677CC genotype. In addition, we were also able to demonstrate a tendency towards an increased risk for the heterozygous 677C>T genotype. Recently, several studies have suggested the association between the 677CT genotype and elevated tHcy levels relative to subjects with the 677CC genotype (Kluijtmans and Whitehead, 2001). This finding might also be explained by the 1298A>C polymorphism in the MTHFR gene.

Combined heterozygosity for the 1298A>C and 677C>T MTHFR polymorphism is associated with reduced MTHFR activity and elevated tHcy levels (Botto and Yang, 2000), and is reported to be a genetic risk factor for schizophrenia (Sazci et al., 2003).

If homocysteine or a disturbed homocysteine metabolism are causal factors in the aetiology of schizophrenia, individuals with the 677TT genotype should be at higher risk of schizophrenia than individuals with the 677CC genotype. Our findings give support to this hypothesis, and is consistent with several studies (Arinami et al., 1997; Joober et al., 2000; Sazci et al., 2003). Our previous study on this subject showed no increased frequency for the 677TT genotype in schizophrenic patients compared to controls (Muntjewerff et al., 2003), probably due to the much smaller sample size. The conclusion seems justified that very large numbers of subjects might be required to generate conclusive evidence for an association between the 677TT genotype and schizophrenia in which the disease risk is moderately associated with the genotype. Our finding of a preponderance of the 677TT genotype in female schizophrenic patients contradicts the results of the study by Joober et al. (2000), who reported an increased frequency of this genotype in male patients compared to controls and only a trend in female patients. These findings need replication before a valid conclusion can be drawn about gender specific prevalence of the 677T allele in relation to schizophrenia.

The pathophysiological mechanism by which homocysteine may be associated to schizophrenia remains obscure. Several *in vitro* studies support the view of direct adverse effects of homocysteine on neuronal cells (Mattson and Shea, 2003). Alternatively, hyperhomocysteinemia might reflect impaired folate transport and subsequently aberrant methylation. Reduced methylation in schizophrenic patients, compared to healthy controls, has been reported in several studies (Antun and Kurkjian, 1982; Sargent et al., 1992; Sharma et al., 1999). The 677C>T polymorphism is also associated with a differential distribution of folate derivatives in the red blood cell (Bagley and Selhub, 1998) and probably other cell types, diminishing folate availability. Data from experimental animal models indicate that insufficient folate metabolism might result in aberrant development of the embryo (Rosenquist and Finnell, 2001). From neurodevelopmental perspective, an aberrant homocysteine-folate metabolism due to reduced MTHFR activity, could be present in mother or unborn child, and therefore might act prenatally. This combined effect of the 677C>T MTHFR genotype of the mother with that of her child reported for NTDs (Botto and Yang, 2000) could also be relevant for the aetiology of schizophrenia. Therefore, future studies should include mothers of schizophrenic patients.

We conclude that elevated tHcy levels and homozygosity for the common 677C>T polymorphism in the MTHFR gene are associated with an increased risk for schizophrenia. The phenotypic expression of the MTHFR genotype varies with folate status indicating that

folate supplementation might have clinical relevance. Strong evidence for the involvement of this metabolism in the aetiology of schizophrenia would be provided by clinical trials of periconceptional folate administration in women showing a reduced risk for this disease in their offspring. Additional evidence could be provided by folate supplementation in schizophrenic patients resulting in symptom reduction, parallel to reduced homocysteine levels.

Chapter 5

Homocysteine, methylenetetrahydrofolate reductase and risk of schizophrenia: a meta-analysis

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Molecular Psychiatry 2006; 11: 143-149

Abstract

Objective: Elevated plasma total homocysteine (tHcy) concentration has been suggested as a risk factor for schizophrenia, but the results of epidemiological studies have been inconsistent. The most extensively studied genetic variant in the homocysteine metabolism is the 677C>T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene, resulting in reduced enzyme activity and, subsequently, in elevated tHcy.

Methods: A meta-analysis of eight retrospective studies (812 cases and 2113 control subjects) was carried out to examine the association between homocysteine and schizophrenia. In addition, a meta-analysis of ten studies (2265 cases and 2721 control subjects) on the homozygous (677TT) genotype of the MTHFR 677C>T polymorphism was carried out to assess if this association is causal.

Results: A 5 µmol/L higher tHcy level was associated with a 70% (95% CI: 27-129) higher risk of schizophrenia. The 677TT genotype was associated with a 36% (95% CI: 7-72) higher risk of schizophrenia compared to the 677CC genotype. The performed meta-analyses showed no evidence of publication bias or excessive influence attributable to any given study.

Conclusion: Our study provides evidence for an association of homocysteine with schizophrenia. The elevated risk of schizophrenia associated with the homozygous genotype of the MTHFR 677C>T polymorphism provides support for causality between a disturbed homocysteine metabolism and risk of schizophrenia.

Introduction

It is commonly accepted that both genetic and environmental factors contribute to the aetiology and clinical phenotype of schizophrenia. Despite numerous family, twin, and adoption studies in which evidence for hereditary and environmental contributions to schizophrenia is presented (Gottesman, 1994; Portin and Alanen, 1997; Cannon et al., 1998; Kety, 1998), no underlying inherited mechanism has yet been identified. Although several putative susceptibility genes for schizophrenia have been identified (Harrison and Weinberger, 2005), the pathogenic mechanisms remain to be resolved. Evidence of an association between schizophrenia and a specific aberrant metabolism could reveal new candidate genes and might provide more insight in the complex aetiology of schizophrenia.

Dysfunctional single-carbon transfer reactions involving the methionine-homocysteine metabolism have been proposed to be relevant in the aetiology of schizophrenia. Evolving from the original transmethylation hypothesis postulating that an abnormally (toxic) methylated metabolite might be the cause of schizophrenia (Osmond and Smythies, 1952), it was suggested subsequently that disturbances in the single-carbon metabolism itself might be causative (Smythies, 1963). Methylation processes are vital for normal cell functioning because of their key role in protein, lipid, and DNA metabolism, gene expression, synthesis of neurotransmitters and detoxification processes (Scott and Weir, 1998). Initial clinical evidence for an impaired methyl-carbon pathway in schizophrenic patients stems from many methionine administration studies ranging from the 1960s until the early 1990s. These studies showed that treatment with high-daily doses of L-methionine resulted in exacerbation of schizophrenic symptoms (Pollin et al., 1961; Cohen et al., 1974). In addition, as compared to controls the rate of whole body methylation was found to be decreased in patients with schizophrenia (Antun and Kurkjian, 1982; Sargent et al., 1992). It was suggested that these findings might reflect an enzymatic defect or a failure of a control mechanism of the single-carbon cycle in schizophrenic patients (Smythies et al., 1997). The finding of a defect in methylation due to severe dysfunction of 5, 10-methylenetetrahydrofolate reductase (MTHFR) resulting in hyperhomocysteinemia in a subject with schizophrenia-like symptoms (Freeman et al., 1975) was therefore of great interest. Homocysteine is a sulphur containing amino acid derived from the demethylation of the essential amino acid methionine, which is the only dietary precursor of homocysteine. The metabolism of homocysteine depends on several B-vitamins including folate, cobalamin, pyridoxine and riboflavin. MTHFR is the essential enzyme in the folate-mediated single-carbon transfer reactions. A common polymorphism exists in the catalytic domain of the MTHFR enzyme. The gene is localized on chromosome 1p36.3 (Goyette et al., 1994), in which a C>T substitution at position 677 results in a substitution of alanine to valine (Frosst et al., 1995). The reported prevalence of

the homozygous 677C>T (677TT) genotype is between 0 and 32% of the population world-wide (Wilcken et al., 2003), ranging from 5 to 15% in Caucasian populations (Brattstrom et al., 1998). The single amino acid substitution results in impaired flavin adenine dinucleotide (FAD) binding, leading to loss of folate resulting, in its turn, in reduced activity of MTHFR (Guenther et al., 1999). Subjects with the 677TT genotype have about 25% higher plasma total homocysteine (tHcy) levels than those with the normal homozygous (677CC) genotype (Brattstrom et al., 1998), whereby the impact of the polymorphism varies according to folate or riboflavin status (Hustad et al., 2000; Van der Put et al., 1995).

Elucidation of an association between a genetic variant associated with elevated tHcy levels and schizophrenia might be informative about the hypothesis that higher levels of tHcy play a causal role in the aetiology of schizophrenia. Individual case-control studies concerning homocysteine or the 677C>T polymorphism included too few cases and controls to produce reliable evidence for an association with schizophrenia.

The aim of this study was to examine the association between homocysteine and schizophrenia by conducting a meta-analysis of case-control studies on this topic. In order to assess whether such association is causal, we have also performed a meta-analysis of all case-control observational studies with available data on the MTHFR 677C>T polymorphism and schizophrenia.

Method

Study identification

Eligible studies were identified by searching the electronic NLM MEDLINE data-base for relevant reports published up to and including December 2004 using the search terms (“homocysteine” or “hyperhomocysteinemia” or “MTHFR”) and (“schizophrenia”). Additionally, the reference lists of original articles on this topic were scanned to identify articles missed by the computerized search.

Study selection

Studies examining total serum or plasma total homocysteine (tHcy) levels or the 677C>T polymorphism in the MTHFR gene were included in the meta-analysis. The following additional criteria were used to select studies for data extraction: (a) a diagnosis of schizophrenia for cases, (b) use of a case-control design, and (c) publication in an English-language, peer-reviewed, indexed scientific journal. Meeting abstracts were not allowed. We also included our recent published data (Muntjewerff et al., 2005).

Data extraction and synthesis

For the meta-analysis of homocysteine and schizophrenia, information was extracted from each study on: the odds ratio (OR) and 95% confidence interval (95% CI) as a risk estimate for the association between elevated homocysteine and schizophrenia and the number of cases and controls. If the OR and 95% CI were not provided, they were calculated from the number of cases and controls above and below a particular homocysteine concentration as a cut-off point from the control distribution in each study using Woolf's method (Woolf, 1955). The homocysteine studies included in our meta-analysis reported different definitions of hyperhomocysteinemia by using different cut-off levels. Most studies provided the 90th percentile of tHcy levels in the control group, while other studies reported the 95th or 75th percentile. For pooling of risk estimates, it is essential that these estimates are based on a single unit of comparison. Previous studies on homocysteine and homocysteine-related diseases reported linear dose-effects relations (Boushey et al., 1995; Wald et al., 2002; den Heijer et al., 2005). In line with these findings, we calculated the OR for a 5 µmol/L increase in tHcy level, a standard reference increment. To estimate the log OR for a 5 µmol/L increase in tHcy, information about the normal distribution was used to calculate the expected mean level of tHcy in the groups being compared. For example, the mean value in the top fifth of a normal distribution is 1.4 standard deviation (SD) above the mean, and the mean in the bottom fifth is 1.4 SD below the mean. Hence, the OR comparing the top to the bottom fifth is also the OR for a 2.8 SD difference in tHcy levels. Wherever possible, the SD in controls was used to estimate the difference in µmol/L between the top and bottom fifth of a study, but if this was not reported, then a weighted average was used of the SD from the studies that did report it. Assuming the association was log-linear, it was then possible to calculate the OR for a unit change in tHcy and hence for a 5 µmol/L increase in tHcy.

For the meta-analysis of the MTHFR 677C>T polymorphism and schizophrenia, data were collected on the frequency of 677CC, 677CT and 677TT genotypes in cases and controls or calculated from the allele frequencies. Subjects with the 677TT genotype have approximately 2.7 µmol/L (25%) higher tHcy levels compared to subjects with the 677CC genotype, while the mean difference of tHcy concentration between the 677CT and 677CC genotype is only about 0.29 µmol/L (Wald et al., 2002). Therefore, the 677TT genotype was assigned as the risk genotype, because of the relatively strong contrast in tHcy level between the 677TT and 677CC genotype. The study-specific ORs and 95% CIs for 677TT compared with 677CC genotype were estimated using Woolf's method (Woolf, 1955).

By combining the pooled risk estimate of the association between the 677TT genotype and schizophrenia (OR) with the published estimate of a mean difference in tHcy between 677TT and 677CC genotype (Δhcy), we derived an equivalent risk estimate for a 5 µmol/L increase in tHcy concentration of $OR^{5/\Delta hcy}$ (raising OR to the power of 5/Δhcy). Subsequently,

we compared the derived and measured risk estimates.

Random effects model meta-analyses were performed according to the methods of DerSimonian and Laird (DerSimonian and Laird, 1986), and 95% CIs were constructed by using Woolf's method (Woolf, 1955). The significance of the pooled ORs was determined by the z-test. Heterogeneity (greater variation among study results than would be expected through change) was assessed using standard chi-squared tests. The influence of individual studies on the pooled ORs was determined by sequentially removing each study and recalculating the pooled OR and 95% CI. The individual studies were ordered by the inverse of the standard error (SE) of the risk estimate to evaluate publication bias (Egger et al., 1997). A symmetrical inverted funnel implies absence of significant selection or publication bias. In addition, publication bias was tested by Egger's test (Egger et al., 1997). If necessary, data were completed by personal communication with the original authors. The type I error rate was set at 0.05. All statistical analyses were conducted by using Stata 8.0.

Results

Homocysteine and schizophrenia

Data for the meta-analysis were obtained from eight published case-control studies (Regland et al., 1995; Susser et al., 1998; Virgos et al., 1999; Levine et al., 2002; Muntjewerff et al., 2003; Applebaum et al., 2004; Goff et al., 2004; Muntjewerff et al., 2005) with a total number of 812 cases and 2113 control subjects. Figure 5.1 shows the ORs and 95% CIs of schizophrenia associated with a 5 µmol/L increase in measured tHcy, separately for individual studies and for all studies when taken together. A 5 µmol/L increase in measured tHcy was associated with an overall 70% (95% CI: 27-129) increased risk of schizophrenia. Sequential removing and replacement of individual studies from the calculation of the pooled OR produced risk estimates ranging from 1.58 to 1.94 with 95% CIs that always encompassed 1.0. This finding indicates that the significance of the pooled OR was not excessively influenced by any single study. There was significant heterogeneity between the results of the studies included (X^2=17.949; df=7; P=0.012), suggesting the presence of some moderating variable. Several characteristics of the included homocysteine studies are presented in Table 5.1, showing heterogeneity of exclusion criteria and of age and sex distributions.

In Figure 5.1 the distribution of the ORs of the individual homocysteine studies are ordered by the inverse of the SE of the risk estimate. It indicates that much of the evidence for an association of homocysteine and schizophrenia comes from two larger studies which deviate significantly from an OR of 1.0 (Levine et al., 2002; Applebaum et al., 2004). The shape of the funnel also suggests that a few smaller studies finding an inverse association

may not have been published. However, the effect of these studies on the overall risk estimate is likely to be small. In addition, Egger's regression asymmetry test showed no evidence of publication bias (a= -0.9; t= -0.73; P=0.49).

Table 5.1. Descriptive characteristics of eight case-control studies on the association of schizophrenia and homocysteine

Study	Study area	Percentage of males	Mean age (years)	Diagnostic Instrument	Exclusion criteria
		Cases/controls	Cases/controls		
Regland et al. (1995)	Sweden	55/55	32/32	DSM-III-R	not reported
Susser et al. (1998)	USA	not reported	not reported	DSM-III-R	substance abuse
Virgos et al. (1999)	Spain	67/89	58/58	ICD-9	cardiovascular disease
Levine et al. (2002)	Israel	78/64	not reported	DSM-IV	cardiovascular disease and substance abuse
Muntjewerff et al. (2003)	NL*	77/29	39/36	DSM-IV	neural tube defects, liver and renal dysfunction, use of folate antagonists or vitamin supplements
Applebaum et al. (2004)	Israel	69/50	not reported	DSM-IV	cardiovascular, renal and endocrinological diseases, and substance abuse
Goff et al. (2004)	USA	70/55	43/57	not reported	renal dysfunction, alcohol abuse, and use of drugs affecting folate or homocysteine levels
Muntjewerff et al. (2005)	NL*	76/43	42/51	DSM-IV	physical illness, relevant homocysteine levels modulating drugs, and use of vitamin supplements

* The Netherlands

MTHFR and schizophrenia

A total of nine published articles (Arinami et al., 1997; Virgos et al., 1999; Joober et al., 2000; Kunugi et al., 1998; Muntjewerff et al., 2003; Sazci et al., 2003; Tan et al., 2004; Yu et al., 2004; Muntjewerff et al., 2005) reported on the relationship between schizophrenia and

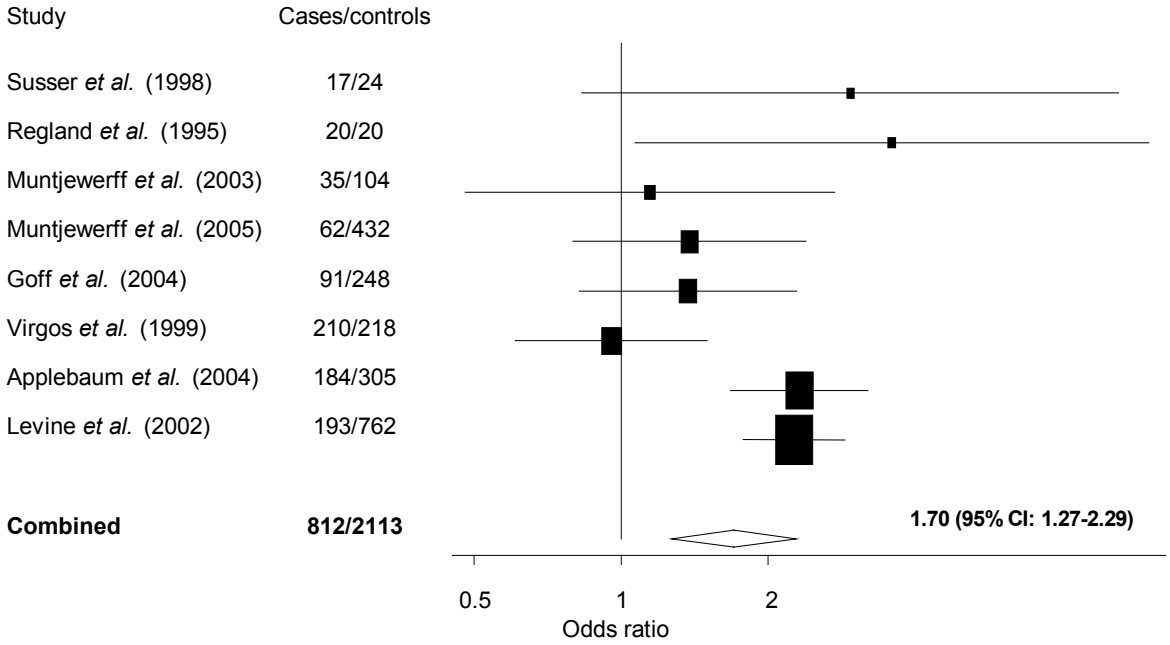

Figure 5.1. Odds ratios (ORs) and 95% confidence intervals (95% CIs) of schizophrenia for a 5 µmol/L increase in plasma total homocysteine concentration for each study separately and combined. Area of each individual study as presented from top to bottom of figure: USA, Sweden, The Netherlands, The Netherlands, USA, Spain, Israel, and Israel. (Studies are ordered by the inverse of the SE of each estimate. The size of the data markers is inversely proportional to the SE. The horizontal lines represent the 95% CIs. The combined OR and 95% CI is indicated by the diamond).

the MTHFR 677C>T polymorphism. One article provided data on two separate studies (Yu et al., 2004). Thus, data were obtained from ten studies involving 2265 cases and 2721 control subjects. All cases were diagnosed according to standard diagnostic systems (DSM-III, DSM-V or ICD-9). All studies used a standardized method to determine the MTHFR 677C>T genotypes. Figure 5.2 shows the results of individual and pooled studies for the 677TT genotype. Overall, the 677TT genotype was associated with a 36% (95% CI: 7-72) increased risk of schizophrenia compared with the 677CC genotype. Sequential removing and replacement of individual studies from the calculation of the pooled OR produced risk estimates ranging from 1.26 to 1.43 with 95% CIs that always encompassed 1.0. This finding indicates that the significance of the pooled OR was not excessively influenced by any single study.

There was no significant heterogeneity among the results of individual studies (X^2=13.973; df=9; P=0.123). The absence of asymmetry in the distribution of the ORs of the individual studies as shown in Figure 5.2, and the outcome of the Egger regression asymmetry test (a= -0.06; t= -0.04; P=0.97) reduce the likelihood of publication bias.

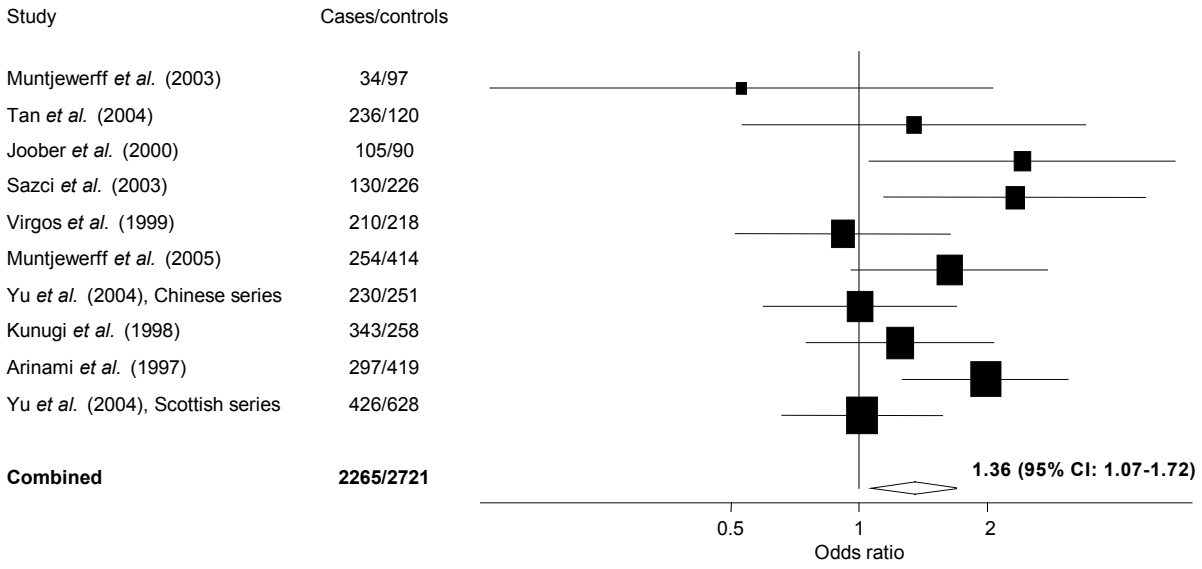

Figure 5.2. Odds ratios (ORs) and 95% confidence intervals (95% CIs) of schizophrenia for 677TT versus 677CC genotype of the MTHFR 677C>T polymorphism of each study separately and combined. Area of each individual study as presented from top to bottom in figure: The Netherlands, Singapore, Canada, Turkey, Spain, The Netherlands, China, Japan, Japan, and Scotland. (Studies are ordered by the inverse of the SE of each estimate. The size of the data markers is inversely proportional to the SE. The horizontal lines represent the 95% CIs. The combined OR and 95% CI is indicated by the diamond).

Homocysteine and MTHFR

Based on the published estimate from a large meta-analysis on homocysteine and MTHFR studies (Wald et al., 2002), we assumed that the average tHcy concentration is 2.7 µmol/L higher in individuals with the 677TT genotype than in subjects with the 677CC genotype. In line with this, the pooled OR of 1.36 (95% CI: 1.07-1.72) for 677TT versus 677CC genotype is equivalent to an OR of 1.77 (95% CI: 1.13-2.73) for the standard 5 µmol/L increase in tHcy concentration (raising 1.36 to the power of 5/2.7). This risk estimate is similar to the observed OR of 1.70 in our meta-analysis.

Discussion

The overall result of the present meta-analysis demonstrates that for each 5 µmol/L increase in tHcy concentration the relative risk of schizophrenia is roughly doubled (OR=1.70 [95% CI: 1.27-2.29]). This finding must be interpreted with some caution because of the observed significant heterogeneity among the homocysteine studies in this meta-analysis. However, the association of homozygosity of the 677C>T polymorphism in the MTHFR gene and schizophrenia with an OR of 1.36 (95% CI: 1.07-1.72) is suggestive for a

causal relation between aberrant homocysteine metabolism and schizophrenia.

A limitation of using a meta-analytic approach for population-based observational studies is the fact that these studies may yield estimates of associations that are influenced by confounding due to age, sex or ethnic admixture (population stratification) either between studies or between cases and controls within each study (Munafò and Flint, 2004). In this meta-analysis, only the unadjusted ORs could be calculated for each study because the adjusted ORs were not routinely provided. There might also be confounding because of the clinical heterogeneity of the patients included. Most studies were restricted to schizophrenia patients, but some studies also included cases with schizophreniform or schizoaffective disorders. It is unlikely that this has been an important moderating variable, because of the low number of included patients with these diagnoses.

Plasma total homocysteine concentrations reflect genetic and environmental factors such as diet, B-vitamin intake, and lifestyle factors like coffee consumption and smoking (Refsum et al., 2004). None of the studies on tHcy levels and risk of schizophrenia explicitly reported on all critical factors in the assessment of tHcy. The distribution of these confounders may have differed among studies and by introducing systematic error it might explain the observed heterogeneity among studies. In order to gain further insight in the association between homocysteine and schizophrenia, it will be of utmost importance to standardize the measurement of tHcy. The most obvious factor that contributes to heterogeneity of tHcy levels is the folate and riboflavin status of the study population (Refsum et al., 2004). In a meta-analysis on MTHFR and venous thrombosis a clear difference was seen between European studies and North-American studies (den Heijer et al., 2005), which could be explained by the implementation of folate enrichment of foods in North-America since 1997. In our meta-analysis, no risk estimates by continent were calculated, because of the low number of included North-American studies.

Studies of genetic variants that affect tHcy levels would reflect long-term exposure to elevated homocysteine, and be independent of confounding and concerns about reverse causality (Davey Smith and Ebrahim, 2003). Apart from the effect modification by dietary intake of folate and riboflavin, it is unlikely that the effects of these genotypes on schizophrenia will be influenced by lifestyle or other confounders. Genetic confounding by linkage disequilibrium is possible, whereby a gene linked to the MTHFR gene controls an unknown risk factor for schizophrenia and also increases the tHcy level. Nevertheless, no such linkage has been found thus far. The studies included in this meta-analysis involved less than a few hundred cases for each study, and so the CIs around the individual ORs were wide. The present study provides more reliable evidence as to the importance of the 677TT genotype for the risk of schizophrenia. The concordance of the risk estimates from homocysteine studies and MTHFR 677C>T polymorphism studies concerning schizophrenia

supports the hypothesis that the association of elevated tHcy levels with this disease is in part causal.

Hyperhomocysteinemia is not specific for schizophrenia, and has been reported as a risk factor for various diseases such as cardiovascular diseases (Wald et al., 2002), neural tube defects (Botto and Yang, 2000), and neuropsychiatric disorders including Alzheimer's disease (Clarke et al., 1998), Parkinson's disease (Allain et al., 1995), and depression (Fava et al., 1997; Bottiglieri et al., 2000; Bjelland et al., 2003), stressing the pivotal role of the single-carbon mechanism. The pathogenic mechanisms underlying these moderate hyperhomocysteinemic diseases are not yet fully understood. Interestingly, accumulating evidence from *in vitro* and animal studies suggests that the nervous system (neuronal homeostasis) is, in particular, sensitive to folate deprivation and raised homocysteine levels (Lipton et al., 1997; Mattson and Shea, 2003). Extra-cellular homocysteine has been found to be toxic to cultured neurons and neuron cells via stimulation of NMDA receptors, enhancing oxidative stress and inducing DNA damage eventually resulting in apoptosis and adverse effects on synaptic and glial function (Lipton et al., 1997; Kruman et al., 2000; Ho et al., 2002). Recently, experiments with mice have shown that these mechanisms are not only important in cultured embryonic pre-mitotic neurons, but in the adult post-mitotic neurons as well (Kruman et al., 2002). The deleterious effect of MTHFR dysfunction on brain development was observed in individuals with a severe deficiency of this enzyme, resulting in delayed psychomotor development, mental retardation and psychiatric symptoms (Rosenblatt, 1995).

The 70% higher risk of schizophrenia for a 5 µmol/L increase in tHcy observed in our meta-analysis suggests a dose-response relationship. This finding provides a rational of using tHcy levels as a biological parameter for intervention studies. A meta-analysis of short-term trials comparing the effects on tHcy levels of different doses of B-vitamins has shown that a daily dose between 0.5 to 5 mg of folic acid was associated with a 25% lower tHcy concentration and that vitamin B12 had an additive effect of about 7% (Homocysteine Lowering Trialist's Collaboration, 1998). This decrease in tHcy of 25% associated with folic acid administration is comparable to the difference in tHcy levels between individuals with the 677TT and 677CC genotype (Brattstrom et al., 1998; Wald et al., 2002). Folate supplementation might therefore contribute to symptom reduction in patients with schizophrenia, as a result of lowering homocysteine levels. Thus far, only one double-blind, placebo-controlled clinical trial with folate supplements in schizophrenic patients has been published (Godfrey et al., 1990). Supplementation of folate improved clinical and social recovery in schizophrenia patients with low red blood cell folate at the start of the trial. Periconceptional folic acid supplementation substantially reduces the risk of neural tube defects (MRC Vitamin Study Research Group, 1991) possibly by protecting the developing

embryo against one or more folate-related metabolic disorders. Evidence is accumulating that an aberrant neurodevelopmental process, starting *in utero* might also be present in the pathogenic pathway of schizophrenia (McGrath et al., 2003). Our finding of the 677TT genotype as a risk factor for schizophrenia supports the view that homocysteine is causally related to this disorder. One can speculate if periconceptional folic acid supplementation reduces the risk of the development of schizophrenia if a disturbance in folate-dependent homocysteine metabolism is present, either in the mother or her child.

The present meta-analysis provides evidence that elevated tHcy levels and the 677TT genotype of the MTHFR 677C>T polymorphism, contribute to the susceptibility of schizophrenia. Trials of folic acid and vitamin B12 supplementation for schizophrenia patients are warranted. Such clinical trials need to include a sufficiently large number of participants to have adequate power to detect improvement in schizophrenic patients. Future research should focus on genetic factors and metabolites associated with the homocysteine pathway in mother and child, because of the possible impact of this metabolism on intra-uterine brain development.

Acknowledgements

Martin den Heijer is recipient of a VENI-grant from the Netherlands Foundation of Scientific Research.

Chapter 6

Maternal and fetal effect of the methylenetetrahydrofolate reductase 677C>T polymorphism on schizophrenia risk

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Submitted for publication

Abstract

Objective: The MTHFR 677C>T polymorphism has been associated with increased risk of schizophrenia in various case-control studies. However, case-control studies are sensitive to population stratification, which is not an issue in family-based studies.

Methods: We conducted a family-based study in 120 families with a schizophrenic family member to explore the association between the parental MTHFR 677C>T polymorphism and schizophrenia risk in offspring.

Results: Transmission Disequilibrium Test (TDT) analysis showed no preferential transmission of the 677T allele from parents heterozygous for the MTHFR 677C>T polymorphism to schizophrenia offspring (P=0.27). The genotype relative risks were 1.43 (95% CI: 0.83-2.47) for the 677TT and 1.42 (95% CI: 0.54-3.78) for the 677CT genotype, relative to the 677CC genotype. A meta-analysis using data from family-based studies yielded no evidence implicating the 677T allele in schizophrenia risk (P=0.58). By applying a log-linear model, we found no evidence that the maternal MTHFR genotype influences the risk of schizophrenia offspring.

Conclusion: Our data provide no evidence that transmission of the MTHFR 677T allele is associated with schizophrenia risk.

Introduction

Schizophrenia is a disabling mental health disorder with a world-wide life-time prevalence of approximately 1.4-4.6 per 1000 population (Jablensky, 2000). Evidence is accumulating that both genetic and environmental factors play a role in the aetiology of schizophrenia, possibly starting during fetal development (Tsuang, 2000). Aberrant homocysteine metabolism has been linked to neurodevelopmental disorders, such as neural tube defects (Van der Put et al., 1995; Blom et al., 2006), Down-syndrome (Gueant et al., 2003; Da Silva et al., 2005), and schizophrenia (Regland et al., 1995; Muntjewerff et al., 2006).

Homocysteine is a sulphur containing amino acid derived from the demethylation of the essential amino acid methionine, which is the only precursor of homocysteine. Plasma total homocysteine (tHcy) concentrations are influenced by various genes and environmental factors, such as dietary intake of folate or vitamin B12 (Refsum et al., 2004). Methylenetetrahydrofolate reductase (MTHFR) is an essential enzyme in the folate-mediated single-carbon transfer reactions of the homocysteine remethylation (Mudd et al., 2001). Homozygosity for the 677C>T mutation (677TT) in the gene coding for MTHFR causes an alanine-to-valine substitution, and results in reduced enzyme activity (Frosst et al., 1995). The MTHFR 677C>T polymorphism is a well-established and the most common inherited cause of elevated homocysteine. Subjects with the 677TT genotype have mildly increased tHcy levels (Brattstrom et al., 1998; Van der Put et al., 1995), whereby the impact of the polymorphism varies according to folate or riboflavin status (Hustad et al., 2000; Van der Put et al., 1995). Folate is a single-carbon donor important for numerous cellular reactions, such as the synthesis of nucleotides, and methylation of DNA, proteins, lipids, and neurotransmitters, and homocysteine (Mudd et al., 2001). In addition, homocysteine itself has been shown to be toxic for neurons (Mattson and Shea, 2003) and endothelial cells (Jacobsen, 2001).

The clinical relevance of the MTHFR 677C>T polymorphism on neurodevelopment was first shown in studies on neural tube defects (NTDs). MTHFR 677C>T was the first identified genetic risk factor of NTDs, showing that not only a genetic defect of the child, but of the mother as well contributed to an increased risk (Van der Put et al., 1995; Whitehead et al., 1995; Botto and Yang, 2000). Upon this genetic predisposition, the risk of NTDs appeared to be greatest when maternal folate levels were low (Molloy et al., 1997). It has been demonstrated convincingly that supplementation of folate during the periconceptional period reduces the risk of having offspring with a NTD (Czeizel, 2000; Czeizel and Dudas, 1992; MRC Vitamin Study Research Group, 1991).

Homozygosity for the MTHFR 677C>T genotype has been associated with an increased risk of schizophrenia in some association studies, but not all (Muntjewerff et al., 2006).

Population stratification, such as ethnic or gender differences of genotype distribution and admixture may account for these conflicting findings. Within-family gene distribution analysis avoids confounding by population stratification (Spielman et al., 1993; Cordell and Clayton, 2005). Thus far, only two family-based studies reported about schizophrenia and MTHFR 677C>T polymorphism, showing equal transmission of 677C and 677T allele from heterozygous parents to offspring in one study (Yu et al., 2004), and preferential transmission of the 677C allele in the other (Wei and Hemmings, 1999). With respect to offspring, maternal genetic effects may behave as environmental risk factors, determining fetal development. Analogous to NTDs, investigations into the genetic component of schizophrenia require analysis of the maternal genotype as well as that of the patient.

In the present study, we examined the prevalence of the MTHFR 677C>T polymorphism in families with schizophrenia offspring, and its impact on schizophrenia risk. We also studied the maternal genotype effect of the MTHFR gene on the risk for schizophrenia.

Methods

Subjects

We enrolled 120 families with schizophrenia offspring recruited from University Medical Centre Utrecht, Utrecht, The Netherlands. Subjects with schizophrenia were diagnosed according to the criteria of DSM-IV (American Psychiatric Association, 1994), and determined by using the Comprehensive Assessment of Symptoms and History (CASH) (Andreasen et al., 1992). All included subjects were of European origin. The population of families consisted of 93 complete triads, 21 mother-child dyads, and 6 father-child dyads. All participants gave their informed consent.

Genetic analysis

Genomic DNA was extracted from lymphocytes of peripheral blood samples. The allelic MTHFR 677C>T polymorphism was assessed with a polymerase chain reaction-based method and digestion with the restriction enzyme *Hin*fI (Frosst et al., 1995). MTHFR genotyping was performed blind to diagnosis, sex or pedigree relationship.

Statistical analysis

The family-based transmission disequilibrium test (TDT) was used to assess the relationship between schizophrenia risk and transmission of the MTHFR 677T allele. In TDT, a significant deviation from random transmission of alleles from heterozygous parents to their offspring can be interpreted as evidence for linkage ànd association (Spielman et al., 1993). The nontransmitted alleles of the heterozygous parents serve as internal controls. For the

TDT only data from complete triads were used. A two-by-two table was constructed in which parental alleles were classified by type (677C or 677T) and transmission status (transmitted or nontransmitted). In addition, a genotype relative risk and its standard-error-based 95% confidence interval (95% CI) was estimated for the 677CT and 677TT genotype (STATA 9 software) (Stata Corporation, College Station, Texas, USA). The data of the present study were also used in a random effect meta-analysis of all available TDT data. We furthermore applied a log-linear model to the complete and incomplete triads data in order to assess the parental effect on offspring phenotype, irrespective of the allele transmission to the offspring (Weinberg et al., 1998; Wilcox et al., 1998). Using this approach each triad is characterized according to the number of high risk alleles (0, 1, 2) in the genotypes of the mother, father, and child. Estimation of maternal or paternal genotypic effects is based on asymmetry in the distribution of reciprocal mating types, and tested for statistical significance with the likelihood-ratio test (LRT). The P values are two-sided, and statistical significance was accepted at $P<0.05$.

Results

The transmission frequencies of the MTHFR 677C>T polymorphism from heterozygous parents to the affected offspring are shown in Table 6.1. The difference in transmission of the 677C allele and the 677T allele was not statistically significant ($X^2=1.22$, df=1, P=0.27). The offspring genotype relative risk was 1.43 (95% CI: 0.83-2.47) for the 677TT and 1.42 (95% CI: 0.54-3.78) for the 677CT genotype relative to the 677CC genotype.

Table 6.1. Transmission frequencies of the C and T alleles of the MTHFR 677C>T polymorphism from heterozygous parents to schizophrenia offspring

Transmitted alleles	Nontransmitted alleles	
	677C	677T
677C	89	36
677T	46	15

A meta-analysis was carried out with the available data on the MTHFR 677C>T polymorphism of two previous published family-based studies (Wei and Hemmings, 1999; Yu et al., 2004), and the results of the present study. All cases were diagnosed according to standard diagnostic systems (DSM-III, or DSM-IV), and all studies used a standardized method to determine the MTHFR 677C>T genotypes. Table 6.2 shows the results of individual and pooled studies. Overall, we observed no preferential transmission of the 677T

or 677C allele (P=0.58). However, there was significant heterogeneity among the results of individual studies (X^2=6.419; df=2; P=0.040).

The statistical analysis using the log-linear model obtained from complete triads and parent-child dyads showed no maternal or paternal effect of MTHFR genotype on schizophrenia risk in offspring (LRT=0.584, df=1, P=0.445) (data not shown).

Table 6.2. Meta-analysis of family-based studies on MTHFR 677C>T polymorphism and schizophrenia

Study	N of triads	N of heterozygous parents	N of preferential transmitted alleles		Study estimate		
			677C	677T	A/B[a]	95% CI	Weights
Wei and Hemmings (1999)	56	51	34	17	0.50	(0.28-0.89)	5.48
Yu et al. (2004)	267	251	129	122	0.95	(0.74-1.21)	9.06
Our study	93	82	36	46	1.28	(0.83-1.98)	6.95
Pooled*	416	384			0.89	(0.58-1.35)	

[a]A/B: Number of 677T alleles/ Number of 677C alleles.
*P=0.58; z value=-0.56.

Discussion

Case-control studies provided evidence for the association between MTHFR 677C>T polymorphism and schizophrenia risk, but these studies are sensitive to population stratification. In the present study, we examined whether transmission of the MTHFR 677T allele is associated with schizophrenia. We found a random transmission of the 677T allele from heterozygous parents to schizophrenia offspring, which was in line with the outcome of the meta-analysis performed with data from three TDT studies, including our data. When we applied a log-linear approach to the case-parent data no asymmetry within parental mating type was found, which does not support a maternal effect.

The question is how these results should be interpreted in relation to the results of a previous meta-analysis of case-control studies (Muntjewerff et al., 2006). A first explanation is that the TDT supposes primarily an effect of the genotype on the occurrence of disease in the patient. If schizophrenia is a developmental disorder that starts already *in utero*, then the maternal genotype might be more important than the genotype of the child. If this is the case for MTHFR and schizophrenia, one would suppose the 677TT genotype to be overrepresented in mothers with schizophrenia offspring and, subsequently, patients may show an enrichment of the 677T allele. However, one would not expect preferential

transmission of the 677T allele, because it is in fact a genetic risk factor in the mother (Labuda et al., 2002). The results of the log-linear model do not support a maternal effect as described, but it should be noticed that the power of this analysis is limited. Larger studies in mothers of patients with schizophrenia are warranted.

A second explanation would be that the case-control studies indeed suffer from population stratification and publication bias, and that the family-based studies are more reliable. In that case the relationship that has been found between MTHFR and schizophrenia, and between homocysteine and schizophrenia (Muntjewerff et al., 2006) turned out to be a spurious association. Also here we should be aware that the power of the meta-analysis on association studies, comprising 2265 cases, is much greater than the power of the currently known family-based studies with a total of 416 triads comprising 383 heterozygous parents. Furthermore, the genotype relative risk of 1.42 (95% CI: 0.54-3.78) for 677TT in our study is, although not significant, in the same magnitude as the estimate from the meta-analysis of case-control studies (odds ratio=1.36).

Recently, data of a birth cohort provided preliminary evidence for an increased risk of schizophrenia following prenatal exposure to elevated homocysteine (Brown and Susser, 2005). The hypothesis that schizophrenia risk might be influenced by maternal homocysteine concentration during pregnancy has important consequences, because it could be easily treated with periconceptional folate supplementation. Folate supplementation decreases plasma total homocysteine levels by 25% (Homocysteine Lowering Trialist's Collaboration, 2005). From epidemiological studies evidence has been provided that a variety of environmental prenatal factors contribute to increased schizophrenia risk, such as maternal nutritional deficiency (Susser et al., 1996) or conception during the Winter months (Torrey et al., 1997; Tochigi et al., 2004). It has been suggested that these environmental factors are associated with low folate status of the mother and may interact with genetic variants of enzymes of the folate-dependent homocysteine metabolism (Susser et al., 1996; Johnson, 1999).

In conclusion, our study showed no preferential transmission of the MTHFR 677T allele from parents to schizophrenia offspring, and no overrepresentation of this allele among mothers with schizophrenia offspring. Further large scale studies including patients and especially their mothers are needed to confirm or exclude the relationship between MTHFR and schizophrenia risk.

Acknowledgements

M. den Heijer is recipient of a VENI-grant from the Netherlands Foundation of Scientific Research. The authors thank A. de Graaf-Hess, D. van Oppenraaij-Emmerzaal, H. Gellekink, and B. Jungerius for the technical assistance.

Chapter 7

Polymorphisms in catechol-O-methyltransferase and methylenetetrahydrofolate reductase in relation to the risk of schizophrenia

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Submitted for publication

Abstract

Objective: Evidence is emerging for the association of aberrant homocysteine-methylation cycle and increased risk of schizophrenia. Catechol-O-methyltransferase (COMT) and methylenetetrahydrofolate reductase (MTHFR) are interconnected enzymes of the dopamine catabolism and homocysteine pathway, respectively. The genes coding for these enzymes each show a common variant associated with a reduced enzyme function, and have been implicated in schizophrenia. The objective of this study was to examine the influence of the COMT 324G>A (Val108/158Met) polymorphism alone and in combination with the MTHFR 677C>T variant on the risk of schizophrenia.

Methods: We examined the prevalence of the COMT 324G>A and MTHFR 677C>T polymorphisms in 252 patients with schizophrenia and 405 control subjects. All subjects were of Dutch ancestry. The impact of each polymorphism and the effect of each gene-gene combination upon the risk of schizophrenia were assessed.

Results: The COMT 324AA genotype was observed in 77 (30.6%) patients and in 113 (27.9%) controls (odds ratio (OR)=1.38 [95% CI: 0.88-2.16]), and the MTHFR 677TT genotype in 31 (12.3%) patients and 35 (8.6%) controls (OR=1.65 [95% CI: 0.97-2.82]). No evidence of statistical interaction between both polymorphisms in the group of controls or patients was found. However, we calculated an increased risk of schizophrenia associated with joint occurrence of the COMT 324AA and MTHFR 677TT genotype (OR=3.08 [95% CI: 1.08-8.76]). In addition, an association was demonstrated between increasing number of low enzyme activity alleles in the COMT and MTHFR genotype combinations and increased risk of schizophrenia (test for trend P=0.017).

Conclusion: Our findings suggest a biologically meaningful interaction between COMT and MTHFR on schizophrenia susceptibility, but confirmation is needed in larger samples.

Introduction

Schizophrenia (MIM 181500) is a complex and severely disabling mental disorder affecting approximately 1% of the world population (Gottesman, 1994). Its causes are determined by genetic and environmental factors (Tsuang, 2000). Although as much as 80% of the liability for schizophrenia may be inherited (Sullivan et al., 2003), no single polymorphism in any candidate gene has been consistently replicated across all studies as a risk factor for schizophrenia (Harrison and Weinberger, 2005; Hirschhorn et al., 2002). It has been proposed that critical combinations of functional polymorphisms each with minor impact on the risk of schizophrenia may be responsible for an increased risk of schizophrenia (Lewis et al., 2003). Revealing these combinations of genetic variations that affect a specific metabolic pathway associated with an increased risk to develop schizophrenia might be a fruitful strategy in the search of schizophrenia genes.

One metabolic pathway that has been extensively related to schizophrenia is the dopaminergic pathway (Kapur, 2003). Catecholamine-O-methyltransferase (COMT; MIM 116790) is one of the major enzymes in dopamine catabolism by influencing cortical dopamine flux (Chen et al., 2004). The gene encoding COMT is located on chromosome 22q11, which several linkage studies have suggested to be a susceptibility locus for schizophrenia (Lewis et al., 2003). A common 324G>A variant in the COMT gene (dbSNP rs4680) causes a valine to methionine substitution at codon 108 of the soluble form and codon 158 of the membrane-bound form (Val108/158Met) in the COMT protein. This polymorphism leads to significant reduction in activity of COMT in liver (Lachman et al., 1996), red blood cell (Syvanen et al., 1997), and brain (Chen et al., 2004). The COMT 324G>A polymorphism has been examined repeatedly in case-control association and family-based studies for an association with schizophrenia. However, a recent meta-analysis showed no significant evidence for an association between the COMT 324G>A polymorphism and schizophrenia (Fan et al., 2005). Recently, Xu et al., (2004) performed a full-scale gene search on the dopamine metabolic pathway, and found that a combination of polymorphisms in the genes encoding for COMT and ALDH3, another enzyme of dopamine catabolism, was associated with the susceptibility of schizophrenia.

A second pathway of interest that has been related to schizophrenia is homocysteine metabolism. Elevated plasma total homocysteine (tHcy) levels are a risk factor for diseases of the central nervous system, such as Alzheimer's disease, Parkinson's disease, and neural tube defects (Mattson and Shea, 2003). THcy levels reflect the cell's ability to methylate compounds such as DNA, lipids, proteins or neurotransmitters (Mudd et al., 2001). Increased homocysteine levels, and its oxidized derivatives directly induce neurotoxicity (Lipton et al., 1997; Mattson and Shea, 2003) and vascular endothelial damage (Jacobsen, 2001).

Elevated tHcy levels may originate from nutritional deficiencies, especially low folate, or from genetic aberrations in enzymes involved in homocysteine metabolism (Refsum et al., 2004). In that respect, homozygosity for the 677C>T polymorphism (dbSNP rs165688) in the methylenetetrahydrofolate reductase (MTHFR; MIM 607093) gene is the most common inherited cause of elevated tHcy levels, especially when folate levels are low (Frosst et al., 1995). A recent meta-analysis of case-control studies performed by us supports the association of elevated tHcy levels or MTHFR 677TT genotype and increased risk to develop schizophrenia (Muntjewerff et al., 2006).

Interestingly, the metabolic pathways of COMT and MTHFR are interconnected (Mudd et al., 2001) (Figure 7.1). COMT catalyses the transfer of a methyl group from S-adenosylmethionine (AdoMet) to catecholamines, including dopamine, noradrenaline and adrenaline, thereby inactivating these neurotransmitters. Upon the methylation of a COMT substrate, AdoMet is converted to S-adenosylhomocysteine (AdoHcy), a strong inhibitor of COMT and is therefore rapidly hydrolyzed to homocysteine in normal conditions. Methyl groups necessary for the successive methylation processes are provided by the folate-dependent conversion of homocysteine to methionine in most cells. MTHFR is a key regulatory enzyme in this remethylation process, thereby determining AdoMet levels. In line with this, one can hypothesize that variants in the COMT and MTHFR genes are related to schizophrenia in a common pathway, influencing the methylation of neurotransmitters.

In the present study we investigated the effect of the 324G>A variant in the COMT gene alone and in combination with the MTHFR 677C>T polymorphism on the risk of schizophrenia.

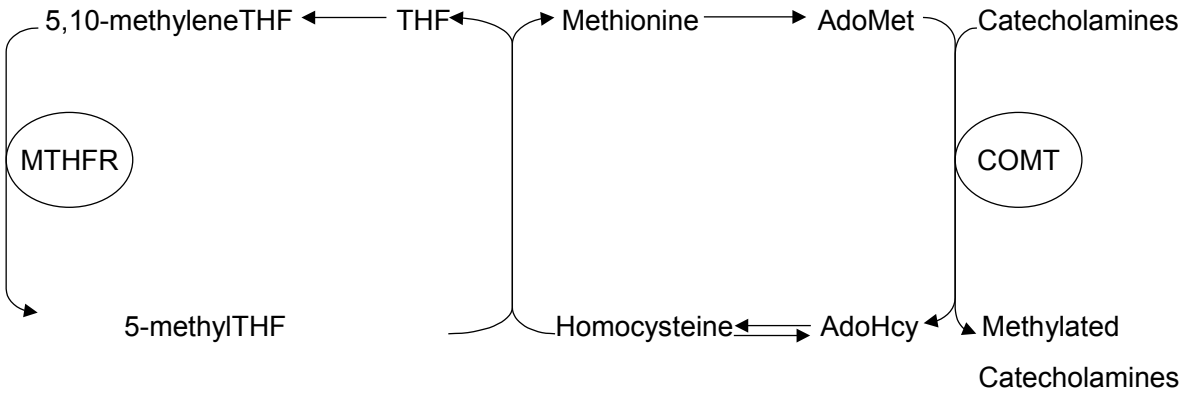

Figure 7.1. Simplified scheme of the interconnection between the homocysteine metabolism and catecholamines catabolism. Abbreviations: COMT, catechol-O-methyltransferase; MTHFR, methylenetetrahydrofolate reductase; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; THF, tetrahydrofolate.

Methods

Study population

The population of schizophrenic patients has been described in detail previously (Muntjewerff et al., 2005). In summary, schizophrenia patients were recruited via the University Medical Centre Utrecht, Utrecht and GGz Nijmegen, Mental Health Institute, Nijmegen, The Netherlands. Diagnosis was established on the basis of a clinical interview, using the Comprehensive Assessment of Symptoms and History (CASH) (Andreasen et al., 1992). All patients met DSM-IV criteria (American Psychiatric Association, 1994) for schizophrenia. Control subjects were recruited from a general practice (den Heijer et al., 1995). For this study only subjects with complete data for COMT as well as MTHFR genotypes were included comprising 252 cases (mean age 41 ± 14 years, 73% male) and 405 controls (mean age 51 ± 13 years, 41% male). Therefore, the number of patients and controls included in the present study is lower than the number reported in our previous study (Muntjewerff et al., 2005). All subjects were Dutch Caucasian in ethnic origin. The protocol was approved by the local ethics committee and written consent was obtained from all participants.

Sample analyses

Genomic DNA was extracted from peripheral lymphocytes. A G>A transition in exon 4 of the COMT gene has been described which creates an additional *Nl*aIII restriction site. The COMT 324G>A polymorphism was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) essentially as described by Lavigne et al. (1997). To detect the MTHFR 677C>T genotypes, the polymerase chain reaction was performed as described by Frosst et al. (1995).

Statistical analyses

Comparison of genotype frequencies was performed by chi-square test. Odds ratios (ORs) and 95% confidence intervals (95% CIs) as estimates of relative risk for schizophrenia associated with different genotypes were calculated with logistic regression. A test for trend was performed for the association of the number (0, 1, 2, 3, or 4) of low activity alleles for each combination of COMT and MTHFR genotypes and the risk of schizophrenia using logistic regression analysis. All statistical analyses were performed two-tailed and with SPSS software package version 12.0. Statistical significance was accepted at $P<0.05$.

Results

Risk of schizophrenia in relation to COMT or MTHFR polymorphism

Genotype frequencies of COMT and MTHFR polymorphisms were in Hardy-Weinberg equilibrium (P>0.05 in control group). The distribution of the COMT 324G>A genotypes and MTHFR 677C>T genotypes observed in control and patient groups, and ORs for risk of schizophrenia are presented in Table 7.1 and Table 7.2, respectively.

Table 7.1. Prevalence of the COMT 324G>A polymorphism in controls and schizophrenic patients and odds ratios (OR) with 95% confidence intervals (95% CI) as risk estimate for schizophrenia

Genotype	Controls (N=405)		Patients (N=252)		OR	(95% CI)
	Number	(%)	Number	(%)		
324GG	99	(24.4)	49	(19.4)	1[a]	
324GA	193	(47.7)	126	(50.0)	1.32	(0.88-1.99)
324AA	113	(27.9)	77	(30.6)	1.38	(0.88-2.16)

[a] Reference category.

As stratification according to sex yielded similar results only crude risk estimates are shown. The COMT 324AA and COMT 324GA genotypes were not associated with a significantly increased risk of schizophrenia compared to the COMT 324GG genotype. When subjects homozygous or heterozygous for the COMT 324G>A polymorphism where combined, an OR of 1.34 (95% CI: 0.91-1.97) for having schizophrenia relative to the COMT 324GG genotype was found. No significant trend of increasing risk with increasing numbers of COMT 324A alleles (1 or 2) was observed (test for trend P=0.182).

Table 7.2. Prevalence of the MTHFR 677C>T polymorphism in controls and schizophrenic patients and odds ratios (OR) with 95% confidence intervals (95% CI) as risk estimate for schizophrenia

Genotype	Controls (N=405)		Patients (N=252)		OR	(95% CI)
	Number	(%)	Number	(%)		
677CC	205	(50.6)	110	(43.7)	1[a]	
677CT	165	(40.7)	111	(44.0)	1.25	(0.90-1.75)
677TT	35	(8.6)	31	(12.3)	1.65	(0.97-2.82)

[a] Reference category.

We found a nearly significant risk estimate for the association between the MTHFR 677TT genotype and schizophrenia (P=0.067). When subjects homozygous or heterozygous for the MTHFR 677C>T polymorphism where combined, an OR of 1.32 (95% CI: 0.97-1.82) for having schizophrenia relative to the MTHFR 677CC genotype was calculated. A significant trend of increasing risk with increasing numbers of MTHFR 677T alleles (1 or 2) was observed (test for trend P=0.046).

Risk of schizophrenia in relation to COMT and MTHFR combinations

Table 7.3 shows the frequencies of the compound genotypes within the groups of controls and patients. Using the chi-square test we found no evidence of a statistical interaction between COMT 324G>A and MTHFR 677C>T variants within the group of controls (X^2=5.991; df=4; P=0.200) or patients (X^2=1.322; df=4; P=0.858). An additional test for interaction within both groups was performed by incorporating the frequencies of the homozygous genotypes for both the COMT324G>A and MTHFR 677C>T variants into a logistic regression model. The results did not reveal an interaction effect within controls (Exp(B)=0.69 [95% CI: 0.24-1.97], P=0.49) or patients (Exp(B)=1.07 [95% CI: 0.35-3.31], P=0.91). In line with this finding, the distribution of compound genotypes showed no significant difference between controls and patients (X^2=8.123; df=8 ; P=0.422). However, coexistence of the low enzyme activity genotypes was associated with an increased risk of schizophrenia when compared to the homozygous wild-type combination (OR=3.08 [95%CI: 1.08-8.76]). No significantly increased risk of schizophrenia for any other composite genotype was found. In addition, we observed a significant trend of increasing risk with increasing numbers of risk alleles (1, 2, 3, or 4) when all COMT and MTHFR gene combinations were taken into account (test for trend P=0.017).

Table 7.3. Distribution of the combinations of COMT 324G>A and MTHFR 677C>T genotypes within control and patient group

COMT 324G>A	MTHFR 677C>T					
	Controls			Patients		
	677CC	677CT	677TT	677CC	677CT	677TT
324GG	47	44	8	21	22	6
324GA	90	84	19	53	59	14
324AA	68	37	8	36	30	11

Discussion

We applied a candidate-gene approach to examine the effect of two common and functional genetic variants of enzymes of the methylation pathway, the COMT 324 G>A and MTHFR 677C>T polymorphisms, on schizophrenia risk. Although our study revealed no evidence of interaction between both polymorphisms within control or patient group, subjects with the COMT 324AA genotype combined with the MTHFR 677TT genotype displayed a higher risk of schizophrenia than subjects with either genotype alone. The increased risk was strong enough to reach significance despite the small numbers of subjects carrying the compound genotype. Moreover, we found a positive association between the number of low enzyme activity alleles of the COMT and MTHFR genotype combinations and risk of schizophrenia.

In the present study, we found a non-significant increased risk associated with the COMT 324AA genotype (OR=1.38 [95% CI: 0.88-2.16]). Genetic association studies are not consistent regarding the involvement of the Val-allele or Met-allele of the COMT 324G>A polymorphism in the aetiology of schizophrenia (Egan et al., 2001; Shifman et al., 2002; Fan et al., 2005). Many factors can contribute to the conflicting findings, such as small sample size or population stratification, and linkage disequilibrium of the Val-allele with a nearby causal variant. In addition, the relationship between the COMT 324G>A polymorphism and schizophrenia is complex as can be denoted from functional studies showing decreased enzyme activity associated with the Met-allele *in vitro* and human brain extracts (Lotta et al., 1995; Chen et al., 2004), whereas the Val-allele has been associated with a lower level of expression in human brain (Bray et al., 2003). Our finding of a nearly significant increased risk of schizophrenia associated with the MTHFR 677TT genotype (OR=1.65 [95% CI: 0.97-2.82]) is in line with the risk estimate provided by our previous study (Muntjewerff et al., 2005), and the recently performed meta-analysis on this subject (Muntjewerff et al., 2006). This meta-analysis comprised data of ten studies, and showed that the MTHFR 677TT genotype is associated with a 36% (95% CI: 7-72) higher risk of schizophrenia compared to the MTHFR 677CC genotype.

The risk of schizophrenia has been investigated for COMT variants in combination with other candidate genes. Patients not responding to neuroleptic treatment showed a higher prevalence of the combination of COMT 324G>A and NOTCH4 variants than controls (Antilla et al., 2004). NOTCH4 plays a role in the development of the central nervous system by influencing the generation of neurons and glia from neural stem cells (Grandbarbe et al., 2003). The combined effect of another COMT variant (dbSNP rs174682) with the gene encoding for ALDH3 on schizophrenia risk (Xu et al., 2004) supports the involvement of the dopamine pathway in the pathogenesis of this disease. An additive effect on schizophrenia

risk was not observed for genetic variants of COMT and monoamine oxidase (MAO) (Norton et al., 2002). MAO, like COMT, is another enzyme which degrades dopamine, noradrenaline and serotonin, but MAO is not involved in methylation reactions. To our knowledge, the present study is the first to examine the combined effect of the COMT 324G>A and 677C>T MTHFR polymorphisms on the risk to develop schizophrenia.

The COMT 324G>A and MTHFR 677C>T polymorphisms are biological relevant and exert their influence through the homocysteine and methylation pathway. We cannot rule out the possible mechanism that elevated homocysteine caused by dysfunctional MTHFR, inhibits COMT function, thereby influencing susceptibility to schizophrenia. An increase in tHcy is associated with an increase in AdoHcy (Yi et al., 2000), a strong inhibitor of COMT. Accumulation of AdoHcy has the potential to inhibit many other methyltransferases, thus further impairing adequate methylation of a variety of molecules, including DNA, neurotransmitters, lipids, and proteins. Reduced MTHFR activity might also cause AdoMet depletion (Finkelstein, 1990), resulting in less methyl groups needed for the numerous methylation processes such as dopamine methylation by COMT. Decreased COMT activity might be related to diminished methylation of dopamine, whereas lowered MTHFR activity is associated with aberrant methylation (Mudd et al., 2001), and homocysteine-induced pathophysiological mechanisms (Mattson and Shea, 2003). These processes related to aberrant COMT and MTHFR function may have a role in the aetiology of schizophrenia (Kapur, 2003; Mattson and Shea, 2003; Abdolmaleky et al., 2004). Determination of AdoHcy, AdoMet, methionine, homocysteine and folate levels stratified by COMT and MTHFR variants might provide more insight into the relation between these mechanisms and schizophrenia risk.

Certain points must be taken in consideration. First, other functional variants of COMT (Xu et al., 2004; Handoko et al., 2005; Lee et al., 2005) or MTHFR (Van der Put et al., 1998), not examined in the present study may also interact and increase the risk of schizophrenia. Second, environmental factors in concert with the COMT 324G>A or MTHFR 677C>T variant may also influence schizophrenia risk. Evidence for this is the finding of a joint effect of the COMT variant and adolescent-onset cannabis use on psychosis risk (Caspi et al., 2005). As the phenotypic expression of the MTHFR genotype varies with folate status (Frosst et al., 1995), folate supplementation in schizophrenia patients might have clinical relevance (Godfrey et al., 1990; Levine et al., 2006), especially for subjects carrying the MTHFR 677TT genotype. Lastly, we observed no significant sex-specific genotype distribution of either COMT or MTHFR. Interestingly, Chen et al. (2004) reported lower COMT activity in human brain of females than males, and this difference was observed in each of the COMT genotypes. It was suggested that estrogens as regulators of COMT promoter activity account for the observed sex-specific effect (Xie et al., 1999), rather than genetic factors.

We postulated that subjects homozygous for the COMT and MTHFR variant exert an increased risk of schizophrenia thereby reflecting a biological interaction between the two corresponding dysfunctional enzymes. Our data show preliminary evidence for an effect of COMT 324G>A and MTHFR 677C>T polymorphism combination on schizophrenia risk, and call for replication in studies with larger sample size. Other genetic determinants of schizophrenia may be revealed by examining genes associated with the methylation pathway and their synergistic effects on schizophrenia risk, metabolic phenotypes, or brain function and structure.

Acknowledgements

M. den Heijer is recipient of a VENI-grant from the Netherlands Foundation of Scientific Research. The authors thank A. de Graaf-Hess, D. van Oppenraaij-Emmerzaal and A. Bardoel for the technical assistance.

Electronic-Database Information

URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

Databank Single Nucleotide Polymorphism (dbSNP),
http://www.ncbi.nlm.nih.gov/projects/SNP/

Chapter 8

Catechol-O-methyltransferase genotype is associated with plasma total homocysteine levels

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Part of this chapter has been submitted as "Catechol-O-methyltransferase genotype is associated with plasma total homocysteine levels and may increase recurrent venous thrombosis risk" by Gellekink H, Muntjewerff JW, Vermeulen SHHM, Hermus ARMM, Blom HJ, den Heijer M.

Abstract

Objective: A disturbed methylation may partly explain the mechanism behind homocysteine-related diseases, such as cardiovascular disease, schizophrenia and breast cancer. Catechol-O-methyltransferase (COMT) is involved in the S-adenosylmethionine-dependent methylation of catecholamines and catecholestrogens and in this way contributes to homocysteine formation. COMT dysfunction has been related to schizophrenia and other diseases as well. We hypothesized that COMT dysfunction, by virtue of functional polymorphisms, may alter plasma total homocysteine (tHcy) and performed haplotype analysis to investigate this association.

Methods: The polymorphisms rs2097603, rs4633, rs4680 (324G>A) and rs174699 were assessed by means of RFLP-PCR followed by agarose gelelectrophoresis and UV-detection. Haplotypes were constructed using Whap and assessed for their effects on tHcy in population-based controls.

Results: The variants rs4633 and rs4680 were in almost complete linkage disequilibrium. The rs4680 (324G>A) polymorphism was associated with tHcy levels and largely explained the observed haplotype effect (-13.3% [95% CI -23.6 to -3.1], P=0.01).

Conclusion: We show that the functional COMT rs4680 variant is associated with tHcy, which may reflect its effect on COMT expression. Hence, COMT dysfunction, as well as the concomitant effect on tHcy may alter the risk for COMT-related diseases, such as schizophrenia, cardiovascular disease and breast cancer.

Introduction

A disturbed homocysteine metabolism has been associated with diseases of the vascular system, both of arterial and venous origin (The Homocysteine Studies Collaboration, 2002; Wald et al., 2002; den Heijer et al., 2005). In addition, high plasma total homocysteine (tHcy) increases the risk of neurological disorders like spina bifida, schizophrenia and Alzheimer's disease (Clarke et al., 1998; Czeizel, 2000; Muntjewerff et al., 2006). The mechanism of how homocysteine is related to disease is still obscure, but there are strong indications that a disturbed transmethylation may partly explain this association (James et al., 2002; Abdolmaleky et al., 2005; Bjorklund and Gordon, 2006). Several studies show that tHcy levels correlate well with plasma S-adenosylhomocysteine (AdoHcy) (Yi et al., 2000; Castro et al., 2003), a strong inhibitor of S-adenosylmethionine (AdoMet)-dependent methylation. Given the importance of methylation of nucleic acids, proteins, lipids but also hormones and neurotransmitterss, it seems plausible that the inhibition or dysfunction of specific methyltransferases affect critical processes and hence confer a higher risk of disease.

The enzyme catechol-O-methyltransferase (COMT, E.C. 2.1.1.6) is one of the methyltransferases that is highly susceptible to inhibition by AdoHcy (Clarke and Banfield, 2001). COMT represents a major pathway in the degradation of catecholamine neurotransmitters, like dopamine and (nor)adrenaline, and catecholestrogens. Hence, dysfunction of COMT enzyme has been implicated in complex diseases like schizophrenia, Parkinson's disease and breast cancer (Goodman et al., 2001; Shifman et al., 2002; Zhu, 2003; Zhu, 2004). Because the vascular system is constantly exposed to circulating catecholamines, COMT dysfunction may also have implications in cardiovascular disease (Zhu, 2002).

The COMT gene has been assigned to chromosome 22q11.2 and spans about 27 kb. COMT enzyme exists as a membrane-bound (MB) and soluble (S) isoform, the expression of which is regulated by two different promotors. A common 324G>A polymorphism (rs4680) in the COMT gene, resulting in a valine-to-methionine substitution at position 108 (S-isoform) and 158 (MB-isoform), has been studied extensively for its effect on enzyme activity, although the data is non consistent (Bray et al., 2003; Chen et al., 2004). This may be due to the presence of another variant, closely located to the 324G>A polymorphism, that is causally related to COMT dysfunction (Shifman et al., 2002). The metabolic pathways of COMT and homocysteine are interconnected as the O-methylation of catecholamines catalyzed by COMT produces homocysteine. Hence, COMT dysfunction may be related to homocysteine levels (Goodman et al., 2001) as a result of its role in catecholamine metabolism.

The aim of our study was to investigate the effect of variation of the COMT gene on tHcy concentration. Considering the controversy whether the 324G>A variant is causally related to decreased enzyme activity or expression, we included three other variants (rs2097603 (Chen et al., 2004), rs4633 and rs174699) in or directly adjacent to the COMT gene and performed haplotype analysis.

Methods

Subjects used in the present study

The study population was recruited via a general practice in The Hague (The Netherlands) and represents the control group of a previously published retrospective study on venous thrombosis (den Heijer et al., 1995). For the current study, DNA was available of 438 individuals. All participants gave their informed consent.

Biochemical parameters

Blood samples were drawn from the antecubital vein in 5 mL Vacutainer tubes and 4.5 mL EDTA vacuum glass tubes for determination of tHcy concentration and for DNA extraction. EDTA samples for homocysteine measurement were placed on ice immediately and centrifuged at 3,500 g for 5 minutes with minimal sured by an automated high-performance liquid chromatography method with reverse phadelay. The plasma was separated and then stored at -20ºC. THcy concentrations were mease separation and fluorescent detection (Gilson 232-401 sample processor, Spectra Physics 8800 solvent delivery system and LC 304 fluorometer) (TePoele-Pothoff et al., 1995). DNA extraction was performed as described previously (Miller et al., 1988) and the DNA was stored at 4 ºC.

Genotype analysis

We genotyped four single-nucleotide polymorphisms (SNPs) distributed over the gene of interest; rs2097603, rs4633, rs4680 (sometimes referred to as rs165688) and rs174699. The SNPs were chosen based on frequency, functionality and location within the gene. All four created or abolished an enzyme restriction site allowing a simple screening based on restriction-fragment length polymorphism (RFLP) analysis. Screening conditions were similar in all analyses. For details see Table 8.1. PCR conditions: 4 minutes at 94°C, 35 cycles of 94°C/60s, 52-60°C/60s, and 72°C/30s, and a final extension of 7 minutes at 72°C. Each PCR reaction mixture contained 50 ng of both forward and reverse primer (Biolegio BV, The Netherlands), 200 μM dNTPs, 10 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 2.0-4.0 mM $MgCl_2$, 0.5 U of recombinant Taq polymerase, 5% DMSO (Invitrogen, The Netherlands) and 75 ng DNA. The resulting PCR product was digested by at least 10 units of restriction

enzyme (all from New England Biolabs, Inc.) and incubated for 3 hours or overnight at 37°C. The digests were analyzed by gel electrophoresis on a 3 or 4% agarose gel, stained with ethidiumbromide and visualized by UV. In all PCR amplifications and restriction analyses, DNA samples from which the genotype had been identified by sequence analysis served as positive controls. Genotype data was available from at least 413 individuals. In addition, data of four other reported variants (i.e. rs6267 [Ala22Ser] (Lee et al., 2005), rs740602, rs13306281 and rs3218737) was obtained from 150 subjects by means of sequence analysis using the ABI Prism 3130XL automated sequencer according to the instructions of the manufacturer (Applied Biosystems, The Netherlands). However, three of them appeared non-polymorphic, while rs740602 was found only once in the heterozygous state (minor allele frequency of <0.01) and were excluded for further analysis.

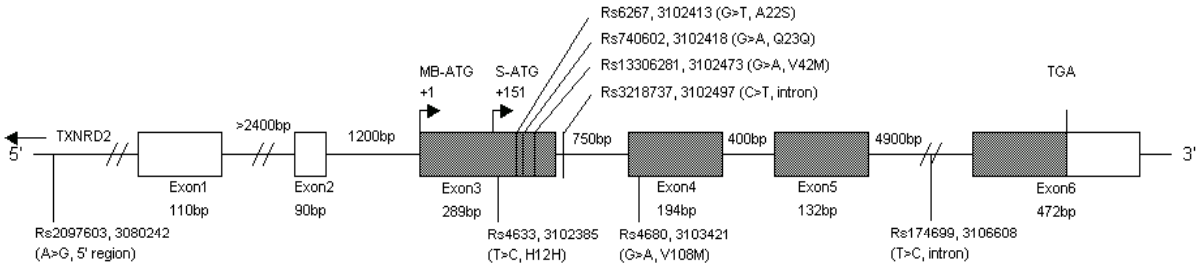

Figure 8.1. Gene structure of the COMT gene. The boxes represent exons and the thin lines in between represent introns. Gray boxes indicate protein-coding regions. The length (bp) of each region is indicated, as are the translation initiation codons for the soluble (S-ATG) and membrane-bound (MB-ATG) COMT isoform. In the lower part of the figure the polymorphic variants assessed in this study are shown (rs number, relative position, type of polymorphism), while four other variants that appeared non-polymorphic are depicted in the upper part. Abbreviation: TXNDR2=thioredoxin reductase 2. Adapted from Zhu (2002).

Statistics

Haploview was used to evaluate linkage disequilibrium (LD) between the SNPs. Haplotype association analysis for homocysteine levels was performed using Whap. Whap takes the ambiguity in individual haplotype estimations into account by applying a weighted likelihood approach. Prior to the haplotype analyses, the log transformed tHcy values were standardized. We used permutation testing as implemented in the Whap program to correct for multiple testing in all analyses. Haplotypes with a frequency of less than 2% were excluded from the analysis. Single-locus genotype effects were evaluated by linear regression analysis (SPSS 12.0). Changes in tHcy levels, in the haplotype and single-locus analysis, are expressed relative to the most frequent homozygous genotype group. Statistical significance was accepted at $P<0.05$.

Results

SNP genotyping and linkage disequilibrium

For details about these SNPs regarding location, function and validation method, see Table 8.1 and Figure 8.1. The four COMT SNPs under study were all in Hardy-Weinberg equilibrium (P>0.4). Linkage disequilibrium coefficients (D') between each of the genotyped variants are shown in Table 8.2. The SNP pair rs4680 (324G>A) and rs4633 (36T>C) were in almost complete LD, with a D' of 0.96 and a squared correlation (r^2) of 0.89. No strong LD was observed for the other SNP pairs.

Table 8.2. Linkage disequilibrium coefficient (D') between SNPs across the COMT gene in controls

Variant	Distance from rs2097603 (bp)	rs2097603	rs4633	rs4680	rs174699
rs2097603	0	-	0.427	0.438	0.50
rs4633	22143	*0.149*	-	0.964	0.627
rs4680	23179	*0.165*	*0.89*	-	0.695
rs174699	26366	*0.016*	*0.033*	*0.038*	-

Above the diagonal the D'-values are shown and below the diagonal their corresponding correlation coefficients (in italics). The distance relative to rs2097603 is also shown

Haplotype analysis

The haplotype frequencies and their relative effects on tHcy are presented in Table 8.3. By omitting the haplotypes with a frequency of less than two percent, 95% of the haplotypes was covered. The omnibus association test using all haplotypes for crude fasting tHcy showed a borderline significant effect (P=0.05). Haplotype specific analysis, i.e. analysis of the effect of the haplotype relative to the (most common) reference haplotype, showed that the effect was mainly due to the G-C-G-T haplotype and was statistically significant associated with low tHcy levels (-13.3% [95% CI -23.6 to -3.1], P=0.01). The omnibus haplotype test was no longer significant when the analysis was conducted conditional on rs4680 or on rs4633 (P=0.28 and P=0.26, respectively) meaning that the haplotype analysis did not reveal major additional effects besides that observed for the single-loci. Furthermore, by comparing a model in which the effects of the 3 haplotypes containing allele C and G at the second and third locus were constrained to be equal to a model in which all haplotype effects were estimated separately, we found that the effects of the N-C-G-N haplotypes (where N represents one of the alleles observed at the first and fourth locus) were not different (P=0.18). This suggests that the haplotype background on which these variants appear had no major influence on tHcy.

Table 8.1. Relevant data of analyzed polymorphisms and method of screening

Variant	Contignr	Reported MAF[a]	DNA	Protein[b]	HW[c] P value	Primers (5'→ 3')	Validation method	[Mg2+] (mM)	Tann (°C)
rs2097603	3080242	0.39 (G)	5' region A>G (P2 promoter)	-	0.87	F aatttggctattgccgtgtc R gtcccataaaaggggattcg	+HindIII	2.0	52
rs4633	3102385	0.39 (T)	36T>C	H12H	0.52	F acaacctgctcatgggtgac R tcctgtaagggctttgatgc	+PmlI	4.0	60
rs4680	3103421	0.36-0.52 (A)	324G>A	V108M	0.40	F tactgtggctactcagctgtgc R gtgaacgtggtgtgaacacc	+NlaIII	2.0	59
rs174699	3106608	0.19 (C)	Intron 3 T>C	-	1.0	F cctctgtgaggctccaactc R gaaagaaggcagcacctgtc	-NcoI	3.0	59

[a] MAF, minor allele frequency (source: NCBI), [b] amino acid change using the (short) soluble form as reference, [c] Hardy-Weinberg (HW) P value

Table 8.3. Haplotype frequencies and relative change in tHcy in controls using the G-T-A-T haplotype as a reference

Haplotype	Frequency	Crude change tHcy % [95% CI]	P value
G-T-A-T	0.355	0[a]	
A-C-G-T	0.312	-3.2 [-9.7 to 3.4]	
A-T-A-T	0.167	1.3 [-7.3 to 9.8]	
G-C-G-T	0.120	-13.3 [-23.6 to -3.1]	0.01
A-C-G-C	0.046	-3.3 [-15.8 to 9.2]	

[a] Reference category

Single-locus effect on tHcy

The single-locus genotypic effects are shown in Table 8.4. As expected from the haplotype analysis we observed that the rs4680 variant (324G>A) was significantly associated with tHcy levels. Compared to the most common genotype 324AA, tHcy decreased by 2.1% (95% CI -10.3 to 6.7) and 10.4% (95% CI -19.0 to -0.8) in 324GA and 324GG subjects, respectively (P for trend=0.036). Adjustment for age, sex, serum creatinine, MTHFR 677C>T polymorphism and plasma folate did not change this point estimate (not shown). The rs4633 variant was also associated with tHcy albeit to a lesser extent (P for trend=0.143). The rs2097603 and rs174699 variants did not affect tHcy.

Discussion

The COMT gene has been investigated extensively as a susceptibility locus for schizophrenia (Shifman et al., 2002; Glatt et al., 2003; Xu et al., 2004; Handoko et al. 2005) and COMT dysfunction has been linked to Parkinson's disease and breast cancer (Lavigne et al., 1997; Goodman et al., 2001; Zhu, 2003; Zhu, 2004). In addition, disturbed homocysteine/folate metabolism is associated with brain pathology (Clarke et al., 1998; Muntjewerff et al., 2006) as well, while high tHcy is an established risk factor for cardiovascular disease (Wald et al., 2002; den Heijer et al., 2005). Because the reaction catalyzed by COMT produces homocysteine, functional variants within the COMT gene may influence tHcy levels and possibly reflect disturbed transmethylation capacity implicated COMT-related diseases.

We screened for the rs4680 variant as well as three other SNPs dispersed over the COMT gene (rs2097603, rs4633 and rs174699) and performed haplotype analysis in order to identify whether a specific haplotype was related to homocysteine levels. We show that rs4680 and rs4633 were singularly associated with tHcy levels in a group of Dutch population-based controls, and were responsible for the observed haplotype effect (-13.3%

[95% CI -23.6 to -3.1], P=0.01). Based on functional studies and the finding that the effect size of the rs4680 variant was larger than that of the rs4633 variant we think that the effect of the latter can be fully explained by its high correlation with rs4680 (Shifman et al., 2002; Handoko et al., 2005).

Table 8.4. COMT single-locus genotype effects on tHcy concentrations in population-based controls

Variant	Genotype	N (%)	MAF[b]	Crude mean tHcy [95% CI] (μmol/l)	Crude change % [95% CI]	P trend
rs2097603 (n=422)	AA	117 (27.7)		10.7 [10.0 to 11.5]	0[a]	
	AG	208 (49.3)		10.3 [9.8 to 10.8]	-4.1 [-12.1 to 4.7]	
	GG	97 (23.0)	0.48 (G)	10.3 [9.5 to 11.1]	-4.2 [-13.6 to 6.3]	0.474
rs4633 (n=413)	TT	120 (29.1)		10.8 [10.4 to 11.5]	0[a]	
	TC	199 (48.2)		10.4 [9.9 to 11.0]	-3.0 [-11.0 to 5.6]	
	CC	94 (22.8)	0.47 (C)	10.0 [9.2 to 10.8]	-7.3 [-16.3 to 2.6]	0.143
rs4680 (n=424)	AA	117 (27.6)		10.8 [10.0 to 11.5]	0[a]	
	GA	202 (47.6)		10.5 [9.6 to 11.5]	-2.1 [-10.3 to 6.7]	
	GG	105 (24.8)	0.49 (G)	9.6 [8.7 to 10.7]	-10.4 [-19.0 to -0.8]	0.036
rs174699 (n=420)	TT	365 (86.9)		10.4 [10.0 to 10.8]	0[a]	
	TC	53 (12.6)		10.2 [9.2 to 11.3]	-2.4 [-12.7 to 9.1]	
	CC	2 (0.5)	6.8 (C)	10.7 [6.3 to 18.3]	2.8 [-39.8 to 75.1]	0.748

[a] reference category, [b] MAF, minor allele frequency

The rs4680 (also denoted 324G>A or Val108/158Met) polymorphism has been extensively studied for its effect at the molecular level, mostly because of its potential role in schizophrenia susceptibility. Functional studies showed that the COMT 324AA genotype is associated with decreased enzyme activity *in vitro* and human brain extracts (Lotta et al., 1995; Chen et al., 2004) although the Val-allele was expressed at a slightly lower level in human brain (Bray et al., 2003). In addition, genetic association studies are not consistent regarding the involvement of COMT in the aetiology of schizophrenia (Glatt et al., 2003; Fan et al., 2005), but haplotypes that include rs4680 were associated with schizophrenia risk (Shifman et al., 2002; Handoko et al., 2005). However, other variants cannot be excluded as a cause for the observed association (Shifman et al., 2002). Our results show that 324GG genotype is significantly associated with decreased tHcy levels, and may support the observation of lower expression of the Val-allele in brain tissue (Bray et al., 2003).

One may raise the question whether it is plausible that the flux through the COMT enzyme is high enough to generate a relatively large difference in tHcy (about 10%) between subjects having the 324GG and 324AA genotype. Studies with Parkinson's disease patients whose

tHcy levels rose upon L-DOPA treatment (Yasui et al., 2003; Lamberti et al., 2005a; Lamberti et al., 2005b), indicate that a higher COMT flux is reflected in plasma tHcy levels. In addition, a recent genome-wide linkage scan performed by Souto and colleagues identified another methyltransferase, Nicotinamide N-methyltransferase (NNMT), as a possible major determinant of tHcy (Souto et al., 2005). This shows that not only methyltransferases with a high flux-rate, like guanidinoacetate- and phosphatidylethanolamine methyltransferase (Brosnan et al., 2004), contribute to homocysteine synthesis, but also methyltransferases with an apparently modest contribution to overall methyltransferase activity.

In conclusion, the functional rs4680 variant is associated with tHcy in the general population. If this reflects a disturbed methylation capacity of catecholamines and catecholestrogens, than an effect on the nervous and vascular system may be proposed. On the other hand, the 324AA genotype may, relative to the 324GG genotype, contribute to higher tHcy and cause an inhibitory effect on methyltransferases, due to an increase in AdoHcy, in peripheral tissues as well. If COMT dysfunction leads to disturbed catecholmethylation, the measurement of plasma AdoMet and AdoHcy levels as a marker of methylation capacity could provide additional evidence. *In vitro* studies should confirm that catecholmethylation is indeed disturbed. These data may give a hint as to what is the high-risk allele in COMT-related disorders and schizophrenia in particular.

Acknowledgements

This study was in part supported by grant 2002B68 from the Netherlands Heart Foundation. Martin den Heijer, MD, PhD, is supported by a VENI-grant from the Dutch Organization for Scientific Research (NWO).

Chapter 9

Summary, Discussion and Future perspectives

Summary

A general introduction on homocysteine metabolism, and clinical implications of hyperhomocysteinemia along with epidemiological evidence of the relationship between homocysteine and schizophrenia is given in chapter 1. Chapters 2-6 concern the effect of B-vitamins, homocysteine, and methylenetetrahydrofolate reductase (MTHFR) 677C>T polymorphism on schizophrenia risk, and chapter 7 and 8 examine the interconnection of catechol-O-methyltransferase (COMT) and homocysteine metabolism.

In chapter 2 we examined the folate, vitamin B6, vitamin B12, and plasma total homocysteine (tHcy) concentrations, and the prevalence of the MTHFR 677C>T polymorphism in 35 schizophrenia patients and 104 control subjects. Schizophrenia patients had significantly lower plasma folate, and elevated red blood cell folate concentrations compared to controls. Plasma folate concentrations below the 10^{th} percentile of controls were associated with an approximate 4 to 7 fold (before and after adjustment of folate concentrations for tHcy, respectively) risk of having schizophrenia. We found a significant dose-response relation between plasma folate and schizophrenia risk, which is suggestive for a protective effect by high folate plasma concentrations. We observed no differences between the plasma vitamin B6, vitamin B12, or tHcy concentrations of patients and controls. Moreover, homozygosity for the MTHFR 677C>T polymorphism was not associated with an increased risk of schizophrenia. These findings are suggestive for a disturbed folate metabolism in schizophrenia patients, independently of homocysteine status.

An association between low folate status and psychopathology was hypothesized already approximately half a century ago, and since then a vast amount of studies has been published on this topic. We reviewed the available literature with data on the association of folate status and schizophrenia examined in a case-control design (chapter 3). Positive findings in this matter may provide a rationale for routine determination of folate in patients, and treating these patients with folate supplements in case of low folate status. A total of seven eligible studies were found with a total number of 325 patients and 560 control subjects. Three out of seven studies reported lower plasma folate concentrations more frequently in schizophrenia patients compared to controls. Unfortunately, all studies included showed a variety of methodological shortcomings. We have therefore concluded that evidence of an association between aberrant folate status and schizophrenia is still limited, and does not justify routine folate determination or supplementation in schizophrenia patients.

In chapter 4 we determined the MTHFR 677C>T genotype distribution in a group of 254 schizophrenia patients and 414 control subjects. The MTHFR 677TT genotype was associated with a nearly significant increased risk of having schizophrenia (odds ratio (OR) of

1.6 [95% CI: 0.96-2.8]), and the MTHFR 677CT genotype accounted for an OR of 1.3 (95% CI: 0.91-1.8). In addition, tHcy concentrations were measured in 62 patients and 432 control subjects. When homocysteine concentrations were stratified into quartiles of the control distribution, we calculated an increased risk for schizophrenia in the fourth and third quartile versus the lowest quartile (OR=3.3 [95% CI: 1.2-9.2], and OR=3.1 [95% CI: 1.2-8.0], respectively). We also observed a dose-response relation of increasing homocysteine concentrations and increasing schizophrenia risk (P=0.036). Schizophrenia patients with the MTHFR 677TT or 677CT genotype showed higher tHcy concentrations than 677CC patients, whereas in the control group only subjects with 677TT genotype had higher tHcy levels compared to wild-type subjects.

In chapter 5 we performed a meta-analysis of the studies published up to 2005 on the prevalence of the MTHFR 677C>T polymorphism or homocysteine concentrations in schizophrenia patients and control subjects. The meta-analysis on homocysteine included 812 patients and 2113 control subjects from ten studies. We found that a 5 µmol/L higher tHcy concentration was associated with an increased risk of schizophrenia (OR=1.70 [95% CI: 1.27-2.29]). This finding must be interpreted with caution because of the heterogeneity observed among the homocysteine studies along with various methodological shortcomings of each study in this meta-analysis. The meta-analysis on the MTHFR 677C>T polymorphism included 2265 patients and 2721 control subjects from ten studies. The association of homozygosity of the MTHFR 677C>T polymorphism and schizophrenia with an OR of 1.36 (95% CI: 1.07-1.72) is indicative for a causal relation between aberrant homocysteine metabolism and schizophrenia based on published studies.

Despite the positive association between homozygosity for the MTHFR 677C>T genotype and schizophrenia risk population stratification, such as ethnic or gender differences of genotype distribution cannot be ruled out. Because within-family gene distribution analysis avoids confounding by population stratification, we examined the prevalence of the MTHFR 677C>T polymorphism in a collection of 120 families with schizophrenia offspring, and its impact on schizophrenia risk (chapter 6). This study showed an equal transmission of 677C and 677T allele from heterozygous parents to schizophrenia offspring. However, genotype relative risks of 1.43 (95% CI: 0.83-2.47) and 1.42 (95% CI: 0.54-3.78) were estimated for the 677TT and 677CT genotype, respectively, relative to the 677CC genotype. A meta-analysis using data from three family-based studies, including our data, yielded no evidence implicating the 677T allele in schizophrenia risk (P=0.58). By applying a log-linear model, we found no evidence that the maternal MTHFR genotype influences the risk of schizophrenia offspring substantially. We concluded that with respect to development of schizophrenia in offspring no evidence was found for an independent maternal genetic effect involving the MTHFR 677C>T polymorphism.

In chapter 7 we analysed the question whether the coexistence of MTHFR 677C>T and COMT 324G>A polymorphism leads to an enhancement of schizophrenia risk. COMT is interconnected with MTHFR in the methylation pathway, and the 324G>A variant in the COMT gene has been studied extensively in relation to schizophrenia risk. In a population-based case-control study of 252 schizophrenia patients and 405 controls, we observed no overrepresentation of any genotype of each mutation in patients compared to controls as expressed by non-significant odds ratios. Although our study revealed no evidence of interaction between both polymorphisms within control or patient group, an increased risk of schizophrenia associated with joint occurrence of the COMT 324AA and MTHFR 677TT genotype (OR=3.08 [95% CI: 1.08-8.76]) was found. In addition, we found a positive association between the number of low enzyme activity alleles of the COMT and MTHFR genotype combinations and risk of schizophrenia. We therefore concluded that our data show preliminary evidence for an effect of COMT 324G>A and MTHFR 677C>T polymorphism combination on schizophrenia risk, possibly reflecting a biological interaction between the two corresponding dysfunctional enzymes. However, further confirmation with a larger sample size is necessary.

COMT is involved in the S-adenosylmethionine-dependent methylation of catecholamines and in this way contributes to homocysteine formation. We investigated the possibility of COMT 324G>A polymorphism, and three other polymorphisms as contributors to fasting tHcy concentration using a large group of 438 control subjects (chapter 8). Homozygotes for the 324G>A variant had higher tHcy concentrations than subjects with the 324GG genotype. Adjustment for well-established determinants of homocysteine concentration, such as age, sex, MTHFR 677C>T polymorphism, and plasma folate levels did not alter the findings. It was concluded that the COMT 324G>A variant is associated with tHcy levels in the general population, possibly reflecting disturbed methylation of catecholamines and catecholestrogens. This interesting observation deserves further exploration, because of the possible implication in COMT-related diseases, such as schizophrenia.

Discussion

Starting in the middle of the last century a role of aberrant methylation in the aetiology of schizophrenia was hypothesized (Osmond and Smythies, 1952). Several studies reported adverse behavioural responses in schizophrenia patients, but not in normal controls, when given high oral loads of methionine (Cohen et al., 1974). Furthermore, a higher incidence of schizophrenia patients was detected among subjects with homocystinuria compared to the general population. These findings were suggestive for a causal role of enzyme defects in the methyl transfer cycle (Smythies, 1963), in which homocysteine is an intermediate

metabolite. This one-carbon cycle hypothesis initiated research of schizophrenia risk in relation to sensitive markers of aberrant homocysteine metabolism, such as B-vitamin concentrations, tHcy concentrations, and genetic determinants. At the time the studies of this thesis were initiated results of previous reports on homocysteine metabolism and schizophrenia were scarce and inconsistent.

B-vitamins and schizophrenia risk

By examining folate and homocysteine metabolism we observed decreased plasma and elevated RBC folate levels as risk factors for schizophrenia, which appeared to be independent of tHcy concentrations (chapter 2). As folate is obtained from the diet, low folate status may be a consequence of poor nutrition (Lucock, 2000). However, folate intake is correlated with vitamin B6 intake (De Bree et al., 2001), but no deficiency of vitamin B6 levels was found in our study. Elevated RBC folate concentration has been observed in patients with NTDs as a result of decreased activity of MTHFR due to the MTHFR 677C>T polymorphism (Van der Put et al., 1995; Van der Put et al., 1996), although not consistently (Molloy et al., 1997). MTHFR dysfunction alters the folate distribution in red blood cells from methylated to formylated folate polyglutamates, the latter folate form accumulating inside the cell (Bagley and Selhub, 1998). We did not observe an increased prevalence of the MTHFR 677C>T variant in patients compared to controls, but other polymorphisms in the MTHFR gene not investigated in this thesis may account for elevated RBC folate concentration. Folate redistribution has also been observed in subjects heterozygous for the 677C>T mutation in combination with heterozygosity of the 1298A>C mutation in the MTHFR gene (Van der Put et al., 1998). The MTHFR 1298A>C mutation was not examined in our study.

Folate deficiency has been reported between 8% and 50% in patients with various psychiatric disorders, including schizophrenia (Young and Ghadirian, 1989). Unfortunately, comparatively few studies provided data on folate levels in schizophrenia patients (chapter 3). These studies showed no substantial support for an aberrant folate metabolism in schizophrenia patients. However, all studies showed a variety of methodological flaws defying a more definite conclusion on the relationship between folate and schizophrenia. Nevertheless, biologically plausible rationales seem to exist for the effect of B-vitamins on mental functioning, because of their role in neurotransmitter metabolism, and methylation processes (Mudd et al., 2001; Bottiglieri, 2005). The only studies that can provide more definitive answers for the causality question, are intervention studies. Beneficial clinical effects of folate supplementation in schizophrenia patients have been reported in schizophrenia patients with low RBC folate (Godfrey et al., 1990) (n=9), or elevated tHcy (Levine et al., 2006) (n=42) levels at entry of the trial. These two double-blind placebo-controlled trials were rather small, but the findings are encouraging. In contrast, vitamin B12

levels in relation to schizophrenia risk have not been subject of extensive study (Silver, 2000), and only anecdotic reports of vitamin B12 administration to schizophrenia patients (Regland et al., 1997; Kemperman et al., 2006) have been published. As reviewed previously, there is no adequate support from controlled trials in favour of an effect of vitamin B6 supplementation on mental function in schizophrenia patients (Kleijnen and Knipschild, 1991). However, there is preliminary evidence that vitamin B6 is effective in the treatment of neuroleptic-induced movement disorders in schizophrenia patients (Lerner et al., 2001; Lerner et al., 2004). In conclusion, our findings on the relationship between folate and schizophrenia need replication in larger samples, and do not justify routine B-vitamin determination or administration in the population of psychiatric patients.

Homocysteine and schizophrenia risk

The results of a meta-analysis of the current literature (chapter 5) support to the conclusion presented in our previous report (chapter 4) that elevated tHcy concentration is a risk factor for schizophrenia. Caution is warranted, because tHcy concentration is determined by numerous environmental factors (Refsum et al., 2004). In line with this, schizophrenia risk associated with hyperhomocysteinemia varied between different studies possibly reflecting differential distribution of confounders. Homocysteine-influencing factors such as poor nutritional status, smoking and coffee consumption have been reported to be present frequently in schizophrenia patients (Brown et al., 1999; Poirier et al., 2002). Remarkably, Stahl et al. (2005) found that only 24% of the variance for tHcy levels in schizophrenia patients was explained by homocysteine determining variables such as gender, serum folate and vitamin B12 levels. Regarding confounding factors like smoking or medication, no effect on tHcy levels was shown (Reif et al., 2005; Stahl et al., 2005). Variance in tHcy proved best explained by folate in male patients, and by vitamin B6 and B12 in females (Kemperman et al., 2006). Further insight into the association between homocysteine and schizophrenia can only be obtained when future studies take into account the homocysteine-influencing factors.

In this thesis tHcy levels were not analysed in subgroups of the subjects included, other than stratification by MTHFR 677C>T genotype. Next to the MTHFR 677TT genotype, we found elevated tHcy concentrations in schizophrenia patients with the heterozygous MTHFR 677C>T genotype compared to patients homozygous for the normal genotype (chapter 4). The MTHFR 677CT genotype itself (Kluijtmans and Whitehead, 2001), or in combination with the MTHFR 1298AC genotype (Van der Put et al., 1998) may result in elevated tHcy concentrations. Combined heterozygosity for the 677C>T and 1298A>C polymorphism has been associated with an increased risk of schizophrenia (Sazci et al., 2003; Sazci et al., 2005), but not consistenly (Vilella et al., 2005). However, we did not investigate the prevalence of MTHFR 1298A>C in any study of this thesis.

Schizophrenia is a heterogeneous disorder with a variety of symptoms. It is possible that increased homocysteine levels are associated with specific subgroups or clinical conditions of patients. For example, tHcy levels were found to be elevated in young male schizophrenia patients (Levine et al., 2002; Applebaum et al., 2004), a finding that needs replication. There is still no convincing explanation for this gender-specific effect of hyperhomocysteinemia in schizophrenia. One can only speculate whether biological or rather socio-economic factors like low vitamin intake due to impoverishment play a role. Elevated tHcy levels were also found to be more prominent in groups of schizophrenia patients with cognitive impairment (Levine et al., 2006), and have been related to psychosis (Monji et al., 2005), and severity of extra-pyramidal symptoms (Goff et al., 2004).

All the studies that reported about hyperhomocysteinemia and schizophrenia risk, as presented in the meta-analysis of this thesis (chapter 5), determined homocysteine concentrations retrospectively. This hampers the discrimination between either a causal or a consequential role for hyperhomocysteinemia in schizophrenia. In this respect, there is an interesting study in progress examining the relationship between prenatal homocysteine levels as determined in mothers, and risk of schizophrenia in adulthood (Brown et al., 2004; Brown and Susser, 2005).

MTHFR and schizophrenia risk

If homocysteine is causally related to schizophrenia determinants of hyperhomocysteinemia are expected to be found more often among cases than controls. In this thesis, we examined the prevalence of the 677C>T MTHFR polymorphism, which is a well-established genetic determinant of tHcy, in schizophrenia patients and controls (chapter 2 and 4). The results of our initial study showed no increased frequency of the 677TT genotype (chapter 2), which may be due to the low number of schizophrenia patients included. Evidence for this line of thought stems from the results presented in chapter 4, which is an enlargement of our previous study, showing a nearly significant increased risk (OR=1.6 [95% CI: 0.96-2.8]) of schizophrenia associated with the 677TT genotype in the MTHFR gene. When these data were incorporated in a meta-analysis based on published studies the MTHFR 677C>T mutation in homozygous state appeared to be a modest, but significant genetic risk factor for schizophrenia with a pooled risk estimate of 1.36 (95% CI: 1.07-1.72) (chapter 5). The MTHFR 677TT genotype has not consistently been associated with schizophrenia (Kunugi et al., 1998; Virgos et al., 1999; Muntjewerff et al., 2003; Yu et al., 2004), and there was great variation in the risk estimates for the MTHFR 677TT genotype between studies. Differences in geographic distribution of the 677T allele (Wilcken et al., 2003), and sample size, but also clinical heterogeneity of the schizophrenia population may contribute to differences between risk estimates. The MTHFR 677TT genotype may be more

prevalent in schizophrenia patients of male gender (Joober et al., 2000; Sazci et al., 2005), or in the group of neuroleptic responders (Joober et al., 2000). Clearly, large case-control studies are needed to detect and replicate reliable risk estimates of the prevalence of the MTHFR 677C>T polymorphism in subgroups of schizophrenia patients.

Most studies that examined the association between prevalence of the 677C>T MTHFR polymorphism and schizophrenia risk used a case-control design. Applying a family-based study design may circumvent confounding by population stratification as detected in case-control studies (Cordell and Clayton, 2005). Moreover, studying the prevalence of this polymorphism may reveal a mother-child interaction *in utero*, if any. It has been demonstrated that NTDs are related to a folate-sensitive genetic defect in homocysteine metabolism (Van der Put et al., 1995; Botto and Yang, 2000). Remarkably, NTD risk was increased if patient or mother were homozygous for the 677C>T polymorphism, but was the strongest if both the mother and child were carrying the 677TT genotype. We observed no transmission distortion of the 677T allele in families with schizophrenia offspring, which is in accordance with the result of the meta-analysis using data from three family-based studies, including our data (chapter 6). However, a genotype relative risk of 1.43 (95% CI: 0.83-2.47) was estimated for the 677TT genotype relative to the 677CC genotype in our family-based study which is in the same magnitude as the pooled odds ratio from our meta-analysis of case-control studies (OR=1.36) (chapter 5). When we applied a log-linear model to the case-parent data no evidence for a strong maternal genetic effect on schizophrenia risk was observed. Nevertheless, it is possible that mothers carrying the MTHFR polymorphism may be predisposed to increased risk of schizophrenia offspring when maternal folate status is low during pregnancy. In order to gain more insight in the relationship between MTHFR and schizophrenia future studies should focus on the maternal MTHFR genotype in combination with maternal folate status related to schizophrenia risk.

COMT, MTHFR and schizophrenia risk

Schizophrenia has a complex pattern of inheritance, and unknown molecular aetiology. It has been thought that schizophrenia is genetically attributable to multiple interactive genes of small effect (Lewis et al., 2003). In chapter 7 the effect of genetic variations of two interconnected enzymes of the methylation pathway, COMT and MTHFR, on schizophrenia risk were subject of study. COMT is involved in the S-adenosylmethionine-dependent methylation of catecholamines, such as dopamine and in this way contributes to homocysteine formation (Figure 1.1). We hypothesized that the combined effect of genes encoding for COMT and MTHFR would confer a significant susceptibility to schizophrenia. Many studies have mentioned a role for COMT in the aetiology of schizophrenia (Fan et al., 2005), because of its key role in dopamine metabolism. Dopaminergic dysfunction has been

suggested as a risk factor for schizophrenia (Kapur, 2003). Despite the low numbers of subjects carrying the combination of the COMT 324AA and MTHFR 677TT genotype an increased risk of schizophrenia was associated with this compound genotype in our study (chapter 7). No other study on the subject of psychiatric disorders thus far has investigated the prevalence of the combination of COMT and MTHFR polymorphisms, and our results await replication in independent data sets.

The common 324G>A polymorphism in the COMT gene has been studied extensively for its effect on enzyme activity (Lotta et al., 1995; Bray et al., 2003; Chen et al., 2004), gene expression (Bray et al., 2003), and association with schizophrenia (Egan et al., 2001; Shifman et al., 2002; Xu et al., 2004; Fan et al., 2005). The COMT related methylation reactions produce homocysteine. Elevated tHcy concentrations appeared in levodopa treated patients with Parkinson's disease (Yasui et al., 2003; Lamberti et al., 2005a), indicating that a higher COMT flux is reflected in plasma homocysteine concentrations. In this thesis, four polymorphisms of the COMT gene, and their effect on tHcy levels in population-based controls were examined (chapter 8). Only the COMT 324G>A variant was associated with homocysteine, although the effect of this polymorphism on tHcy concentration is lower than that of the MTHFR 677C>T polymorphism. The increase of tHcy concentration associated with the COMT 324 G>A variant might reflect an increased methylation capacity resulting in higher total plasma homocysteine concentrations. Determination of AdoMet and AdoHcy concentrations, as markers of methylation capacity, may contribute to unravel the complexity of COMT dysfunction in the aetiology of schizophrenia. Moreover, relating COMT activity and gene expression to brain function, including cognitive function and emotional processing, might provide additional insight into the complex relation between functional COMT loci and schizophrenia symptoms (Tunbridge et al., 2006). In analogy with the effect modification of the MTHFR 677TT genotype on tHcy levels by plasma folate status (Van der Put et al., 1996) COMT variants may be influenced by environmental factors, including folate (Goodman et al., 2001) or cannabis use (Caspi et al., 2005).

Future perspectives

The studies presented in this thesis provide evidence of an association between aberrant homocysteine metabolism and schizophrenia as reflected by hyperhomocysteinemia and increased prevalence of the MTHFR 677TT genotype. Schizophrenia as a disease entity has been proven to be clinically heterogeneous with a multifactorial aetiology in which multiple genes interact influenced by environmental factors. Future studies should take these factors into consideration.

Heterogeneity of study groups

In this thesis, we have not attempted to stratify schizophrenia patients according to clinical subgroups. It is possible that an aberrant homocysteine metabolism is more prevalent in certain subgroups of the schizophrenia population as reflected by the heterogeneity of the phenotype. Thus far, some clinical conditions which are commonly present in the schizophrenia phenotype have been related with markers of aberrant homocysteine metabolism. Increased tHcy or decreased folate concentrations have been related to cognitive impairment (Garcia and Zanibbi, 2004; Kado et al., 2005 Durga et al., 2006), psychotic symptoms (Monji et al., 2005), tardive dyskinesia or extrapyramidal symptoms (Goff et al., 2004), and depressive symptoms (Bottiglieri et al., 2000; Bjelland et al., 2003). Schizophrenia is reported to be more prevalent in males (Aleman et al., 2003), a finding that lacks a solid explanation. Remarkably, there is some evidence for an association of elevated tHcy concentrations in male schizophrenia patients compared to male control subjects (Levine et al., 2002; Applebaum et al., 2004). However, this finding of gender-specificity needs replication in larger populations, and adjustment of several potential confounders of homocysteine metabolism are needed.

Genetic studies

During the course of the studies of this thesis, many genetic variations in genes encoding enzymes or proteins involved in the folate-dependent homocysteine pathway have been identified (Gellekink et al., 2005). Thus far only the MTHFR 677C>T polymorphism and to a lesser extent the 1298A>C polymorphism have been studied in relation to homocysteine metabolism and schizophrenia risk. As schizophrenia is a common disorder with a prevalence of 1:100, the frequency of variants in candidate genes in the general population, next to MTHFR, must be high in order to constitute a significant effect on schizophrenia risk. Genetic variants that modulate homocysteine or folate levels (Table 9.1) are genes of particular interest to study in relation to the risk of schizophrenia. Several kinetic studies showed decreased activities of enzymes of homocysteine metabolism, such as methionine-adenosyltransferase (MAT) and serine-hydroxymethyltransferase (SHMT) in erythrocytes (Carl et al., 1978, Kelsoe et al., 1982; Smythies et al., 1986; Gomes-Trolin et al., 1998) in schizophrenia patients compared to control subjects. Although a decrease of MAT activity was also found in cortex gyrus frontalis of patients with schizophrenia (Gomes-Trolin et al., 1998), this change of enzyme activity may be attributed to the use of neuroleptic medication of the patients. Glutamate carboxypeptidase II (GCPII), an enzyme regulating intestinal folate absorption, and activating NMDA receptors in the brain, may also have a role in the aetiology of schizophrenia (Goff et al., 2001). In the hippocampus and prefrontal cortex of patients with schizophrenia decreased activity of GCPII, and low brain levels of GCPII were observed

(Tsai et al., 1995; Coyle et al., 2003). Recently, a common variant in the GCPII gene, a 1561C>T polymorphism, has been reported to decrease the enzyme activity and has been associated with lower levels of serum folate and elevated homocysteine levels (Devlin et al., 2000). Genetic association studies are needed to examine the effect of the polymorphisms encoding these candidate enzymes on schizophrenia risk. Although several genetic variants of hyperhomocysteinemia have been identified, and the heritability of hyperhomocysteinemia is about 50%, only about 10% can be explained by variants including the MTHFR 677C>T polymorphism (Kluijtmans et al, 2003). This indicates that a significant proportion of the heritability of hyperhomocysteinemia still needs to be resolved.

Table 9.1. Several genetic determinants studied for their effect on plasma total homocysteine*

Enzym	Locus	Polymorphism	Amino acid substitution	Allele frequency in % [mutant allele]
Cystathionine γ-lyase	1p31.1	1364G>T	S403I	29 [T]
Methylenetetrahydrofolate reductase	1p36.3	677C>T	A222V	30-40 [T]
Methionine synthase	1q43	2756A>G	D919G	20 [G]
Methionine synthase reductase	5p15.3-15.2	66A>G	I22M	46-59 [G]
Glutamate carboxypeptidase II	11q11.2	1561C>T	H475Y	6 [T]
Serine-hydroxymethyltransferase (cytoplasmic form)	17p11.2	1420C>T	L474F	30 [T]
Cystathionine β-synthase	21q22.3	31 bp VNTR		~77 [18xrpt]
Reduced folate carrier 1	21q22.3	80G>A	R27H	38-51 [A]
Transcobalamin	22q12.2	776C>G	P259R	35-47 [G]

* The data are based on the systematic review of Gellekink et al. (2005)

Another topic of interest for future studies may be the role of gene-gene and gene-environment interactions in the aetiology of schizophrenia in the context of homocysteine metabolism. Several gene-gene interactions already have been related to the aetiology of NTD, such as MTHFR 677TT and MTHFR 1298A>C genotypes (Van der Put et al., 1998; Botto and Yang, 2000), MTHFR 677TT and methionine synthase reductase (MTRR) 66A>G genotypes (Wilson et al., 1999; Relton et al., 2004), MTRR 66A>G and GCPII 1561C>T genotypes (Relton et al., 2004), and MTRR 66A>G and methionine synthase (MTR) 2756A>G genotypes (Doolin et al., 2002). These polymorphisms are associated with hyperhomocysteinemia in carrier individuals, and could lead to an increased schizophrenia risk. Moreover, maternal genetic effects, exerted by these genetic variants involved in homocysteine metabolism may give rise to an additional increase of schizophrenia risk. Given the interconnection between homocysteine metabolism and methylation processes

numerous genes encoding enzymes in these processes may interact, thereby influencing schizophrenia risk. Application of rapid screening techniques in which many SNP's of one or more pathways in relation to schizophrenia risk can be examined simultaneously (Erali et al., 2003) is warranted. These SNP's arrays facilitate the analysis of gene-gene interactions in relation to schizophrenia risk, although large samples are required for this approach.

Supplementation studies

Dietary supplementation with folic acid, the synthetic form of folate, and vitamin B12 lowers tHcy concentrations by about 25% to 30% in populations without routine folic acid fortification of food and by about 10% to 15% in populations with such fortification. (Homocysteine Lowering Trialists' Collaboration, 1998). Homozygotes for the MTHFR 677C>T polymorphism have higher tHcy levels compared to subjects with the 677CC genotype, especially when folate status is low. The clinical relevance of the finding that elevated tHcy levels and the MTHFR 677TT genotype are risk factors for schizophrenia depends on the question whether supplementation with folate or other B-vitamins will influence either schizophrenia symptoms or risk.

Although folate supplementation has shown beneficial clinical effects in schizophrenia patients (Godfrey et al., 1990; Levine et al., 2006), the evidence is still limited. Larger and more long-term double-blind, randomised and placebo-controlled trials are needed to assess a possible beneficial effect of homocysteine lowering therapy on schizophrenia symptoms. The effects of different dosages of B-vitamins should also be subject of study in these trials. A combined analysis of future trials should have adequate power to determine the effects of homocysteine lowering by B-vitamins on schizophrenia disease outcomes. However, clinical trials will be difficult to obtain, because of the folate food fortification programs in some countries, such as United States of America (United States Food and Drug Administration, 1996). In case of irreversible pathological neurodevelopmental damage due to elevated homocysteine, the beneficial effects of B-vitamin supplements in schizophrenia patients would be limited.

The ultimate proof for a causal relation between hyperhomocysteinemia and schizophrenia risk is a decrease in the incidence of schizophrenia after periconceptional folate supplementation. Currently, no studies are available examining periconceptional folate supplementation and risk of schizophrenia offspring. However, as folate-food fortification of cereal products in the USA since 1998 has proven to lower tHcy levels (Jacques et al., 1999) one can hypothesize that this will effect the incidence of schizophrenia in the USA in the near future. The impact of folic acid fortification on decrease of the occurrence of NTDs was estimated by almost 20% to 50% (Honein et al., 2001; Ray et al., 2002). It seems worthwhile to investigate if dietary folate supplementation will reduce the risk of neurodevelopmental

disorders, including schizophrenia. Vitamins B12 and B6 supplementation might provide additional protection.

Pathophysiology

Although several biological mechanisms have been proposed through which elevated homocysteine or low folate influence the endothelial (Jacobsen, 2001) or neuronal (Mattson and Shea, 2003) cell function, the relation of these mechanisms to schizophrenia remains unclear (Picker and Coyle, 2005). A major task for future research is to provide evidence that these mechanisms also play a role in the aetiology of schizophrenia.

Firstly, homocysteine could be toxic to the brain, either during the period of neurodevelopment, or after that. Thus far, evidence for the neurotoxic effects of homocysteine comes largely from in vitro studies. Examinations of potential adverse effects of increased homocysteine or folate depletion in neuronal cell cultures showed induction of DNA breakage, apoptosis, adverse effects on synaptic and glial function, potentiation of glutamate neurotoxicity, and oxidative damage to neurons (Lipton et al., 1997; Kruman et al., 2000; Ho et al., 2002; Mattson and Shea, 2003). Interestingly, extra-cellular homocysteine has also been found to be toxic to cultured neurons and neuron cells via stimulation of NMDA receptors (Lipton et al., 1997). Dysfunction of the NMDA receptor has been found in schizophrenia patients (Coyle et al., 2003).

Secondly, decreased intracellular methylation reactions can result from either a decrease in formation of AdoMet or an increase of AdoHcy, both related to elevated tHcy concentrations (Mudd et al., 2001). COMT has been implicated in schizophrenia risk, because of its role in momoamine metabolism, and its influence on cortical interneuronal signalling (Harrison and Weinberger, 2004). In addition to COMT, many other methyltransferases have been identified which may contribute to schizophrenia risk. Hypomethylation of DNA has been suggested to play a role in the pathogenesis of schizophrenia (Abdolmaleky et al., 2004), but evidence is still lacking. Future studies should not only examine methylation of DNA, but methylation of proteins, phospholipids (Sharma et al., 1999) and neurotransmitters in schizophrenia patients as well.

Thirdly, hyperhomocysteinemia is known to induce subtle damage to the placental vasculature (Nelen et al., 2000), and is also associated with pregnancy complications, such as pre-eclampsia (Ray and Laskin, 1999), and adverse birth outcomes, such as low birth weight, and preterm delivery (Scholl and Johnson, 2000). Various studies have shown that obstetric complications such as fetal hypoxia and pre-eclampsia are likely risk factors for schizophrenia (Cannon et al., 2002). By utilizing birth cohorts in which follow-up investigations take place the relation between pre-natal conditions and risk of schizophrenia in adulthood can be examined (Susser et al., 1996; Brown and Susser, 2005). By conducting

systematic assessment of pregnancy, perinatal and neonatal conditions more insight in the role of aberrant homocysteine metabolism may be obtained. Prenatal serum specimens of markers of homocysteine metabolism should be drawn from mothers during first, second and third trimester in order to elucidate the most critical period during gestation. The third trimester of pregnancy may be the most relevant period, because most of the growth of placenta and fetus occur during this period.

In order to elucidate the pathophysiological mechanism of hyperhomocysteinemia or hypofolataemia *in vitro* and *in vivo* studies are needed. *In vitro* models are needed to study the effect of homocysteine on differences in gene expression, assessed by mRNA transcription or enzyme function, within the neuronal cells. Different homocysteine concentrations may be applied to mimic mild hyperhomocysteinemia. *In vivo* studies should include transgenic mouse model to study the effects of hyperhomocysteinemia, low folate and hypomethylation on neuronal structures, central nervous structures, and neurotransmission. Currently, raised tHcy has been associated with atrophic changes in the brain in healthy elderly (Williams et al., 2002; den Heijer et al., 2003; Sachdev et al., 2004). These findings are consistent with the hypothesis that hyperhomocysteinemia induces neurodegeneration. Various studies using visual imaging techniques, such as CAT and MRI, have consistently reported a volume reduction in several brain structures of schizophrenia patients (Tamminga and Holcomb, 2005). By applying MRI, either cross-sectional or longitudinal, a correlation between homocysteine levels and brain atrophy in schizophrenia patients can be examined.

Concluding remarks

In conclusion, our findings of an association between homocysteine, or MTHFR 677TT genotype and schizophrenia risk suggest a causal relationship. The MTHFR polymorphism, by its impact on tHcy levels, may also interact with many other variants of genes of the methylation cycle, including COMT polymorphisms, and with environmental factors such as folate status, thereby contributing to schizophrenia risk. However, it is still not known whether homocysteine itself is pathogenic or whether increased homocysteine levels are a reflection of a disturbed metabolism. By utilizing birth cohorts in which follow-up investigations take place the relation between pre-natal conditions, such as folate status and homocysteine levels, and risk of schizophrenia in adulthood can be examined. Further large scale case-control and family-based studies are needed in order to obtain more insight into the relationship between homocysteine and schizophrenia risk.

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Samenvatting

Dit proefschrift met als titel “Homocysteine metabolisme en het risico op schizofrenie” beschrijft de resultaten van onderzoek naar een mogelijke rol van stoornissen in de homocysteine stofwisseling bij het ontstaan van schizofrenie. Een algemene inleiding betreffende het homocysteine metabolisme, de klinische gevolgen van hyperhomocysteinemie en de epidemiologische aanwijzingen voor een relatie tussen homocysteine en schizofrenie worden beschreven in hoofdstuk 1 van dit proefschrift. In de hoofdstukken 2 tot en met 6 worden de concentraties van B vitaminen en homocysteine, en de veel voorkomende genetische variant van het MTHFR enzym (MTHFR 677C>T polymorfisme) in relatie tot het risico op schizofrenie bestudeerd. De hoofdstukken 7 en 8 beschrijven het onderzoek naar een mogelijk verband tussen het enzym catechol-O-methyltransferase (COMT) en de homocysteine stofwisseling.

Schizofrenie is een veelvuldig voorkomende psychiatrische aandoening, bij 1 op de 100 mensen wordt deze diagnose gesteld. Karakteristiek is een ontstaan van klachten tussen het 20^{e} en 35^{e} levensjaar, maar ook op latere leeftijd kan de diagnose gesteld worden. De klachten betreffen veelal stoornissen in de waarneming, bijvoorbeeld auditieve hallucinaties (horen van stemmen in het hoofd) of wanen zoals ziekelijke achterdocht (paranoïde waan). Alhoewel de oorzaak van schizofrenie niet bekend is zijn er steeds meer aanwijzingen dat de ziekte door zowel erfelijke als omgevingsfactoren bepaalt wordt.

De stofwisseling van homocysteine, een aminozuur, voltrekt zich in de lichaamscel. Na opname van methionine, een essentieel aminozuur dat via de voedingseiwitten opgenomen moet worden, wordt in de lichaamscel methionine omgezet in homocysteine. Met behulp van enzymen en B vitaminen zoals foliumzuur (folaat), vitamine B6 en vitamine B12 wordt homocysteine verder afgebroken of weer omgezet naar methionine. De functie van deze stofwisselingscyclus is het leveren van bepaalde bouwstenen (methyl groepen) om een diversiteit aan stoffen voor het menselijk lichaam aan te maken, zoals vetten, eiwitten, DNA, of neurotransmitters. De hoeveelheid homocysteine in de lichaamscel wordt exact gereguleerd. Een overschot aan homocysteine wordt vanuit de lichaamscel naar het bloedplasma getransporteerd. Als de hoeveelheid van homocysteine in het bloed te hoog wordt, noemt men dit hyperhomocysteinemie. Veel factoren beïnvloeden de homocysteine stofwisseling zoals een verminderde werking van een van de enzymen of een tekort aan B vitaminen. Een belangrijk enzym van de homocysteine stofwisseling is het methyleentetrahydrofolaat reductase (MTHFR). Er bestaat een veelvoorkomende erfelijke variant van het MTHFR enzym, het MTHFR 677C>T polymorfisme dat een verminderde enzym werking tot gevolg heeft. Bij een gelijktijdig tekort aan foliumzuur in het lichaam

resulteert de verminderde MTHFR functie in hyperhomocysteinemie.

Aanwijzingen voor een mogelijk verband tussen een gestoorde homocysteine stofwisseling en schizofrenie werden al vanaf 1961 beschreven. Echter, de studies betroffen veelal gevalsbeschrijvingen of hadden vele methodologische tekortkomingen. De bepaling van de erfelijke MTHFR variant is pas sinds 1995 beschikbaar. Momenteel zijn diverse andere ziektes van het centrale zenuwstelsel, zoals neurale buis defecten (“open ruggetje”), de ziekte van Alzheimer en de ziekte van Parkinson, in verband gebracht met een gestoorde homocysteine stofwisseling.

In hoofdstuk 2 worden concentraties van diverse B vitaminen en homocysteine onderzocht evenals de frequentie van de MTHFR 677C>T variant in het bloed van 35 patiënten met schizofrenie en 104 controle personen. Opmerkelijk is de bevinding dat voor patiënten de concentraties folaat in plasma lager maar in rode bloedcellen hoger waren dan die in de groep van controle personen. Overeenkomstig hieraan was het aantal schizofrene patiënten met een verlaagde concentratie van plasma folaat (folate lager dan de waarde die gevonden wordt bij 10% van de controles) groter dan het aantal controles. De concentraties van de vitaminen B6 en B12 evenals die van homocysteine waren in de groep van patiënten vergelijkbaar aan die van de controle groep. De frequenties van de MTHFR variant in beide groepen verschilden niet significant. Onze bevindingen suggereren een gestoorde foliumzuur stofwisseling in patiënten met schizofrenie, onafhankelijk van de homocysteine stofwisseling.

In hoofdstuk 3 worden de studies besproken die het verband bestudeerden tussen folaat concentraties en schizofrenie. Het verband tussen een tekort aan folaat en psychische aandoeningen, zoals schizofrenie wordt al decennia verondersteld, echter zonder solide bewijs. Slechts drie studies die zowel patiënten als controle personen onderzochten, rapporteerden een verband tussen verlaagd folaat in het bloed en schizofrenie. Echter, de besproken studies vertoonden allen diverse tekortkomingen in de studieopzet en laten dan ook geen definitieve conclusies toe.

In hoofdstuk 4 wordt de frequentie van de MTHFR variant in een groep van 254 patiënten met schizofrenie en 414 controle personen bestudeerd. Aanwezigheid van het MTHFR 677TT genotype bleek gerelateerd aan een verhoogd risico op schizofrenie (odds ratio=1.6 en 95% betrouwbaarheidsinterval (95% BI): 0.96-2.8). In een subgroep van 62 patiënten en 432 controle personen werd onderzocht hoe vaak een verhoogde homocysteine concentratie (hyperhomocysteinemie) voorkomt bij patiënten met schizofrenie in vergelijking met de controle personen. Het bleek dat hyperhomocysteinemie gerelateerd was aan een drie keer verhoogd risico op schizofrenie.

In hoofdstuk 5 worden de resultaten van de onderzoeken zoals beschreven in de hoofdstukken 2 en 4 vergeleken met de huidige literatuur over schizofrenie, hyperhomocysteinemie en de MTHFR 677C>T mutatie. Deze meta-analyse toont aan dat de

conclusie gerechtvaardigd is dat zowel hyperhomocysteinemie als het genetische defect in het enzym MTHFR beschouwd moeten worden als risicofactoren voor het ontstaan van schizofrenie. Een stijging van 5 µmol/l in homocysteine concentratie was gerelateerd aan een 70% (95% BI: 27-129%) hogere kans op schizofrenie. Het door ons aangetoonde verband tussen de MTHFR mutatie en de 36% (95% BI: 7-72%) grotere kans op schizofrenie veronderstelt dat er mogelijk sprake is van een oorzakelijk verband tussen een gestoorde homocysteine stofwisseling en schizofrenie.

In hoofdstuk 6 wordt de aanwezigheid van de MTHFR 677C>T mutatie onderzocht in 120 families waarin een kind met schizofrenie voorkomt. Resultaten van studies met alleen patiënten en controle personen kunnen vertekend zijn door verschillen in groepskenmerken zoals etniciteit of geslacht (populatiestratificatie). Familiestudies hebben dit bezwaar niet. Naast overerving van de MTHFR mutatie kan een moeder met deze mutatie in de baarmoeder een schadelijke invloed uitoefenen op de foetus. Als de MTHFR mutatie bij de moeder aanwezig is, kan het, in geval van een foliumzuur tekort, niet alleen homocysteine concentraties in het bloed van de moeder verhogen, maar ook via de placenta die van het ongeboren kind. Opmerkelijk genoeg werden er in onze studie geen aanwijzingen gevonden voor een verhoogde aanwezigheid van het genetische defect in MTHFR in moeders of vaders van de kinderen met schizofrenie. Ook het samenvoegen van onze bevindingen met die van twee andere familiestudies (meta-analyse) leverde hiervoor geen bewijs.

In hoofdstuk 7 wordt de aanwezigheid van de mutatie in MTHFR gen en een genetische variant van het enzym COMT onderzocht in 252 patiënten met schizofrenie en 405 controle personen. COMT is van belang voor de afbraak van dopamine, een neurotransmitter. Stoornissen in de stofwisseling van dopamine worden al decennia lang in verband gebracht met de symptomen van schizofrenie. De COMT 324G>A variant resulteert in een verminderde enzym werking, en wordt in diverse studies in verband gebracht met een verhoogd risico op schizofrenie. MTHFR en COMT hebben beide een functie in de overdracht van methyl groepen (methyleringsproces). De combinatie van beide enzym mutaties leidt tot een drie keer verhoogd risico op schizofrenie, hetgeen hoger is dan het berekende risico voor elke mutatie afzonderlijk. Het is van belang om dit onderzoek in grotere groepen te herhalen, omdat het aantal personen met de combinatie van COMT en MTHFR mutaties in onze studie laag was.

In hoofdstuk 8 wordt, als vervolg op het onderzoek in hoofdstuk 7, het effect van de COMT 324G>A variant, en enkele andere COMT varianten, op homocysteine concentratie onderzocht in een groep van 438 controle personen. Bij de afbraak van dopamine draagt COMT bij aan de vorming van homocysteine. Personen die homozygoot waren voor de COMT variant (COMT 324AA genotype) hadden een verhoogde homocysteine concentratie in het bloed vergeleken met personen met het COMT 324GG genotype. Het verschil bleef

aanwezig ook na correctie voor diverse factoren die homocysteine concentratie kunnen beïnvloeden zoals leeftijd en geslacht, de aanwezigheid van de MTHFR 677C>T variant, en de folaat concentratie. Geconcludeerd wordt dan ook dat de rol van COMT in de context van de homocysteine stofwisseling en het risico op schizofrenie nader onderzoek verdient.

In hoofdstuk 9 worden de resultaten van dit proefschrift samengevat, bediscussieerd, en worden aanbevelingen gedaan voor verder onderzoek. In dit proefschrift is aangetoond dat de aanwezigheid van een verhoogde homocysteine concentratie een bescheiden risicofactor is voor schizofrenie. De bevinding dat de MTHFR 677C>T mutatie vaker voorkomt bij patiënten met schizofrenie (hoofdstuk 5) dan in de groep met controle personen is een aanwijzing dat er een oorzakelijk verband bestaat tussen hyperhomocysteinemie en schizofrenie. De homocysteine stofwisseling wordt door veel genetische en omgevingsfactoren factoren beïnvloed. Uit vervolg onderzoek zal bijvoorbeeld moeten blijken of er een wisselwerking tussen de MTHFR mutatie en de mutatie in het gen voor COMT bestaat. Behandeling van grote groepen schizofrene patiënten met vitamine B supplementen kan inzicht geven in het verband tussen hyperhomocysteinemie en de symptomen van de ziekte. In geval de schadelijke werking van homocysteine reeds in de baarmoeder heeft plaatsgevonden zal er weinig tot geen positief effect van de vitamine B toevoeging te verwachten zijn. Daarom dient vervolgonderzoek zich ook te richten op afwijkingen in de homocysteine of foliumzuur stofwisseling gedurende de zwangerschap.

Dankwoord

Het boekje is af. Het onderzoek beschreven in dit proefschrift was geen eenmansactie, maar kwam tot stand met de hulp van veel mensen. Die personen wil ik dan ook via deze weg nadrukkelijk bedanken.

Prof. dr. R.S. Kahn, beste René, bedankt voor het vertrouwen dat je in me stelde toen ik bij je aanklopte met het verzoek mij te begeleiden bij het doen van onderzoek. Je gaf me alle ruimte, maar hield de voortgang goed in de gaten en je stuurde bij waar nodig. Ik heb er veel van geleerd en zal de kennis zeker toepassen in het opzetten van nieuwe onderzoeksprojecten. Nogmaals bedankt.

Dr. H.J. Blom, beste Henk, jij was vanaf het begin erbij betrokken en gaf zo ook richting aan het onderzoekstraject. Ook al was ik als psychiater een vreemde eend in de bijt van “Homocysteine-land”, je gaf me alle steun in het opzetten van het onderzoek. Ik zal de autoritjes naar Utrecht missen, waarbij niet alleen de voortgang van het onderzoek, maar een heel arsenaal aan onderwerpen ter sprake kwam.

Dr. M. den Heijer, beste Martin, je stroomde wat later in het onderzoekstraject, maar speelde met je methodologische kennis een cruciale rol bij het succesvol afronden van meerdere studies zoals beschreven in dit proefschrift.

Aan de “wieg” van dit proefschrift stond oud-hoogleraar Obstetrie en Gynaecologie prof. dr. T.K.A.B. Eskes die jarenlang onderzoek verrichtte en begeleidde betreffende het homocysteine metabolisme bij moeder en kind. Zonder uw initiatief om contact te leggen met de afdeling psychiatrie van het UMC St Radboud zou dit proefschrift nooit ontstaan zijn. Prof. dr. F.G. Zitman, beste Frans, bedankt dat je mij in de beginfase van dit onderzoek hebt willen begeleiden. Eveneens personen van het eerste uur, die deel uit maakten van het toenmalige werkgroepje “homocysteine en schizofrenie”, zijn dr. N.M.J. van der Put, dr. B.A. Ellenbroek en prof. dr. E.A.P. Steegers. Nathalie, Bart en Eric bedankt voor jullie inzet en discussies om dit onderzoek ook daadwerkelijk vorm te geven en te starten. Ook de steun van Nelleke Hamel als ondersteunend onderzoeksverpleegkundige in de begin fase van de studies van dit proefschrift was van grote waarde.

Veel dank ben ik verschuldigd aan Mechteld Hoogendoorn en Maartje Aukes vanwege hun hulp bij het verzamelen en testen van patiënten en familieleden. Jullie steun vanuit Utrecht was geweldig en ik wens jullie veel sterkte bij de afronding van jullie eigen promotieonderzoek.

Bij deze wil ik dr. R. J. Sinke, van UMC Utrecht, bedanken die het mogelijk maakte om meerdere genetische bepalingen te verrichten, evenals Alfons Bardoel en Bart Jungerius voor het uitvoeren van deze bepalingen en aanleveren van de monsters.

Uiteraard wil ik ook de medewerkers van het Laboratorium voor Kindergeneeskunde en Neurologie van harte bedanken voor hun niet aflatende inzet de vele bloedmonsters te verwerken en in het bijzonder Leo Kluijtmans, Addy van der Graaf en Dinny van Oppenraaij en Henkjan Gellekink. Henkjan veel succes bij het verdedigen van je eigen proefschrift binnenkort.

De directie van GGz Nijmegen ben ik veel dank verschuldigd voor de ondersteuning bij het tot stand komen van dit proefschrift. Vanuit GGz Nijmegen heb ik ook veel steun ervaren van mijn collega's, in het bijzonder binnen het CAB-LZ, die regelmatig informeerden naar de stand van zaken.

De leden van de manuscriptcommissie, de hoogleraren H.G.M. Westenberg, J.E.J.M. Hovens, G.F. Koerselman, J. Lindemans en Y.M. Smulders, wil ik via deze weg bedanken voor hun inzet om mijn manuscript te beoordelen.

In de laatste fase van het tot stand komen van dit proefschrift ben ik veel dank verschuldigd aan Jan Hertman voor het corrigeren van de engelse tekst, Harrie Eijkemans voor het vervaardigen van de prachtige omslag en mijn lieve broer Henk voor de onvermoeibare hulp bij het instellen van de lay-out.

Mijn paranimfen, Atse van der Hoek en Arne Zschage, die ik al zo lang ken. Met jullie aan mijn zijde voelt het heel vertrouwd.

Lieve Pa en Ma, jullie stelden regelmatig de vraag aan mij waar het proefschrift over ging. Hopelijk dat dit proefschrift bijdraagt aan het antwoord op die vraag, maar ik leg het graag nog een keer uit.

Elke, Stijn en Veerle, jullie vinden het vast jammer dat dit boekje af is. Stiekem langer spelen (Playmobil, Gamecube of Sims) of later naar bed terwijl ik nog "even" iets moest nakijken vonden jullie in ieder geval geen enkel probleem. En ik eigenlijk ook niet lieverds.

Lieve Philou. Jou zelf bedachte zin voor dit dankwoord begint met "Ondanks mijn echtgenote heb ik dit proefschrift toch maar afgerond". Niets is minder waar. Je zag vanaf het begin dat het doen van onderzoek geen kortstondig gebeuren zou zijn, maar gaf me toch alle ruimte en steun. Bedankt lieverd.

Curriculum vitae

Jan-Willem Muntjewerff werd op 1 januari 1961 te Elburg geboren. Van 1973 tot 1979 werd het Atheneum B doorlopen aan het Newman College te Breda. Van 1980 tot 1981 studeerde hij psychologie aan de Universiteit van Amsterdam (UvA) alwaar de propedeuse werd behaald. Aansluitend startte hij met de studie geneeskunde, eveneens aan de UvA, hetgeen afgerond werd met het behalen van het artsexamen in 1989. Hierna was hij werkzaam als arts-assistent psychiatrie in het toenmalige psychiatrisch ziekenhuis “Het Groot Graffel” te Warnsveld. Gedurende deze periode werd vanaf 1990 gestart met de opleiding tot psychiater, waarbij hij voor de basis opleiding achtereenvolgens werkzaam was op de afdeling deeltijdbehandeling te Doetinchem (opleider: dr. J. Hofstra) en op de psychiatrische universiteitskliniek en polikliniek van het UMC St Radboud te Nijmegen (opleiders: prof. dr. G.J. Zwanikken en prof. dr. F.G. Zitman). Hierna werd de stage sociale psychiatrie vervuld bij het toenmalige Riagg Nijmegen (opleider: prof. dr. P.P.G. Hodiamont). Van maart 1994 tot maart 1995 werd de opleiding afgerond met een keuze stage, waarbij hij werkzaam was als arts-assistent op de afdeling Neurologie van het UMC St Radboud (opleider: prof. dr. G.W.A.M. Padberg). Aansluitend is hij tot op heden werkzaam als psychiater bij de Stichting Geestelijke Gezondheidszorg Nijmegen. Jan-Willem is getrouwd met Philou Rypma en is vader van drie kinderen: Elke, Stijn en Veerle.