Homocysteine related Nutritional and Genetic Risk Factors for Human Congenital Heart Defects

Homocysteine related Nutritional and Genetic Risk Factors for Human Congenital Heart Defects
Anna Corina Verkleij-Hagoort
Thesis Erasmus MC, University Medical Centre – with ref. – with summary in Dutch

The work presented in this thesis was performed at the Department of Obstetrics and Gynaecology/Division of Obstetrics and Prenatal Medicine of Erasmus MC, University Medical Centre, Rotterdam, the Netherlands, in close collaboration with the Departments of Paediatric Cardiology of the same hospital and of Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam, and the child health centres of 'Thuiszorg Nieuwe Waterweg Noord' in the surroundings of Rotterdam.

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Homocysteine related Nutritional and Genetic Risk Factors for Human Congenital Heart Defects

Homocysteine gerelateerde voedings- en genetische risicofactoren voor humane congenitale hartafwijkingen

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Uw ogen hebben mijn ongevormde klomp gezien

Psalm 139:16 (Statenvertaling, 1637)

Contents

List of abbreviations

ANOVA	Analysis of Variance
BMI	body mass index
CHD	congenital heart defect
CI	confidence interval
CLP	cleft lip with or without cleft palate
CV	coefficient of variation
CX43	connexin 43
DNA	deoxyribonucleic acid
DRI	dietary reference intake
en%	percentage of total energy intake
et al.	and others
FFQ	food frequency questionnaire
GATA4	GATA binding protein 4
H	hour
L	litre
mg	milligram
min	minute
mL	millilitre
MS	methionine synthase
MTHFR	5,10-methylenetetrahydrofolate reductase
MTRR	methionine synthase reductase
n	number
NA	not available
NKX2.5	NK2 transcription factor 2.5
nmol	nanomol
NTD	neural tube defect
OFC	orofacial clefting
OR	odds ratio
P	probability
PCR	polymerase chain reaction
pmol	picomol
RBC	red blood cell
RDA	recommended dietary allowance
RFLP	restriction fragment length polymorphism
SEM	standard error of the mean
TBX1	T-Box 1
TBX5	T-Box 5
TC	transcobalamin
TDT	transmission disequilibrium test
tHcy	plasma total homocysteine
THF	tetrahydrofolate
µg	microgram
µmol	micromol
^{o}C	degrees Celsius
24HR	24-hour recall

Chapter 1

General Introduction, objectives and outline of the thesis

Congenital heart defects (CHDs) originate from abnormal organogenesis during the embryonic period. They belong to the most common group of major congenital malformations in newborns. Cardiac outflow tract defects are the most prevalent CHDs, such as membranous ventricular septal defects, pulmonary valve stenosis, tetralogy of Fallot and transposition of the great arteries.[1] CHDs are associated with other anomalies or occur as part of a syndrome, but are generally present as an isolated malformation. Worldwide, one million children per year are born with a CHD.[2] In the Netherlands about six per 1,000 live births are affected with a CHD and these malformations occur in approximately nine per 1,000 live births in the United States of America.[3, 4] The reported rate of CHDs increased nearly two-fold since the early 1970s.[4] The increase is assumed to result partially from improved case ascertainment and reporting. In addition, it might be caused by changes in the distribution of risk factors in the population.[5] At present, birth defects are the leading cause of infant mortality[6] with CHDs accounting for one in every three birth defect-related infant deaths.[7] Over the last decades the overall CHD related mortality declined as well as the mortality for most specific CHDs.[8] From 1979 through 1998 the decline of CHD mortality was 38.3% in the United States, but it did not decrease as rapidly as the overall infant mortality decrease of 44.9%.[9]

The increasing number of children with a CHD that now survive into adolescence and adulthood stresses the need for the health-care community to prepare for the challenging and often complex needs and life-long surveillance of adults with a CHD.[4] A substantial part of these surviving children has to undergo surgery and complex medical treatments and they often suffer from serious physical and psychological problems. Moreover, parental distress and financial problems contribute to a reduced quality of life in families of CHD children.[10] In addition, the estimated average lifetime cost was $344,500 per new case of the most clinically important CHDs in 1992 in the United States of America.[11] Therefore, prevention of CHDs can substantially reduce the paediatric morbidity and mortality, and related health care costs as well. However, knowledge about cardiovascular development and pathogenesis is necessary for primary prevention of CHDs.

Embryonic cardiovascular development in human occurs between the third and eighth weeks after conception.[12] Thus, heart development is essentially completed when most women enter antenatal care. Therefore, detailed information about embryonic cardiovascular development generally originates from animal models. The heart primordia are derived from the cardiogenic mesenchyme where angioblasts aggregate and develop into angioblastic cords. These cords form a pair of lateral endocardial tubes, which are brought together by folding of the embryo during the early fourth week and fuse into the single primitive heart tube (*Figure 1, panel A*). Between weeks five and eight of development, the primitive heart tube undergoes processes of looping, remodelling, and septation, starting at the inflow end and progresses towards the outflow end of the heart (*Figure 1, panel A-D*).[13]

During cardiac looping localized parts of the endocardial epithelium will undergo epithelial-to-mesenchymal transformation, thereby giving rise to mesenchymal cells that migrate into the endocardial cushions.[14] These cushions are named according to the region in which they develop: the outflow tract cushions and the atrioventricular cushions. The formation of the atrial septum and

ventricular septum together with rearrangement of the inflow and outflow tract separates the heart into four different chambers. The definitive structures that separate the atrial and ventricular cavities are almost entirely muscular tissue. The small part of the septum that is not muscular, i.e., the membranous septum, is partly formed from the atrioventricular cushions, and in part from the proximal end of the fused outflow cushions.[15]

The common outflow lumen is separated into a pulmonary trunk and an aorta by outflow tract septation. This process involves the cardiac neural crest cell population that contributes to the condensed mesenchyme of the aorticopulmonary septum.[16, 17] Precise control of processes such as neural crest cell migration, differentiation, proliferation, apoptosis and intracellular signalling are essential for correct formation and modelling of the heart and vascular system. The outflow tract cushions are remodelled into arterial valves.[18] Finally, the aorta with its aortic valves is continuous with the left ventricle, and the pulmonary trunk with its pulmonary valves originates from the right ventricle.[13]

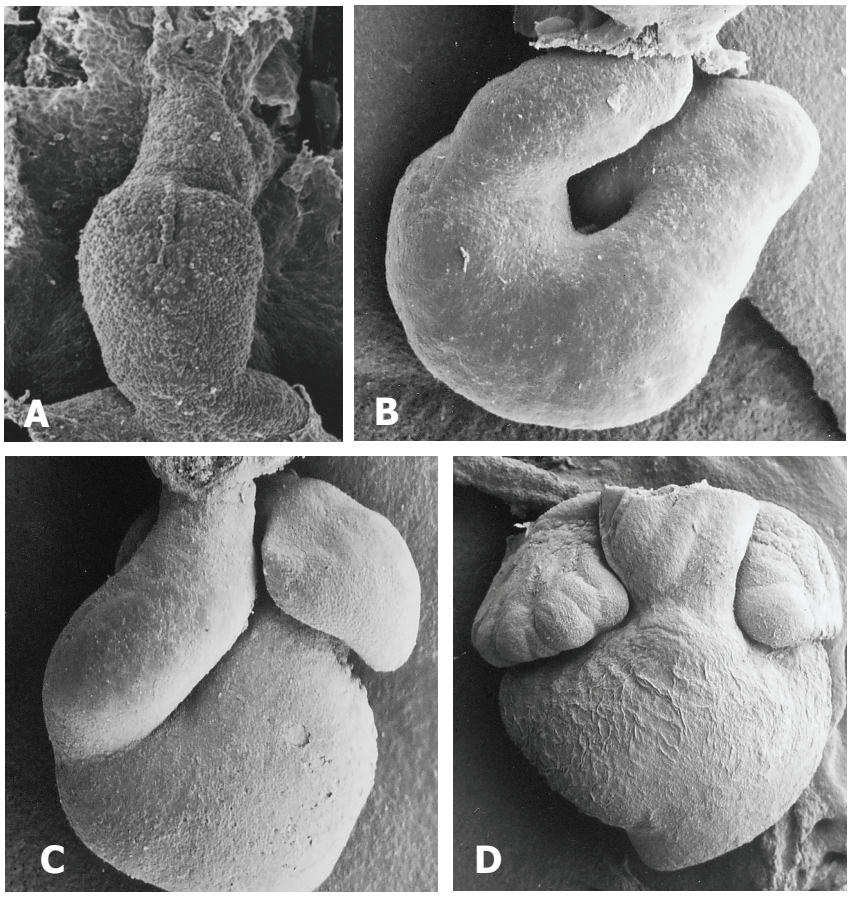

Figure 1. Embryonic cardiovascular development in the chicken. *The heart primordia develop into angioblastic cords, which form a pair of lateral endocardial tubes. The single primitive heart tube is formed by folding of the embryo and fusion of the endocardial tubes (panel A). Thereafter, the primitive heart tube undergoes processes of looping and remodelling (panel B-D). Reproduced with permission from B. Hogers and R.E. Poelmann. Dept. Anatomy, LUMC, the Netherlands.*

With regard to aetiology, only approximately 15% of the CHDs can be attributed to a known cause, even when including genetic and chromosomal conditions with environmental exposures. The strongest genetic contributors to CHDs are the chromosomal abnormalities, e.g., trisomy 21, 18, 13, and the 22q11 deletion.[4] Genetic causes related to human CHDs comprise of mutations in NKX2.5, CX43, GATA4, JAGGED-1, TBX1 and TBX5 genes.[19, 20] Known maternal environmental risk factors are diabetes mellitus, use of medications, pesticides and solvents, obesity, and febrile illnesses.[1] Most

CHDs, however, are considered as complex diseases with a multifactorial aetiology, which are thought to result from interactions between both genetic and environmental factors.[21]

The first studies showing that B-vitamins might be involved in the pathogenesis of congenital anomalies originate from the 1950s. Multiple congenital malformations including cardiovascular defects were observed in rats when a folic acid deficient diet was given to female rats in early pregnancy.[22, 23] Some decades later, the role of B-vitamins in vascular pregnancy complications was reported. Mothers of a child with a congenital malformation and women with placental abruption showed a folate deficiency.[24, 25] Interestingly, Raymond *et al*. demonstrated an association between placental abruption and congenital anomalies, particularly CHDs.[26] These data support the hypothesis that vascular disruptions in early pregnancy may lead to abnormal placental development and birth defects.[21] Thereafter, several epidemiological studies reported that the use of folic acid containing supplements in the periconceptional period reduces the risk of having a child with a CHD.[27-30]

Natural folates occur in foods and differ chemically from the synthetic form, i.e., folic acid, that is used in supplements and fortified foods. Food folate has a 50% lower bioavailability compared with that of synthetic folic acid. The term folate represents the many derivatives of this B-vitamin found in food, blood and tissues. It functions as a one-carbon donor in several important cellular reactions, including the synthesis of nucleotides and methyl transfer reactions important for methylation of DNA, proteins, and lipids. Moreover, folate is an important substrate of the homocysteine metabolism (*Figure 2*).[31] Plasma total homocysteine (tHcy) levels are maintained within narrow ranges by the efficient functioning of genes encoding enzymes that participate in the homocysteine metabolism, such as methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MS), methionine synthase

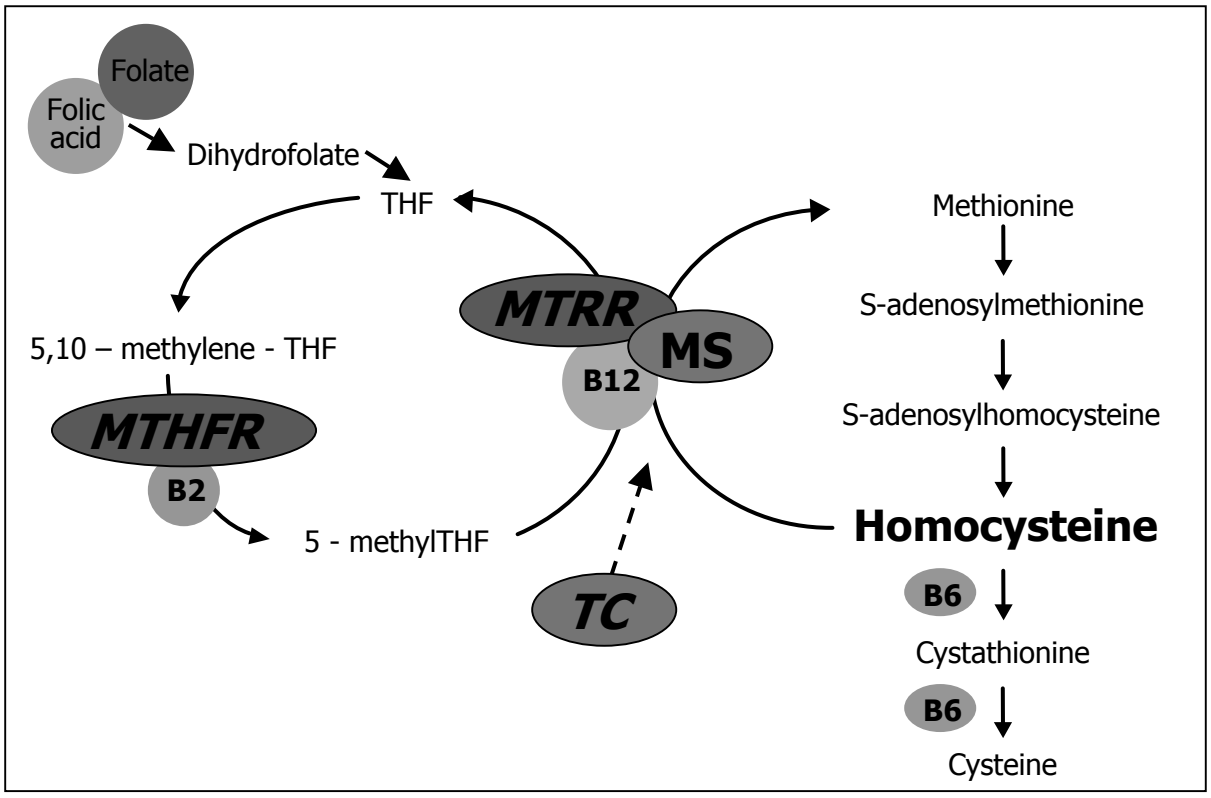

Figure 2. B-vitamin dependent homocysteine metabolism.

reductase (MTRR), and cystathionine β-synthase. Moreover, tHcy concentrations depend on the intake and concentrations of folate, vitamin B12, vitamin B6 and vitamin B2, because these micronutrients are cofactors or substrates in this metabolism.[31, 32] In addition, genetic determinants of B-vitamin transport proteins and receptors may interfere in the homocysteine metabolism by affecting the cellular availability of B-vitamins. For instance, it has been shown that a polymorphism in the transcobalamin gene (TC) is a determinant of the TC and tHcy concentrations.[33]

Mild to moderate hyperhomocysteinaemia results from inadequate intake of B-vitamins as well as from functional polymorphisms in key enzymes involved in the homocysteine metabolism or transport of B-vitamins. Abnormalities in the homocysteine metabolism or the one-carbon metabolism, e.g., hyperhomocysteinaemia or DNA hypomethylation, may contribute to the development of congenital anomalies.[31, 34-37] Animal studies have indicated the involvement of the homocysteine metabolism in heart development. It was shown that homocysteine stimulates the proliferation and migration of avian neural crest cells and inhibits their differentiation.[38] Most chicken embryos exhibited CHDs after injection with homocysteine concentrations comparable to mild hyperhomocysteinaemia in man. It was suggested that homocysteine affects cardiac neural crest cell behaviour and, thereby, leads to the development of CHDs.[39] Interestingly, folic acid supplementation partially rescues these detrimental effects of homocysteine on neural crest cell outgrowth.[38] In addition, our group showed the association between maternal hyperhomocysteinaemia and having a child with a CHD.[40]

The mother is the environment of the developing child in utero and, therefore, the maternal nutritional status will influence the homocysteine metabolism in the embryonic tissues as well. Expression of functional polymorphisms in B-vitamin related genes derived from both parents might also be important in the development of the embryonic heart. In addition, interactions should be considered between the maternal nutritional status and the polymorphisms in B-vitamin related genes of mothers and children. Therefore, we hypothesize that derangements in the homocysteine metabolism affecting the developing primitive cardiovascular system are partially responsible for CHDs.

Objectives

In order to develop and implement effective primary prevention programs, risk factors for CHDs have to be identified. Preconceptional screening of these risk factors and the development of (nutritional) interventions will contribute to future risk assessment and, thereby, prevention of CHDs. The maternal nutritional status has been shown to be involved in the pathogenesis of related birth defects, such as neural tube defects and orofacial clefting. The overall aim of this thesis is to explore the impact of nutritional factors and their interactions with some B-vitamin related genes in the aetiology of CHDs.

Therefore, our objectives are:

1) To investigate associations between maternal B-vitamin and homocysteine status determined by biomarkers and dietary intake, and the risk of having a child with a CHD (Part I).
2) To study gene-nutrient interactions between biomarkers, dietary intake, the B-vitamin related genes MTHFR, MTRR and TC and CHD risk (Part II).

Outline of the thesis

In *Chapter 2* a meta-analysis is presented of published studies that examined maternal hyperhomocysteinaemia or polymorphisms of MTHFR genes in association with the risk of a child with a CHD or orofacial cleft, which is a related malformation with regard to aetiology. We did not include studies of neural tube defects in this meta-analysis, because the folate-dependent pathway has already been investigated and reviewed extensively for these congenital anomalies.[41, 42]

This thesis presents results of the ongoing HAVEN study, which is a Dutch acronym for the study of congenital heart anomalies and the role of genetic and nutritional factors. This case-control study is designed to investigate determinants in the pathogenesis and prevention of CHDs and it has been conducted from June 2003 onwards at the Department of Obstetrics and Gynaecology/Division of Obstetrics and Prenatal Medicine of Erasmus MC, University Medical Centre, Rotterdam, the Netherlands, in close collaboration with the Departments of Paediatric Cardiology of the same hospital and of Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam, and with the child health centres of 'Thuiszorg Nieuwe Waterweg Noord' in the surroundings of Rotterdam. The HAVEN study is funded by the Netherlands Heart Foundation (grant 2002.B027).

The first part of this thesis describes the maternal nutritional status of B-vitamins in association with the risk of having a child with a CHD. Among other factors, the nutritional status is partly determined by the metabolism and intake of nutrients. To study the B-vitamin status we measured the concentrations of serum vitamin B12, serum and red blood cell folate, and tHcy, because these biomarkers partly reflect the effects of the nutritional intake on the homocysteine metabolism (*Chapter 3*). An existing food frequency questionnaire was adapted by the Division of Human Nutrition of Wageningen University in Wageningen to estimate the intake of folate and vitamin B12. Therefore, we performed a validation study for the estimation of the dietary intake of these B-vitamins (*Chapter 4*). We assessed the maternal dietary intake of macronutrients and B-vitamins in case and control-mothers in relation to CHDs in their children (*Chapter 5*).

In Part II, gene-nutrient interactions are described regarding folate and vitamin B12 related genes. In *Chapter 6* we present data of the interactions between nutritional and lifestyle factors, and two polymorphisms in the MTHFR gene whereas *Chapter 7* shows an analysis of these environmental factors in combination with polymorphisms in the MTRR and TC genes. In the general discussion, the results of this case-control study are discussed and our objectives are evaluated (*Chapter 8*). Furthermore, we recommend future research and discuss the implications regarding clinical practice and public health care.

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Chapter 2

Hyperhomocysteinaemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects. A meta-analysis

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Abstract

Several studies have reported an association between hyperhomocysteinaemia, 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphisms and cleft lip with or without cleft palate (CLP) and congenital heart defects (CHDs). However, findings have been inconsistent. A meta-analysis was performed of published studies until September 2006 investigating these associations in both mothers and children. Homocysteine data were provided in two CLP and three CHD studies, and MTHFR polymorphisms were reported in ten CLP and eight CHD studies. Data were analysed using the random effects model in the Cochrane Review Manager.

The pooled odds ratio (OR) of maternal hyperhomocysteinaemia was 2.3 (95% CI 0.4-11.9) for CLP and 4.4 (2.6-7.3) for CHDs. The MTHFR C677T polymorphism and CLP showed pooled ORs of 1.2 (0.9-1.5) in mothers and 1.0 (0.9-1.2) in children, whereas these estimates for the A1298C polymorphism were 1.0 (0.7-1.2) in mothers and 0.9 (0.6-1.2) in children. The MTHFR C677T polymorphism in CHD studies demonstrated a pooled OR of 1.0 (0.8-1.3) for mothers and 1.1 (0.9-1.5) for children. Two studies investigating the maternal A1298C polymorphism in CHDs demonstrated a pooled OR of 1.2 (0.8-1.8). Only one CHD study reported an OR of 1.3 (0.8-2.1) for this polymorphism in children.

In conclusion, this meta-analysis demonstrates that maternal hyperhomocysteinaemia is a risk factor for CHDs. The MTHFR polymorphisms C677T and A1298C in both mothers and children are not independently associated with CLP or CHDs. Future studies should be performed to investigate the interactions between maternal hyperhomocysteinaemia, B-vitamin intake, related polymorphisms and the risk of CLP and CHDs.

Introduction

Orofacial clefting (OFC) and congenital heart defects (CHDs) develop during the first weeks after conception. These defects are common congenital anomalies of multifactorial origin influenced by both genetic and environmental factors.[1, 2] Various epidemiologic studies have shown the protective effect of maternal use of multivitamins in the periconceptional period on the risk of having a child with OFC[3] and a child with a CHD.[4-6]

However, it is unknown which ingredient(s) in multivitamins are responsible for this risk reduction. Indirect evidence that folic acid is a key factor in orofacial and cardiovascular development has been suggested by a study of Hernandez-Diaz *et al.*,[7] in which folic acid antagonists were shown to increase the risk of a child with OFC or a CHD. Mothers who used multivitamins containing folic acid in addition to dihydrofolate reductase inhibitors showed a five-fold lower risk of having a child with OFC or a CHD compared with mothers who did not concomitantly use multivitamins.[7] Folate contributes to the transfer of one-carbon groups as part of nucleotide synthesis, the remethylation of homocysteine to methionine, and the subsequent methylation of DNA, proteins, and phospholipids. Hyperhomocysteinaemia and DNA hypomethylation contribute to the development of complex congenital disorders.[8] Therefore, an optimal maternal and embryonic folate status is important for normal embryogenesis.

The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in the homocysteine metabolism and catalyses the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the predominant circulating form of folate. The MTHFR gene has at least two functional polymorphisms, C677T and A1298C. The MTHFR 677 T-allele is associated with reduced enzyme activity, decreased concentrations of folate in serum, plasma, and red blood cells, and mildly increased plasma total homocysteine (tHcy) concentrations.[9, 10] The second polymorphism, MTHFR A1298C, also affects MTHFR activity but without biochemical changes.[11] Normal MTHFR activity is crucial to maintain the pool of circulating folate and methionine and to prevent the accumulation of homocysteine.[9] Homocysteine can be considered as a useful and important metabolic marker of the overall folate status.

Several studies have reported inconsistent findings on associations between hyperhomocysteinaemia, MTHFR polymorphisms, and both OFC and CHD risk. Therefore, we performed a meta-analysis of all published studies until September 2006 investigating in mothers and children the associations between hyperhomocysteinaemia, MTHFR C677T and A1298C polymorphisms, and the risk of both OFC and CHDs.

Materials and methods

Studies

Potential relevant studies were identified by using MESH terms and text words in a search of PubMed at the National Library of Medicine, Web of Science, Cochrane library, Scopus and the Genetic Association Database through September 1, 2006. The main search terms were 'cleft lip', 'cleft palate', 'heart defects, congenital', 'homocysteine', 'methylenetetrahydrofolate reductase (nadph2)' and 'MTHFR'.

We also conducted searches on congenital anomalies and malformations in general, because OFC and CHDs may not be specified when a study is related to several congenital malformations. Furthermore, we performed manual searches of reference lists in articles found during the electronic searches. If studies presented overlapping data, only the study with the largest number was included. All studies were published in German or English language. Authors were contacted by email asking them to provide data if the content of the paper was insufficient. This meta-analysis is limited to case-control and cohort studies that include data of homocysteine concentrations and/or the MTHFR polymorphisms. It does not include animal studies and studies of case series.

Genetics and embryology suggest that clefts of the primary palate that involve the lip and/or palate are different in aetiology from clefts that affect the secondary palate and are, therefore, developmentally distinct entities.[12, 13] Moreover, patients with cleft lip with or without cleft palate (CLP) represent the largest and most homogeneous group of oral clefts. Therefore, we included only those studies that investigated CLP and excluded the studies concerning isolated cleft palate.

In total, we identified two CLP and five CHD studies investigating tHcy concentrations, and 23 CLP and 11 CHD studies that reported on MTHFR polymorphisms. We excluded two CHD studies on tHcy concentrations, because one study population[14] was part of a larger included study[15] and the other

study used a different cut-off level for the tHcy concentrations and included older participants as well.[16] Twelve studies on CLP and MTHFR polymorphisms were excluded, because seven studies used a family-based design,[17-23] two studies were part of a larger included study,[24, 25] and three studies only reported allele frequencies[26, 27] or incomplete genotype frequencies.[28] In respect of MTHFR polymorphisms in CHD studies, we excluded three studies that included only cases.[29-31] Therefore, the meta-analysis was performed on tHcy data provided in two CLP[32, 33] and three CHD studies[15, 34, 35] and on MTHFR polymorphisms reported in ten CLP[36-45] and eight CHD studies.[41, 46-52]

Statistical Analysis

Hardy-Weinberg equilibrium (HWE) was examined in all studies that included MTHFR genotype frequencies. The available tHcy and polymorphism data for the meta-analysis were entered in Review Manager (RevMan [Computer Program], version 4.2 for Windows. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2003) and analysed with RevMan Analyses (version 1.0 for Windows). This program uses the method of moments to calculate the odds ratio (OR) and 95% confidence interval (CI) for the pooled data. The genetic dominant model was used for comparisons of the polymorphism data. Heterogeneity was assessed using the Q test.[53] Nevertheless, we used the random effects model in all analyses. Funnel plots were used to investigate publication bias.

Results

The included studies were all case-control studies, except for the cohort study of Nurk *et al*.[41] In the CLP meta-analysis, we included 263 mothers for tHcy data, 16,772 mothers and 3,341 children for the C677T polymorphism, and 15,584 mothers and 1,379 children for the A1298C polymorphism (*Table 1*). For the CHD meta-analysis, those numbers were 771, 15,599, 2,167, 14,777 and 303, respectively (*Table 2*). The OR and 95% CI for the individual studies and the pooled analyses are presented in *Figures 1 to 3*. In all tHcy studies, blood samples were drawn after an overnight fast with the exception of the study of Hobbs *et al*.[15] The pooled OR (95% CI) for maternal hyperhomocysteinaemia and CLP was 2.3 (0.4-11.9) and 4.4 (2.6-7.3) for CHDs (*Figure 1*). Based on the data provided in the study of Kapusta *et al*., an OR of 4.8 was computed instead of the OR of 5.1 that is mentioned in their article.[34] Only two studies have investigated hyperhomocysteinaemia in children with respect to CLP[33] and CHDs.[35] Therefore, we could not estimate a pooled OR for hyperhomocysteinaemia in children with CLP or a CHD.

The MTHFR genotype frequencies were consistent with HWE in all studies except for the group of case-children in the study of Junker *et al*.[49] and the control-children in the study of Wenstrom *et al*.[48] The MTHFR 677CT + TT genotype and CLP revealed pooled ORs of 1.2 (0.9-1.5) in mothers and 1.0 (0.9-1.2) in children. For the MTHFR 1298AC + CC genotype, these estimates were 1.0 (0.7-1.2) in mothers and 0.9 (0.6-1.2) in children, respectively (*Figure 2*). In CHD-studies, the MTHFR C677T polymorphism showed a pooled OR of 1.0 (0.8-1.3) for the CT + TT genotype in mothers and 1.1 (0.9-1.5) in children. We considered excluding the studies of Junker *et al*. and Wenstrom *et al*., because the case and the control-group, respectively, were out of HWE.[48, 49] The CT + TT genotype in children then demonstrated a pooled OR of 1.0 (0.8-1.2). Two studies investigated the maternal

References	Subjects	Frequency in mothers, *n* (%)			Frequency in children, *n* (%)		
C677T polymorphism		CC	CT	TT	CC	CT	TT
Shaw *et al*. [1998]	Case	NA	NA	NA	143 (46)	127 (41)	40 (13)
California, USA	Control	NA	NA	NA	156 (41)	178 (46)	49 (13)
Tolarova *et al*. [1998]	Case	39 (42)	37 (40)	17 (18)	43 (39)	49 (44)	19 (17)
Argentina	Control	39 (47)	33 (39)	12 (14)	46 (43)	52 (49)	8 (8)
Martinelli *et al*. [2001]	Case	14 (22)	36 (57)	13 (21)	22 (34)	30 (47)	12 (19)
Italy	Control	46 (43)	43 (41)	17 (16)	46 (43)	43 (41)	17 (16)
Grunert *et al*. [2002]	Case	NA	NA	NA	34 (52)	26 (39)	6 (9)
Germany	Control	NA	NA	NA	90 (49)	69 (37)	25 (14)
Shotelersuk *et al*. [2003]	Case	46 (69)	19 (28)	2 (3)	84 (77)	25 (23)	0 (0)
Thailand	Control	154 (76)	46 (23)	2 (1)	154 (76)	46 (23)	2 (1)
van Rooij *et al*. [2003]	Case	78 (53)	55 (37)	15 (10)	54 (51)	45 (43)	6 (6)
the Netherlands	Control	84 (49)	74 (44)	12 (7)	70 (55)	54 (42)	4 (3)
Gaspar *et al*. [2004]	Case	174 (52)	131 (39)	31 (9)	327 (51)	269 (42)	48 (7)
Brazil	Control	213 (50)	172 (41)	39 (9)	213 (50)	172 (41)	39 (9)
Nurk *et al*. [2004]	Case	12 (55)	8 (36)	2 (9)	NA	NA	NA
Norway	Control	7153 (49)	6029 (42)	1280 (9)	NA	NA	NA
Pezzetti *et al*. [2004]	Case	27 (26)	47 (45)	30 (29)	28 (25)	58 (53)	24 (22)
Italy	Control	95 (33)	151 (52)	43 (15)	95 (33)	151 (52)	43 (15)
Mostowska *et al*. [2006]	Case	60 (50)	46 (38)	15 (12)	NA	NA	NA
Poland	Control	42 (52)	33 (41)	6 (7)	NA	NA	NA
A1298C polymorphism		AA	AC	CC	AA	AC	CC
Tolarova *et al*. [1998]	Case	56 (65)	27 (31)	3 (4)	67 (62)	39 (36)	2 (2)
Argentina	Control	50 (64)	25 (32)	3 (4)	63 (61)	33 (32)	7 (7)
Grunert *et al*. [2002]	Case	NA	NA	NA	28 (43)	30 (46)	7 (11)
Germany	Control	NA	NA	NA	77 (42)	80 (43)	27 (15)
Shotelersuk *et al*. [2003]	Case	30 (45)	33 (49)	4 (6)	55 (50)	48 (44)	6 (6)
Thailand	Control	108 (53)	80 (40)	14 (7)	108 (53)	80 (40)	14 (7)
van Rooij *et al*. [2003b]	Case	57 (46)	52 (41)	16 (13)	48 (51)	34 (36)	12 (13)
the Netherlands	Control	76 (48)	67 (42)	16 (10)	61 (53)	43 (37)	11 (10)
Nurk *et al*. [2004]	Case	9 (41)	10 (45)	3 (14)	NA	NA	NA
Norway	Control	6598 (46)	6332 (44)	1522 (10)	NA	NA	NA
Pezzetti *et al*. [2004]	Case	57 (55)	36 (35)	11 (10)	56 (51)	46 (42)	8 (7)
Italy	Control	121 (42)	130 (45)	38 (13)	95 (33)	151 (52)	43 (15)

Table 1. MTHFR genotype frequencies in CLP studies.

MTHFR A1298C polymorphism in association with CHDs and the pooled OR was 1.2 (0.8-1.8) for the AC + CC carriers (*Figure 3*). Only one CHD study reported of this polymorphism in children and showed an OR of 1.3 (0.8-2.1).[46] The funnel plots were asymmetrical for tHcy concentrations in both CLP and CHD studies, the C677T polymorphism in CHD studies, and the A1298C polymorphism in CLP and CHD studies (data not shown).

References	Subjects	Frequency in mothers, *n* (%)			Frequency in children, *n* (%)		
C677T polymorphism		CC	CT	TT	CC	CT	TT
Junker *et al*. [2001]	Case	NA	NA	NA	51 (45)	42 (37)	21(18)
Germany	Control	NA	NA	NA	129 (57)	78 (34)	21 (9)
Wenstrom *et al*. [2001]	Case	NA	NA	NA	17 (65)	8 (31)	1 (4)
Alabama, USA	Control	NA	NA	NA	104 (90)	9 (8)	3 (2)
Storti *et al*. [2003]	Case	27 (26)	53 (52)	23 (22)	28 (27)	55 (53)	20 (20)
Italy	Control	52 (26)	108 (54)	40 (20)	52 (26)	108 (54)	40 (20)
Nurk *et al*. [2004]	Case	12 (48)	12 (48)	1 (4)	NA	NA	NA
Norway	Control	7153 (49)	6025 (42)	1281 (9)	NA	NA	NA
Lee *et al*. [2005]	Case	NA	NA	NA	110 (51)	89 (42)	14 (7)
Taiwan	Control	NA	NA	NA	114 (58)	68 (35)	13 (7)
Shaw *et al*. [2005]	Case	NA	NA	NA	69 (45)	68 (44)	16 (11)
California, USA	Control	NA	NA	NA	180 (42)	202 (46)	52 (12)
Hobbs *et al*. [2006]	Case	127 (46)	118 (43)	30 (11)	NA	NA	NA
Arkansas, USA	Control	48 (41)	56 (47)	14 (12)	NA	NA	NA
van Beynum *et al*. [2006]	Case	72 (46)	68 (43)	18 (12)	79 (48)	66 (40)	20 (12)
the Netherlands	Control	131 (50)	107 (41)	23 (9)	98 (45)	104 (47)	18 (8)
A1298C polymorphism		AA	AC	CC	AA	AC	CC
Storti *et al*. [2003]	Case	49 (48)	46 (45)	8 (8)	45 (44)	47 (45)	11 (11)
Italy	Control	101 (50)	86 (43)	13 (7)	101 (50)	86 (43)	13 (7)
Nurk *et al*. [2004]	Case	9 (36)	13 (52)	3 (12)	NA	NA	NA
Norway	Control	6598 (46)	6329 (44)	1522 (10)	NA	NA	NA

Table 2. MTHFR genotype frequencies in CHD studies.

Discussion

In this meta-analysis we used the results from studies published until September 2006 to calculate a pooled estimate of the reported associations between hyperhomocysteinaemia, MTHFR polymorphisms and the risk of CLP and CHDs. Maternal hyperhomocysteinaemia was significantly associated with a 4.4-fold increased risk of having a child with a CHD. This finding substantiates the hypothesis that maternal hyperhomocysteinaemia is a risk factor for CHDs. The association was not significant for CLP, but no firm conclusion can be made based on the results of only two published studies. The point estimates for the MTHFR polymorphisms in mothers and children had small confidence intervals and were not significant for both CLP and CHDs. These results suggest that the mutant MTHFR alleles independently do not contribute to the risk of a child with either CLP or a CHD.

It is widely recognized that the origin of these congenital malformations is complex and multifactorial in nature, with genetic and environmental factors affecting various developmental pathways. Of interest, therefore, is that factors like diet and lifestyle can modify the effects of certain genetic polymorphisms. Heterozygosity for both MTHFR polymorphisms can result in a lower MTHFR activity than heterozygosity for either of the MTHFR mutations separately.[11] Two studies showed that tHcy

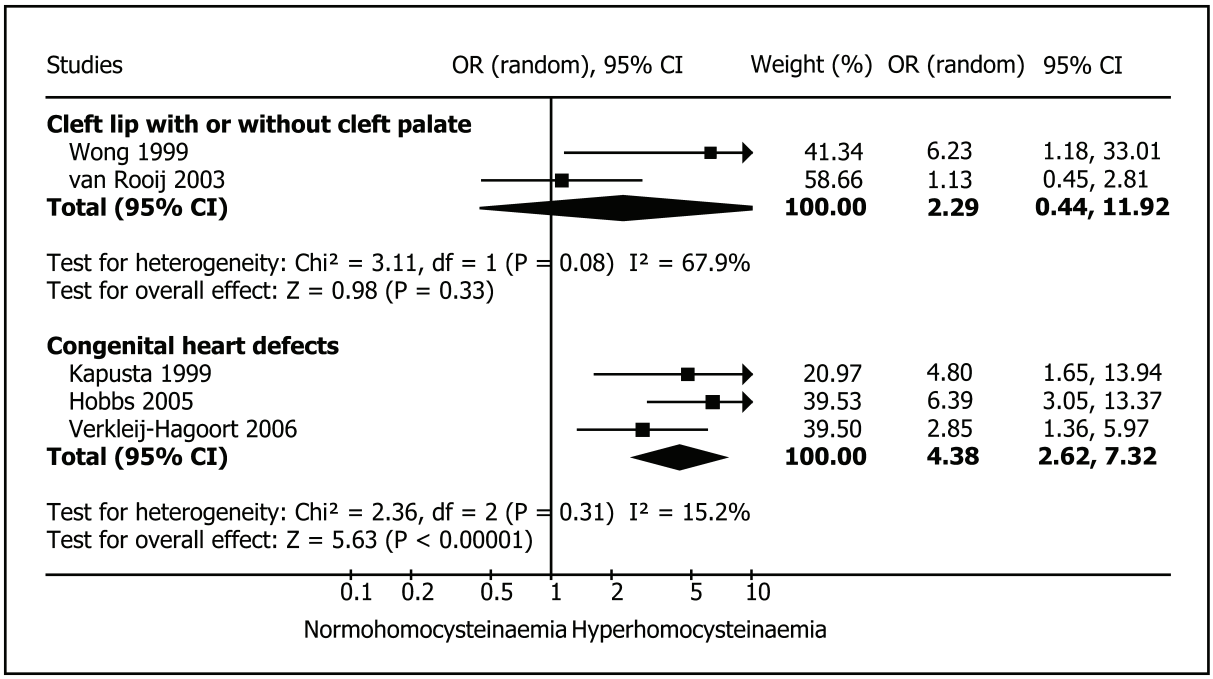

Figure 1. Association between maternal hyperhomocysteinaemia and the risk of cleft lip with or without cleft palate and congenital heart defects.

concentrations were significantly higher in individuals with a combined heterozygosity for the MTHFR polymorphisms than those who were heterozygous for either the C677T or the A1298C genotype.[11, 54] Shotelersuk *et al*. demonstrated a significant increased risk of having a child with CLP if the mother was heterozygous for both MTHFR polymorphisms.[38] The risk of a child with CLP[39] or a CHD[47] was only significantly increased if mothers, carrying the MTHFR 677TT or MTHFR 1298CC genotype, also had a low periconceptional intake of dietary folate and/or folic acid supplements. These examples of gene-gene and gene-nutrient interactions explain why neither of the two MTHFR polymorphisms is an independent risk factor for CLP and CHDs. Moreover, these previously published articles give insight in the protective effect of maternal use of multivitamins containing folic acid in the periconceptional period on the risk of having a child with CLP[3] or a CHD.[4-6] Dietary intake and use of B-vitamin supplements can compensate for the reduced activity of the MTHFR enzyme and lower tHcy concentrations, thereby decreasing the risk of these congenital defects.

We have to consider some strengths and limitations of this meta-analysis. The use of a meta-analysis can overcome the low power of small sample size studies, and, therefore, reconcile previously conducted studies with inconsistent results. Although, the number of included tHcy studies in this meta-analysis is quite low for both congenital defects, all tHcy concentration measurements were performed by the high performance liquid chromatography method. The risk estimates for maternal hyperhomocysteinaemia in CLP and CHDs are consistent and, therefore, laboratory errors are not likely. In the pooled analysis of hyperhomocysteinaemia in mothers and CLP risk only two studies were included with evidence of heterogeneity. This heterogeneity might be caused by the pilot study of Wong *et al*.[32] In this study the cut-off value of the 97.5th percentile was used instead of the 90th

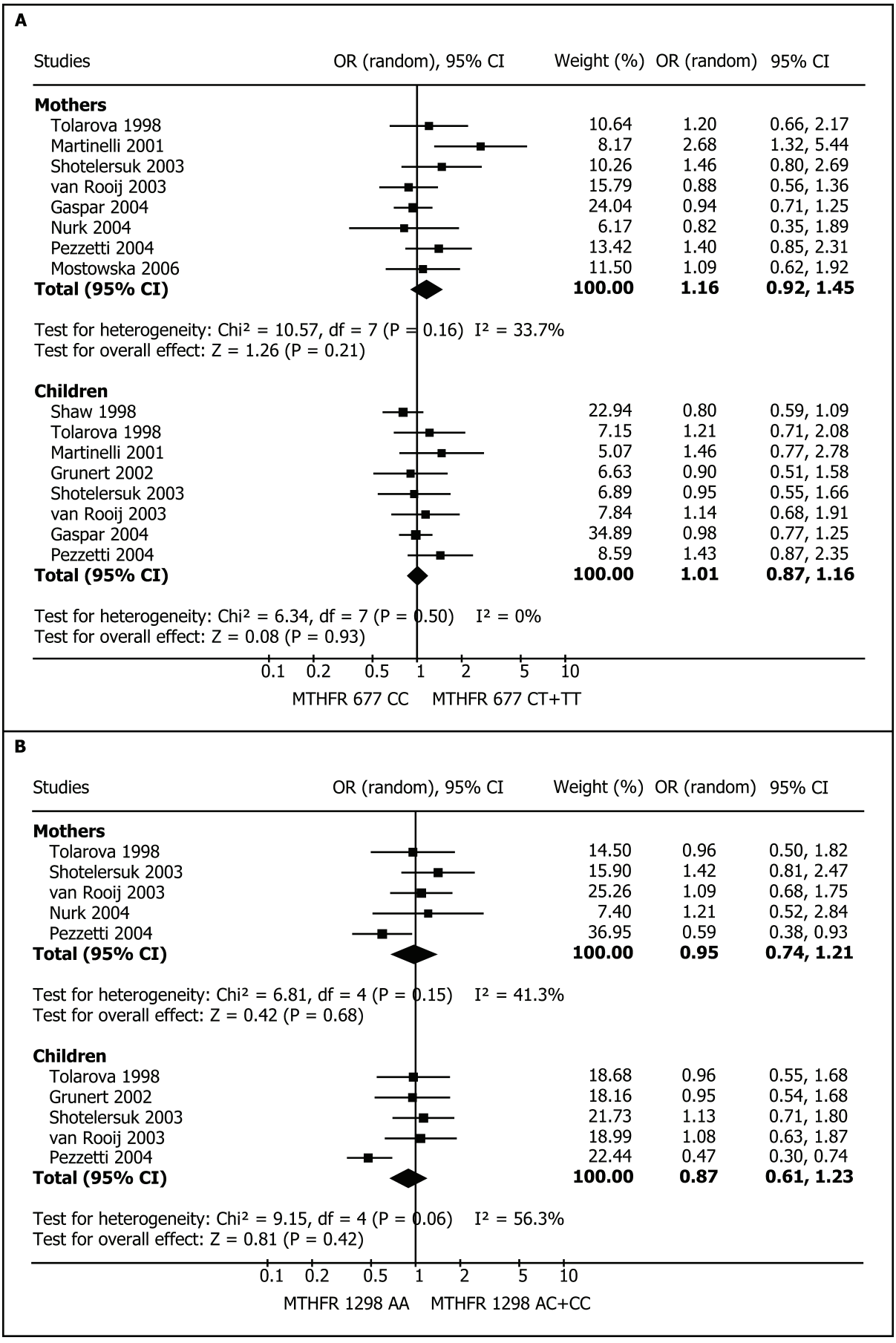

Figure 2. Association between the MTHFR C677T (A) and A1298C (B) polymorphism in mothers and children and cleft lip with or without cleft palate.

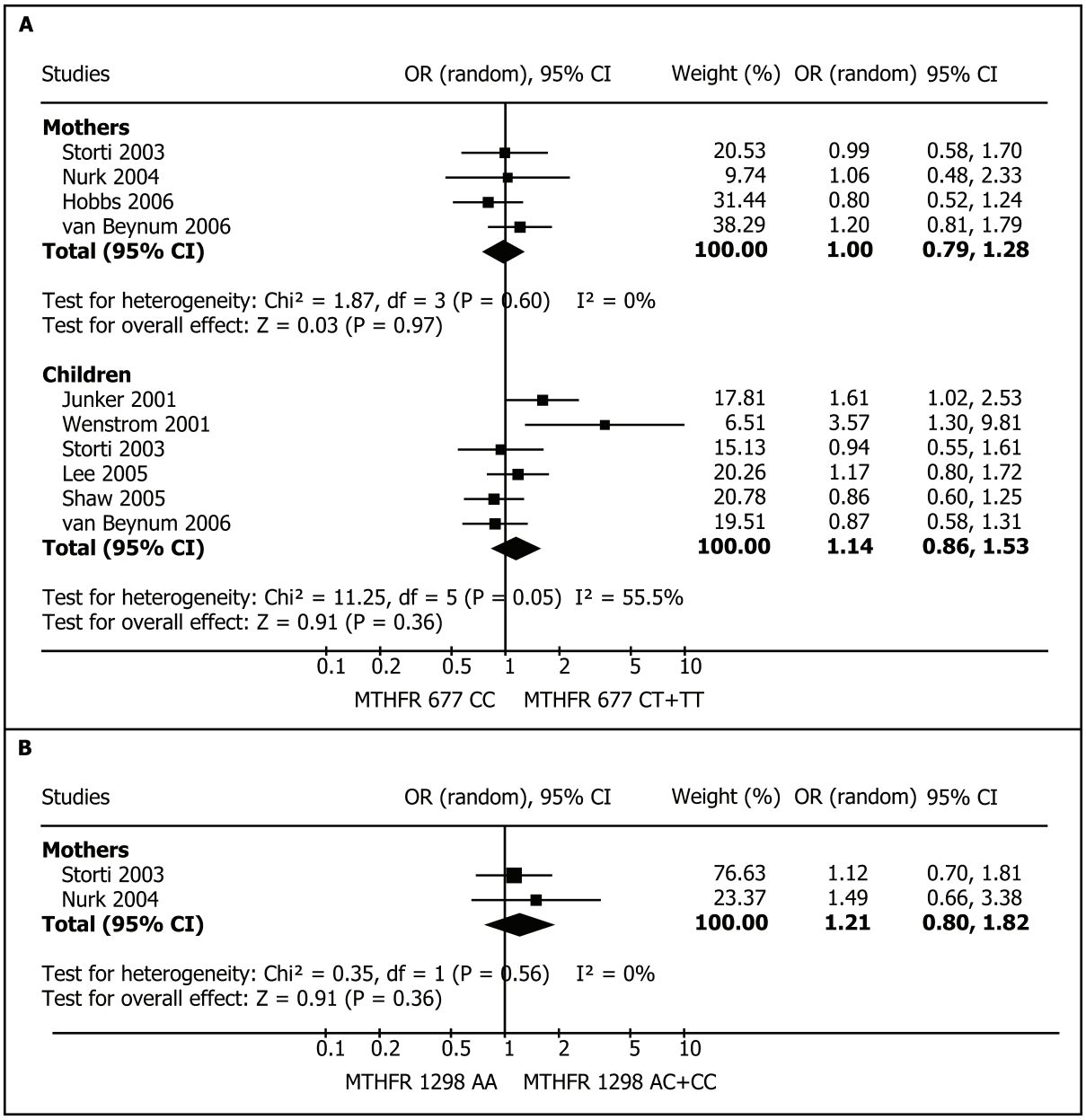

Figure 3. Association between the MTHFR C677T (A) and A1298C (B) polymorphism in mothers and children and congenital heart defects.

percentile that was used by the other tHcy studies, resulting in a low number of hyperhomocysteinaemic mothers. Moreover, they included both methionine afterload and fasting tHcy concentrations.

With concern to the genotyping of MTHFR polymorphisms, laboratory errors are not likely because the genotyping has been done using standard protocols with polymerase chain reactions and restriction enzyme digestion in all studies. Moreover, all studies showed genotype frequencies that were consistent with HWE for both cases and controls with an exception of the cases in the study of Junker *et al*. and the controls in the study of Wenstrom *et al*.[48, 49] The study of Wenstrom *et al*. in particular was the source of heterogeneity in the analysis of CHDs and the MTHFR C677T genotype in children,

possibly because their control-group was out of HWE. We considered exclusion of both studies, but the point estimates did not substantially change. The analysis of CHDs and MTHFR C677T polymorphisms in children, and the analysis of CLP and A1298C polymorphisms in children demonstrated P values less than 0.10 for the Q test for heterogeneity (*Figures 2 and 3*). We used the random effects model in all analyses, thereby accounting for the heterogeneity. In addition, the point estimates of the fixed and the random effects model are nearly identical, which suggests that heterogeneity is not a big issue.

Differences in risk estimates for MTHFR polymorphisms can also be caused by aetiologic heterogeneity between populations, geographical variations of the studied populations, different selection of controls or even by the folate intake of the population.[55] The 677TT and 1298CC genotype frequencies of the included studies demonstrate the known geographical variations.[56-59] Regarding the selection of controls, most studies included unrelated and unaffected controls. We considered the influence of the studies that used another selection of controls, but these studies did not significantly alter the point estimates.

Publication bias has to be addressed in meta-analyses because it can be a substantial cause of bias. The funnel plots are asymmetrical for the A1298C polymorphism and tHcy concentration in both CLP and CHDs, and the C677T polymorphism in CHDs suggesting publication bias towards overestimation of the results. Asymmetry might be due to the low number of published studies. Furthermore, asymmetric funnel plots can also be caused by language and citation bias, if studies with non-significant results are published in non-English languages, and are thereby less likely to be cited. However, the studies with non-significant findings have also been included in the analyses, thereby reducing the chance of publication bias.

In conclusion, we demonstrated in this meta-analysis that maternal hyperhomocysteinaemia is a risk factor for CHDs. The MTHFR polymorphisms C677T and A1298C did not show to be significantly associated with CLP or CHDs. Further research should be performed to investigate the interactions between maternal hyperhomocysteinaemia, B-vitamin intake, related polymorphisms and the risk of CLP and CHDs.

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Part I

B-vitamin and homocysteine status

Chapter 3

Maternal hyperhomocysteinaemia is a risk factor for congenital heart disease

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Abstract

Objective: To investigate the inter-relation between mother and infant homocysteine, folate and vitamin B12 status, and the risk of a child with congenital heart disease (CHD).

Design: Case-control study.

Setting: Erasmus MC, University Medical Centre, Rotterdam, the Netherlands.

Population: Participants were 149 case-mothers and their children with CHD (*n* = 151) and 183 control-mothers with their children (*n* = 175).

Methods: Approximately 17 months after the index-pregnancy maternal fasting and children's random venous blood samples were drawn to measure plasma total homocysteine, serum and red blood cell folate, and serum vitamin B12 concentrations. Data were compared between cases and controls using the Mann-Whitney *U* test. The biochemical parameters were dichotomised according to the cut-off value of the 10th percentile of vitamin concentrations and the 90th percentile of homocysteine concentrations based on control data. Risk estimates for the association between CHD and the biochemical parameters were estimated in a logistic regression model.

Main outcome measures: Medians (minimum-maximum) and odds ratios (95% confidence intervals).

Results: The odds ratio (95% confidence interval) of having a child with CHD was 2.9 (1.4-6.0) for maternal hyperhomocysteinaemia (> 14.3 µmol/L). This finding is substantiated by a significant concentration-dependent risk (P_{trend} = 0.004). Hyperhomocysteinaemic case-mothers showed significantly lower serum folate and vitamin B12 concentrations than normohomocysteinaemic case-mothers. Serum and red blood cell folate concentrations were significantly higher in case-children than in control-children.

Conclusions: Maternal hyperhomocysteinaemia is associated with an increased risk of CHD partially due to low folate and vitamin B12 status. The folate status of children warrants further investigation.

Introduction

Congenital heart disease (CHD) occurs in six per 1,000 live births in the Netherlands and in approximately nine per 1,000 newborns in the United States of America.[1, 2] These malformations are amongst the most common congenital malformations in newborns and account for more than one-third of infant deaths due to congenital anomalies.[2] The most common CHD comprises of outflow defects, such as membranous ventricular septal defects, pulmonary valve stenosis, tetralogy of Fallot and transposition of the great arteries.[3]

Although genetic and environmental factors are involved in the aetiology of CHD, only approximately 15% can be attributed to a known cause.[2] Previous epidemiological studies showed that periconceptional use of multivitamins containing folic acid reduces the risk of having a child with CHD.[4] Shaw *et al*. were the first to show that the maternal use of these vitamins during the sensitive period of heart development reduced the risk of conotruncal heart defects in particular.[5] Hernandez-Diaz *et al*. suggested that folate is a key factor in cardiovascular development by showing an increased risk of CHD after maternal exposure to folate antagonists during the first trimester of pregnancy. In addition, the use of multivitamin supplements containing folic acid diminished this risk.[6]

The B-vitamins folate and vitamin B12 are involved in the remethylation of homocysteine into methionine. A limited supply of folate impairs the methylation cycle, and thereby the methylation of DNA, proteins and lipids. Moreover, a compromised folate and/or vitamin B12 status also results in a mild hyperhomocysteinaemia. Homocysteine can be harmful to cells because it evokes oxidative stress through the production of reactive oxygen species, binds to nitric oxide, or leads to the accumulation of its precursor, S-adenosylhomocysteine, a potent inhibitor of biological transmethylations.[7] Studies in chicken embryos showed 83% subarterial ventricular septal defects after injection of 30 μM homocysteine into the neural tube lumen at the time point of neural crest cell migration.[8, 9] This homocysteine concentration resembles mild hyperhomocysteinaemia in human. Dietary deficiencies in folate have been observed to decrease genome-wide methylation both in humans and animal models, which is corrected after folate repletion. Moreover, derangements in the DNA methylation patterns can lead to developmental malformations.[10]

These findings support a critical role for homocysteine and B-vitamins in the development of CHD. The embryo and fetus are dependent on the maternofetal nutrient transfer. Thus, it can be expected that the maternal homocysteine and B-vitamin status before conception and during the first weeks following conception, e.g. periconceptional, influences pregnancy outcome. Therefore, we performed a case-control study to investigate whether the mother and infant homocysteine, folate and vitamin B12 status influences the risk of CHD.

Materials and methods

Study population

The HAVEN study, a Dutch acronym for the study of heart anomalies and the role of genetic and nutritional factors, is a case-control study designed to investigate determinants in the pathogenesis and prevention of CHD. The study has been conducted from June 2003 onwards at the Department of Obstetrics and Gynaecology/Division of Obstetrics and Prenatal Medicine of Erasmus MC in Rotterdam. Children with CHD and both parents were recruited in collaboration with the Departments of Paediatric Cardiology of the same hospital, and of Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam. Healthy control-children and both parents were enrolled in collaboration with the child health centres of 'Thuiszorg Nieuwe Waterweg Noord' nearby Rotterdam. The domain population comprised case and control-children born from June 2002 onwards and living in the Western part of the Netherlands. The paediatric cardiologists diagnosed and recruited case-children with tetralogy of Fallot (n = 19), transposition of the great arteries (n = 28), atrioventricular septal defect (n = 9), perimembranous ventricular septal defect (n = 44), coarctation of the aorta (n = 14), aortic valve stenosis (n = 5), pulmonary valve stenosis (n = 30), and hypoplastic left heart syndrome (n = 2). Diagnoses were confirmed after birth by echocardiography, and/or cardiac catheterization and/or surgery. Children were eligible as controls if they did not have a major congenital malformation or chromosomal defects according to the medical record and regular health checks by the physician of the child health centre. There was no familial relationship between cases and controls. Invited cases and controls were all aged 11 to 18 months and both groups had

corresponding proportions of males and females. Participating parents had to be familiar with the Dutch language in writing and reading.

Between October 2003 and January 2005 183 control-families and 151 case-families participated in the study. We evaluated the biochemical and questionnaire data of mothers and children that were collected during the hospital visit at Erasmus MC in Rotterdam. The Central Committee of Research in Human and the Medical Ethics Committees of the participating hospitals approved the study protocol. In addition, written informed consent was obtained from both parents.

Study timing

In general, metabolism and nutritional habits are rather constant and do not change except for episodes of illnesses, dieting and increased needs during pregnancy and breastfeeding.[11-13] The study timing between 11 and 18 months after the index-pregnancy is sufficient to mimic the preconceptional biochemical status of the mother and to minimise the risk of undiagnosed less severe CHD in the control-group. At the study moment the general questionnaire was filled out and blood samples were obtained to determine the biochemistry of the mother and her child.

Questionnaire data and anthropometrics

We extracted the following information from the questionnaire administered. The parameters at the study moment included maternal age, body mass index (BMI), educational level, ethnicity, cigarette smoking, use of alcohol, oral contraceptives and medication, age and gender of the child. Ethnicity was classified according to the definitions of Statistics Netherlands.[14] Mothers were classified as Dutch natives, Western or non-Western immigrants. The questionnaire data have been checked on completeness and consistency by the researcher during the hospital visit. Standardised anthropometric measurements were performed, which included maternal height (anthropometric rod, SECA, Hamburg, Germany) up to 0.1 cm accuracy and weight (weighing scale, SECA, Hamburg, Germany) with 0.5 kg accuracy.

Blood sampling and biochemistry

Venous blood samples were drawn from all mothers and children to measure the concentrations of red blood cell (RBC) and serum folate, serum vitamin B12 and plasma total homocysteine (tHcy). Blood sampling was done as described before.[15] The concentrations of serum folate and vitamin B12 were routinely determined by immunoelectrochemiluminescence assay on the Roche Modular E170 (Roche Diagnostics GmbH, Mannheim Germany). Out of an ethylenediamine tetraacetate (EDTA)-treated Vacutainer tube (BD Biosciences, Plymouth, UK) 0.1 mL full-blood was haemolysed with 0.9 mL freshly prepared 1.0% ascorbic acid directly after blood sampling. Subsequently, the haematocrit of the EDTA full-blood was determined on an ADVIA 120 Haematology Analyser (Bayer Diagnostics, Leverkusen, Germany).

The haemolysate was centrifuged at 1,000 x g for five minutes at 18 ^{o}C shortly before folate measurement. The haemolysate folate concentration was recalculated in RBC folate using the following formula: (nM haemolysate folate*10/hematocrit) - (nM serum folate*{1-haematocrit}/haematocrit) = nM RBC folate. The tHcy concentration was routinely measured by high performance liquid chromatography with reverse phase separation and fluorescence detection.[15]

The between-run coefficient of variation for vitamin B12 was 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; for folate these coefficients of variation were 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L and for tHcy 5.9% at 15.3 µmol/L and 3.4% at 39.3 µmol/L. All samples were analysed anonymously within three months after collection. Up to the moment of measurement, samples were kept frozen at –80 ^{o}C.

Data analysis

Maternal age and BMI, and age of the children are presented as medians and compared between cases and controls by using the Mann-Whitney *U* test. Differences in frequencies were tested using the Chi-square test.

We present the biochemical parameters as medians and ranges and the differences between cases and controls were evaluated by the Mann-Whitney *U* test, because of the skewed distributions even after transformation. The parameters considered as confounders comprised of pregnancy, non-fasting state and use of folic acid containing vitamin supplements at the study moment. Therefore, we excluded all pregnant, non-fasting and vitamin supplement using mothers as well as children using vitamin supplements. Thus, the final analysis was based on data of 98 case and 134 control-mothers, and 149 case and 173 control-children. Missing results occurred in approximately 2% of the participants because of failures in blood sampling or laboratory testing.

The risk of CHD was estimated using odds ratios (OR) and 95% confidence intervals (CI) in a logistic regression model. We created the 10th percentile of vitamin concentrations and the 90th percentile of tHcy concentrations based on the data of the control-group. Quintiles of maternal tHcy concentration were computed from the data of the control-mothers. Risk estimates were calculated in a logistic regression model with the lowest quintile as a reference. We performed a trend analysis across the quintiles after modelling the quintiles into five categories ranging from one (reference) to five (highest quintile of tHcy). Probability values $P < 0.05$ were considered statistically significant. All analyses were performed using SPSS for Windows software (version 11.0; SPSS Inc, Chicago, IL, USA).

Results

The median ages of mothers and children were comparable in both cases and controls, 32.1 and 32.8 years for mothers and 17.1 and 17.9 months for children, respectively. At the study moment case-mothers demonstrated a significantly higher median BMI than controls (25.0 and 24.4 kg/m^2, $P < 0.05$). The distributions of ethnicity (78% Dutch native, 5% Western and 17% non-Western immigrant for cases and 81%, 5% and 14% respectively for controls) and educational level (low in 64% of cases and 65% of controls) were comparable in case and control-mothers. Moreover, there were no significant differences in the maternal use of alcohol (cases and controls 44% and 56%, respectively), cigarettes (21% and 22%), oral contraceptives (52% and 48%) and medication (18% and 20%), and the gender of the children (63% and 53% male) at the standardised study moment.

The median tHcy concentration was significantly higher in case-mothers than in control-mothers ($P < 0.01$). The other biochemical parameters were comparable in both groups (*Table 1*). Stratified analysis of the biochemical concentrations for the several types of CHD did not reveal significant diffe-

Mothers	Cases (*n* = 98) [a]	Controls (*n* = 134)
Folate, serum (nmol/L)	15.0 (6.2-27.8)	14.1 (6.4-40.3)
Folate, RBC (nmol/L)	646 (299-1343)	630 (163-1549)
Vitamin B12, serum (pmol/L)	263 (82-602)	248 (89-777)
Homocysteine, plasma (µmol/L)	11.2 (5.1-31.2)	10.3 (5.6-42.0) [b]
Children	Cases (*n* = 146)	Controls (*n* = 167)
Folate, serum (nmol/L)	32.2 (11.3-114.8)	28.7 (11.2-99.6) [b]
Folate, RBC (nmol/L) [c]	1046 (397-2353)	921 (522-1954) [b]
Vitamin B12, serum (pmol/L)	508 (149-1147)	492 (135-1232)
Homocysteine, plasma (µmol/L) [d]	6.3 (3.9-12.3)	6.0 (3.7-12.1)

Table 1. Biochemical parameters at the study moment of mothers and children with CHD and controls. Values are given in median (minimum-maximum).

[a] Serum vitamin B12, serum and RBC folate, n = 97.

[b] P < 0.05 (Mann-Whitney U test).

[c] Control-children n = 166.

[d] Case-children n = 149, control-children n = 172.

rences. The serum folate and vitamin B12 concentrations were significantly lower in hyperhomocysteinaemic case-mothers than in normohomocysteinaemic case-mothers, whereas RBC folate concentrations were comparable. The median (range) serum folate concentrations were 11.1 (6.2-24.1) and 15.1 (8.0-27.8) nmol/L ($P = 0.01$), and vitamin B12 concentrations were 178 (96-411) and 275 (82-602) pmol/L ($P < 0.01$), respectively. In 12 out of 23 hyperhomocysteinaemic case-mothers, serum folate, RBC folate and/or serum vitamin B12 concentrations were below the 10th percentile based on the data of the control-group. In the remaining 11 case-mothers, educational level was significantly lower ($P = 0.01$) and the percentage of smoking mothers was significantly higher ($P < 0.01$) compared with the normohomocysteinaemic case-mothers.

Serum and RBC folate concentrations were significantly higher in children with CHD than in controls (11%, $P = 0.02$ and 12%, $P = 0.001$). However, significantly more cases than controls were formula-fed (36% and 21%, $P < 0.01$). After pooling of case and control-children, serum (37.7 and 27.6 nmol/L, $P < 0.001$) and RBC folate concentrations (1076 and 953 nmol/L, $P = 0.002$) were significantly higher in the 85 formula-fed children than in the 228 children who were fed otherwise. Fifty case-children who were formula-fed still showed significantly higher median serum folate (42.4 and 33.5 nmol/L, $P = 0.02$) and RBC folate concentrations (1146 and 1015 nmol/L, $P = 0.01$) compared with formula-fed controls ($n = 35$).

A high maternal tHcy concentration above 14.3 µmol/L was associated with an increased risk of CHD in their children (OR 2.9, 95% CI 1.4-6.0) (*Table 2*). The risk increased with increasing maternal tHcy concentrations ($P_{trend} = 0.004$, *Table 3*). Significantly more mothers were smoking in the subgroup of hyperhomocysteinaemic case-mothers with the normal vitamin concentrations. After adjustment of

Total group of mothers	Cut-off value	Cases/Controls (*n* = 98/134) [a]	OR	95% CI
Folate, serum (nmol/L)	< 10.1	11/12	1.3	0.5-3.1
Folate, RBC (nmol/L)	< 431	10/13	1.1	0.4- 2.6
Vitamin B12, serum (pmol/L)	< 159	16/13	1.8	0.8-4.0
Homocysteine, plasma (µmol/L)	> 14.3	23/13	2.9	1.4-6.0
Non-supplement users in the periconceptional period [b]	Cut-off value	Cases/Controls (*n* = 38/53)	OR	95% CI
Folate, serum (nmol/L)	< 10.1	6/5	1.8	0.5-6.6
Folate, RBC (nmol/L)	< 431	5/8	0.9	0.3-2.9
Vitamin B12, serum (pmol/L)	< 159	11/6	3.3	1.1-10.0
Homocysteine, plasma (µmol/L)	> 14.3	10/4	4.4	1.3-15.3

Table 2. Associations between the risk of having a child with CHD and the lowest 10th percentile of maternal vitamin concentrations or the highest 10th percentile of maternal plasma total homocysteine concentration.

[a] Serum vitamin B12, serum and RBC folate, case-mothers n = 97.

[b] No use of vitamin supplement containing folic acid in the period of 4 weeks before until 8 weeks after conception

the tHcy concentration for maternal smoking the OR was 3.0 (95% CI 1.4-6.5). Stratification for maternal use of vitamin supplements containing folic acid in the periconceptional period demonstrated even higher risk estimates for a high tHcy and a low serum vitamin B12 concentration in non-supplement users (*Table 2*). Risk estimates for vitamin and homocysteine concentrations in children were 0.6 (0.3-1.4) for tHcy and serum vitamin B12, 1.2 (0.6-2.4) for serum folate and 0.5 (0.2-1.2) for RBC folate concentrations.

Homocysteine, plasma (µmol/L) [a]	Cases/Controls *n* = 98/134	OR	95% CI
5.6-8.6	13/28	1.0	Reference
8.6-9.7	14/26	1.2	0.5-2.9
9.7-10.9	17/29	1.3	0.5-3.1
10.9-12.5	16/26	1.3	0.5-3.3
12.5-42.0	37/25	3.2	1.4-7.3
P_{trend}		0.004	

Table 3. CHD risk in association with maternal plasma total homocysteine concentration.

[a] Quintiles of plasma homocysteine concentrations based on the control-mothers.

Discussion

In our study based on the population of children with CHD and controls with a median age of 17 months living in the Western part of the Netherlands, we demonstrated that a high maternal fasting tHcy concentration is associated with an approximately three-fold higher risk of a child with CHD in a concentration-dependent manner. This finding is in line with the five and ten-fold increased risk of CHD as demonstrated by Kapusta *et al*. and Hobbs *et al*., respectively.[16, 17] The much higher OR in the latter study can partly be explained by the fact that they determined the maternal tHcy concentrations in random blood samples, thereby not considering the confounding by the non-fasting state. Moreover, we standardised the investigation at a fixed study moment for both cases and controls, which is in contrast to their design in which controls were investigated at around 24.5 months and cases at 14.9 months after the index pregnancy. In addition, the included diagnoses were not described in detail. In addition to the differences that will exist between the Dutch and Arkansas populations, these arguments may explain the differences in ORs. In both studies controls were excluded if the medical record indicated any congenital malformation. Echocardiograms, however, were not required to rule out a cardiac defect, which may have underestimated the risk in both studies.

The hyperhomocysteinaemia in the case-mothers can largely be explained by the significantly lower folate and/or vitamin B12 status, even after adjustment for smoking. Therefore, malnutrition and polymorphisms in folate and vitamin B12 genes may play a role. Other causes of the maternal hyperhomocysteinaemia however, have to be considered as well, such as low vitamin B2 or B6 levels, high BMI, or glucose intolerance. The BMI has to be considered as an independent risk factor, because BMI was not only significantly higher in all case-mothers compared with controls but also in the subgroup of hyperhomocysteinaemic case-mothers compared with normohomocysteinaemic case-mothers. Obese women are more prone to an impaired glucose tolerance or overt diabetes. Both phenotypes are associated with an increased risk of CHD in the offspring.[18-21] In our study, only two case-mothers suffered from diabetes and both were normohomocysteinaemic. In addition, the correlation between BMI and tHcy was not significant, which is in contrast to Panagiotakos who showed that BMI is associated with homocysteine concentration.[22]

This is the first study investigating biochemical vitamin and tHcy parameters of children in an attempt to unravel its contribution to the development of CHD. Children with CHD showed significantly higher serum and RBC folate concentrations than controls. This is not due to the use of folic acid containing vitamins, because they were excluded from the analysis. However, significantly more cases than controls were formula-fed. Although formula feeding contains folic acid, this only partially explained the differences between cases and controls, because the formula-fed case-children still showed higher folate concentrations than the formula-fed controls. Nevertheless, the individual B-vitamin status is not only determined by food intake, but also by metabolism, clearance and genetic polymorphisms encoding enzymes in the folate and homocysteine metabolism. The higher folate concentrations in both serum and red blood cells may be explained by the random blood samples taken in children and alterations in the binding of folate to transport proteins and receptors. Other explanations are

differences in the prevalence of genetic polymorphisms encoding these proteins and the enzymes involved in the folate and homocysteine metabolism. In addition, changed concentrations of cofactors, variations in the activity of liver enzymes and renal function affecting folate clearance may play a role as well.

Our findings contribute to the knowledge of the vitamin and homocysteine status in the pathogenesis of CHD in man. However, we have to consider the strengths and weaknesses of our study. Our assumption is that the maternal nutritional status at the study moment is the best reflection of the nutritional status in the preconceptional period of the index-pregnancy. This hypothesis is supported by previous research.[13, 23] The value of a fixed study moment to increase the validity of the biochemical and questionnaire data is shown in our previous studies.[11, 12] We have chosen for a study moment between 11 and 18 months after the index-pregnancy for the following reasons. It takes approximately 12 months after pregnancy and breastfeeding to return to the preconceptional hormonal, metabolic and nutritional status. In general, there is no difference in preconceptional dietary habits and those of one year post partum.[11, 12, 23] Moreover, the seasons of the pre- and periconceptional period and the moment of blood sampling, i.e., approximately two years after conception, are taken into account. Other factors that could have influenced the biomarkers were excluded from analysis, such as vitamin use, pregnancy and non-fasting state at the study moment. However, still some misclassification of vitamin supplement use could have occurred. Therefore, we repeated the analyses after exclusion of serum folate concentrations above 22.5 nmol/L. This cut-off value was based on the study of Brouwer *et al*. showing that folate concentrations increased to a mean value of 22.5 nmol/L in women of reproductive age receiving 250 µg folic acid daily during four weeks.[24] These additional analyses substantiate our findings by showing comparable results.

The potential for recall and selection bias is another issue inherent to the case-control design of studies on congenital malformations. To minimise the recall, we have chosen for one fixed study moment relatively soon after pregnancy in both cases and controls compared with other studies. An earlier study moment after birth would imply a significant interference of the maternal physiology and endocrinology on the biomarkers as well as some misclassification of the CHD and controls, because most malformations are detected and completely diagnosed during the first postnatal year. These issues have been raised in other studies that collected blood samples between three and six months or even up to 4.5 years post partum.[16, 17] Importantly, the concentrations of tHcy, folate and vitamin B12 in our study were comparable to the concentrations measured in the preconceptional period as described by Cikot *et al*.[25]

Our study is unique in the way that we only included patients with a type of CHD that has been associated with folate or other environmental factors before in order to increase the homogeneity of the case group.[4] Furthermore, the number of cases included was comparable to the number of controls. This is in contrast to the study of Hobbs *et al*. who included relatively more cases than controls.[17] The sample size of the study of Kapusta *et al*. was much smaller than ours.[16]

In conclusion, we demonstrate that a high maternal tHcy concentration is associated with an increased risk of having a child with CHD in a concentration-dependent manner, partially due to low

serum folate and vitamin B12. The folate status of the child might be involved as well and should be investigated together with other pathways implicated in CHD.

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Chapter 4

Validation of the assessment of folate and vitamin B12 intake in women of reproductive age: the method of triads

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Abstract

Objective: To validate the folate and vitamin B12 intakes estimated by a food frequency questionnaire (FFQ) designed to be used in a case-control study on the association between maternal dietary intake and the risk of having a child with a congenital heart defect.

Design and subjects: The FFQ was filled out by 53 women of reproductive age. Immediately thereafter, blood samples were taken to determine serum and red blood cell (RBC) folate and serum vitamin B12 concentrations. Subsequently, three dietary 24-hour recalls (24HR) were completed during a period of three successive weeks and used as a reference method. The recalls comprised two weekdays and one weekend day. Using the method of triads validity coefficients were calculated by comparing nutrient intakes derived from the FFQ and 24HR with the corresponding nutritional biomarkers in blood. The validity coefficient is the correlation between the dietary intake reported by the FFQ and the unknown 'true' dietary intake.

Results: The comparison of B-vitamin intakes reported by the FFQ and the mean of the 24HR revealed deattenuated correlation coefficients of 0.98 for folate and 0.66 for vitamin B12. The correlation coefficients between the B-vitamin intakes estimated by the FFQ and concentrations of serum folate, RBC folate and serum vitamin B12 were 0.20, 0.28, and 0.21, respectively. The validity coefficients for serum folate, RBC folate and serum vitamin B12 were 0.94, 0.75 and 1.00, respectively. The estimated folate and vitamin B12 intakes were comparable with the results of the most recent Dutch food consumption survey.

Conclusions: The adapted FFQ is a reliable tool to estimate the dietary intake of energy, macronutrients, folate and vitamin B12 in women of reproductive age. Therefore, this FFQ is suitable for the investigation of nutrient-disease associations in future.

Introduction

Accurate and objective estimates of dietary intake are necessary in order to assess any effects of nutritional status in epidemiologic studies. In general, validation studies of food frequency questionnaires (FFQ) are based on comparisons with reference methods, such as multiple food records or dietary recalls. Traditionally, the validity coefficient between dietary questionnaire data and the unknown 'true' habitual intake is assessed by the correlation between the intakes achieved by the FFQ and the mean value obtained from reference methods. However, the correlation between the reference method and the FFQ may be overestimated, if the sources of errors in these two methods are related. Therefore, the use of biomarkers of nutritional intake is increasingly important in dietary validation studies. Nutritional biomarkers are objective measures of exposure to a certain nutrient and they are not influenced by factors related to misreporting. Therefore, Kaaks described that at least two additional measurements are necessary to determine the validity coefficient of the FFQ measurements, for instance, nutritional biomarkers and 24-hour recalls (24HR). The validity coefficient is the correlation between the dietary intake reported by the FFQ and the unknown 'true' dietary intake.[1] The method of triads is a triangular approach that uses the correlations between each of the three methods to estimate the validity coefficient. Moreover, this model corrects for bias due to

correlated errors in the repeated measurements from the reference method.[2]

The aim of this study was to validate a FFQ, which was adapted for the estimation of B-vitamin intake in women of childbearing age. We examined the association between the biomarkers and two dietary assessment methods for the intake of folate and vitamin B12. The method of triads was applied to validate the intake of folate and vitamin B12, estimated by the FFQ and 24HR and the corresponding biomarkers of nutritional intake.

Materials and methods

A subset of women of reproductive age was recruited from an ongoing case-control study designed to investigate determinants in the pathogenesis and prevention of congenital heart defects. Exclusion criteria were pregnancy, breastfeeding, use of vitamin supplements and non-fasting state at the moment of blood sampling, which was approximately 17 months after the index-pregnancy. From October 2004 until January 2005, 53 women fulfilled the study protocol, which included filling out the questionnaires at home, an immediate blood sample during a hospital study visit thereafter, followed by the completion of three 24HR by phone.

The National Central Committee of Research in Human and the Medical Ethics Committees of all participating hospitals approved the study protocol and written informed consent was obtained of every participant.

Food frequency questionnaire

The FFQ is developed at the Division of Human Nutrition of Wageningen University to estimate the dietary intake of energy, fat and fatty acids.[3] It is a 104-item questionnaire in which participants report their dietary intake during the previous four weeks. Preparation methods, portion sizes and additions can be indicated as well as the frequency of using foods per day, per week, per month or not at all. The FFQ has been updated twice based on data of Dutch national food consumption surveys in 1992 and 1998.[4, 5] This FFQ is validated for energy and fat intake and has been adapted to estimate the intake of folate and vitamin B12. Food items rich in these B-vitamins were added when they contributed more than 0.1% to the intake of each of the nutrients of interest according to the food consumption survey of 1998.[5] In a final step, foods were clustered into food groups and foods were added to guarantee face validity. After all, the FFQ consisted of 121 items and covered the daily intake of each nutrient or food of interest for at least 90% of the population mean intake. The average daily nutrient intake was calculated by multiplying the frequency of consumption of food items by portion size and nutrient content per gram based on the 2001 Dutch food composition table.[6] The existence of underreporting was evaluated by calculating the physical activity level defined by the ratio of reported energy intake (EI) and mean basal metabolic rate (BMR).[7, 8] The BMR was estimated according to the Schofield equations.[9]

Twenty four hour recall

After training by a research dietician, a researcher of the Division of Human Nutrition of Wageningen University contacted the participants for a 24HR within one week after the hospital visit. The standardised telephone interview took on average 20 min. Women reported the dietary intake from

breakfast of the day before till breakfast the next morning. Each subject was contacted three times for a 24HR of two days during the week and one weekend day covering a period of three weeks. Pictures of household measures were used for specific information on portion sizes. The reported foods in the 24HR were coded according to standardised coding procedures. Codes for new foods were added in consultation with a research dietician. The dietary intakes were calculated using the computer programs Komeet and Orion (BaS Nutrition Software, the Netherlands), which are based on the 2001 electronic version of the Dutch food composition table.[6]

Biomarkers

Venous blood samples were taken after an overnight fast. Blood was collected in an 8.5 mL Vacutainer Serum Separator Tube and in a four mL Vacutainer ethylenediaminetetraacetic acid (EDTA) tube (BD Diagnostics, Plymouth, UK) for the determination of serum folate and vitamin B12, and of red blood cell (RBC) folate concentrations, respectively. Directly after blood sampling, the haemolysate was prepared by diluting 0.1 mL full blood in 0.9 mL freshly prepared 1.0 % ascorbic acid. Subsequently, the haematocrit of the remaining EDTA full blood was determined on an ADVIA 120 Haematology Analyser (Bayer Diagnostics, Leverkusen, Germany). The concentrations of folate and vitamin B12 were determined as described before.[10] The haemolysate was centrifuged at 1,000 x *g* for five min at 18 ^{o}C, just before the folate measurement. The haemolysate folate concentration was recalculated into RBC folate concentration using the following formula: (nM haemolysate folate * 10 / haematocrit) - (nM serum folate * {1- haematocrit} / haematocrit) = nM RBC folate. All samples were analysed within three months after collection. Up to the moment of measurement, samples were kept frozen at –80 ^{o}C.

General and anthropometric information

The general questionnaire provided data on maternal age, educational level, and the use of a diet, vitamin supplements or cigarettes. Anthropometry was performed without shoes and jacket, including height (anthropometric rod, SECA, Hamburg, Germany) and weight (weighing scale, SECA, Hamburg, Germany) to the nearest 0.1 cm and 0.5 kg, respectively.

Statistical analysis

The dietary intakes and biomarker concentrations of B-vitamins were log-transformed. The mean dietary nutrient intake was adjusted for total energy intake using the residual method.[11] Differences in intake between the FFQ and 24HR were compared using the paired *t* test. Pearson correlation coefficients were calculated to evaluate the linear association between the data of the FFQ and the 24HR, and the biomarkers. We deattenuated the crude correlation coefficients by multiplying them with the factor $(1 + (\sigma^2_{intra} / \sigma^2_{inter}) / n)^{1/2}$, where n is the number of repeated 24HR, σ^2_{intra} is the intraindividual variance and σ^2_{inter} is the interindividual variance between the 24HR.[12] The method of triads was used to calculate the validity coefficient of the FFQ for folate and vitamin B12 intake. The validity coefficient is the correlation between the dietary intake reported by the FFQ and the unknown 'true' dietary intake. The estimate is interpreted as the upper limit whereas the correlation coefficient between the FFQ and biomarker is considered as lower limit of the true validity coefficient. Confidence intervals were estimated using bootstrap sampling where 1,000 samples of equal size (n = 53) were

obtained by random sampling with replacement.[2] All analyses were performed using SPSS for Windows version 11.0 (SPSS Inc, Chicago, IL, USA).

Results

The study group consisted of 21 case and 32 control-mothers aged 24-44 years with a median body mass index of 23.6 kg/m^2. Other general characteristics and the biomarker concentrations are presented in *Table 1*. All women fulfilled the whole protocol, but 11 women completed the 24HR on three weekdays.

General characteristics	
Age (years)	32.0 (27.8-36.3)
Weight (kg)	71.0 (66.0-87.2)
Height (m)	1.71 (1.66-1.76)
Body mass index (kg/m^2)	23.6 (21.9-29.3)
Low energy diet	4 (8)
Vegetarian diet	4 (8)
Current smokers [a]	7 (13)
Low education level [b]	25 (47)
Biomarkers	
Serum folate (nmol/L)	13.5 (11.5-18.3)
RBC folate (nmol/L)	596 (476-745)
Serum vitamin B12 (pmol/L)	245 (190-339)

Table 1: General characteristics and biomarker co*ncentrations of the 53 women. Values are median (interquartile range) or number (percentage).*

[a] Defined as ≥ 1 cigarette per day.

[b] Primary/lower vocational/intermediate secondary/intermediate vocational education.

The FFQ produced significantly higher estimates of energy and fat intake than the average of the three 24HR (*Table 2*). The mean difference was 12% for energy intake and 19% for fat intake. The calculated ratio of EI and BMR was 1.45 for the cases and 1.44 for the control-group. In general, the dietary intake met the Dutch dietary reference intakes, but folate intake was considerably lower.

The correlation coefficients of the dietary intakes estimated by the FFQ and the average of the three 24HR are shown in *Table 3*. After energy-adjustment, all correlation coefficients decreased, except for carbohydrates. Correlation coefficients ranged from 0.60 to 0.98 after correction for day-to-day variation, with a lower correlation of 0.41 for carbohydrates. The correlations between the FFQ data and the biomarkers were 0.20 for serum folate, 0.28 for RBC folate, and 0.21 for serum vitamin B12, respectively (*Table 4*).

The validity coefficient of the FFQ was 0.94 for serum folate and 0.75 for RBC folate. The validity coefficient between the FFQ and the 'true' intake was 1.66 for vitamin B12 (*Table 4*).

Nutrients	Units/day	FFQ	24HR	DRI [a]
Energy	MJ [b]	9.1 (7.7-10.8) [c]	8.6 (7.1-10.0)	9.7-10.2
Total Fat	g	87.2 (68.7-112.3) [c]	73.8 (61.6-97.1)	
	en%	36.8 (32.7-39.2) [c]	35.6 (31.2-38.2)	20-40
Total Protein	g	77.6 (67.7-90.5)	81.1 (64.1-87.7)	50-52
	en%	14.6 (13.1-15.6) [c]	14.9 (13.3-17.1)	9-25
Total Carbohydrates	g	262 (210-299)	247 (209-281)	
	en%	47.6 (43.2-50.8)	48.4 (43.5-51.4)	40
Folate [d]	µg	177 (138-222)	169 (148-199)	300
Vitamin B12 [d]	µg	3.9 (2.7-4.7)	3.4 (2.6-4.3)	2.8
Vitamin B6 [d]	mg	1.7 (1.5-1.9)	1.6 (1.3-1.9)	1.5

Table 2: The daily intakes estimated by the FFQ and the repeated 24-hour recalls and the dietary reference intakes. Values are median (interquartile range).

[a] Dutch dietary reference intakes for non-pregnant women between 19 and 50 years of age indicate the estimated average requirement for energy, adequate intake for fat, recommended dietary allowance (RDA) with the upper level for proteins, and RDA for carbohydrates and B-vitamins.[18, 19]

[b] 1 kcal = 4.184 kJ.

[c] Comparison of the estimates of the FFQ and 24HR by the paired t test, $P < 0.05$.

[d] Data are log-transformed for statistical analysis.

	Pearson correlation coefficient		
Nutrient	Crude	Energy adjusted [a]	Deattenuated [b]
Energy (MJ)	0.45 [c]	-	0.60
Fat (g)	0.53 [c]	0.35 [c]	0.82
Protein (g)	0.55 [c]	0.40 [c]	0.81
Carbohydrates (g)	0.31 [c]	0.36 [c]	0.41
Folate (µg) [d]	0.40 [c]	0.36 [c]	0.98
Vitamin B12 (µg) [d]	0.49 [c]	0.39 [c]	0.66

Table 3: Correlations between the estimates of the dietary intake by the FFQ and 24HR.

[a] Adjusted for energy by the residual method.[11]

[b] Deattenuation implies correction for day-to-day variation of the repeated 24HR.[12]

[c] $P < 0.05$.

[d] Data are log-transformed.

	Correlation coefficients			Validity coefficient [a]		
	r_{QM}	r_{RM}	r_{QR}	ρ_{QT}	95% CI	Range [b]
Serum folate	0.20	0.22	0.98	0.94	0.36-1.00	0.20-0.94
RBC folate	0.28	0.49	0.98	0.75	0.30-1.00	0.28-0.75
Serum vitamin B12	0.21	0.05	0.66	1.00	0.30-1.00	0.21-1.00

Table 4: Correlation coefficients between each of the dietary assessment methods and the validity coefficient calculated by the method of triads.

r_{QM}, correlation between FFQ and biomarker; r_{RM}, correlation between 24HR and biomarker; r_{QR}, correlation between FFQ and 24HR; ρ_{QT}, validity coefficient of the questionnaire.

[a] Validity coefficients and confidence interval limits above 1 were set to 1.00.

[b] The lower limit is r_{QM} and the upper limit is calculated with the method of triads.[2]

Discussion

The aim of this study was to validate the adapted FFQ for the assessment of folate and vitamin B12 intake in women of reproductive age. Therefore, we collected intake data by two dietary assessment methods, determined concentrations of nutritional biomarkers and applied the method of triads.

The differences between the FFQ and 24HR for estimation of energy and fat intake are shown by others as well[3, 13] and can be explained by underreporting in the 24HR.[14-16] The correlation coefficients between the FFQ and 24HR for energy and fat were lower[3] or comparable with other studies.[13, 15] The correlation coefficient for carbohydrates was higher[13, 15, 17] and the coefficient for proteins was similar to[13] or even higher than the coefficients reported by others.[15, 17] The macronutrient intakes estimated by both methods were in line with the Dutch dietary reference intakes for women between 19 and 50 years of age[18, 19] and the data of the Dutch national food consumption survey of 2003[20] and, therefore, we conclude that the FFQ is a valid method to estimate macronutrient intakes.

The correlation coefficients between the FFQ and 24HR for folate were comparable with other studies (0.40 versus 0.37 and 0.49, respectively)[13, 21] or even higher (0.29)[17] and the correlation coefficient for vitamin B12 was slightly lower, i.e., 0.49 versus 0.58.[13] In the latter study, more than three 24HR have been applied through which the overall day-to-day variation of B-vitamin intake is minimised, resulting in a higher correlation coefficient. Despite the high day-to-day variation of these B-vitamin intakes that is demonstrated by the increased correlation coefficients after correction for day-to-day variation in the 24HR, the correlation coefficients between the FFQ and 24HR were significant. Moreover, these B-vitamin intakes were similar to the Dutch dietary reference intakes with the exception of folate,[18, 19] which is known to be low in the Dutch diet.[22-24]

The correlation between the FFQ and the biomarkers is slightly higher for RBC folate than reported by others (0.28 versus 0.08 and 0.25, respectively).[25, 26] Higher correlation coefficients of 0.38 and 0.39 were reported for serum folate.[25, 26] These differences can be explained by the better reflection of the recent dietary intake by serum folate, whereas the RBC folate concentration is a measure of the long-term folate status in particular.[27-29] Our FFQ covers the intake of a reasonably long period of four

weeks and may, therefore, demonstrate a higher correlation with RBC folate than with serum folate. For vitamin B12, the correlation was comparable with the report (0.19) of Green *et al.*[25]

To our knowledge, this FFQ validation study is the first to assess the validity of the folate and vitamin B12 intake using the method of triads. The validation coefficients are rather high, suggesting that the adapted FFQ is valid for both the assessment of folate and vitamin B12 intakes. Nevertheless, we emphasize that small differences in low sample correlations may result in rather large differences in the estimated validity coefficients.[2] Furthermore, we cannot rule out the presence of a positive correlation between the random errors of the questionnaire and 24HR measurements. Thus, the validity coefficients might be overestimated. Therefore, the estimates must be interpreted as the upper limits of the unknown 'true' validity coefficients.[2] However, the FFQ adequately assesses the intake of folate and vitamin B12 according to the Dutch national food consumption survey of 2003.[20]

The validity coefficient for vitamin B12 is greater than one, which is known as a Heywood case. A Heywood case occurs if the product of two correlation coefficients is much larger than the third correlation. For vitamin B12, the correlation coefficient between the biomarker and 24HR was much lower than the other two correlation coefficients. An explanation for this finding is that vitamin B12 is mainly bound to the metabolically inert protein haptocorrin. Only about 20% of vitamin B12 is bound to transcobalamin, which represents the biologically active fraction that can be delivered to all tissues of the body. Moreover, it has a rapid turnover with a half-life of one to two hours.[30] Since blood sampling was done in the morning after an overnight fast, this may explain the lower correlation between the 24HR and the vitamin B12 concentration as well. The differences in correlation coefficients, however, can still be due to random sampling fluctuation between measurements of both 24HR and biomarkers.

Despite the demonstrated validity of the adapted FFQ, we have to consider the strengths and limitations of our study. The size of the study population is comparable with other validation studies (53 versus 34 and 36, respectively).[21, 26] However, validation studies have been performed in 100 till 200 subjects, but in those studies the method of triads was not applied.[13, 17] The sample size was relatively small with wide confidence intervals of the validity coefficients as a consequence. Validity studies with several hundreds of subjects, more accurate biomarkers or both are, therefore, needed to estimate validity coefficients with a higher precision. It should be kept in mind, however, that Heywood cases can still occur as a result of relatively small sampling fluctuations if the validity coefficient of one of the measurements is close to either one or zero.[2]

The 24HR estimate the intake of a few days and might, therefore, give an inappropriate assessment of the habitual diet. On the other hand, the 24HR were performed during a period of three weeks, which is quite comparable with the reference period of the FFQ. Moreover, the 24HR and FFQ estimates may contain correlated errors. The advantage of the triangular approach of the method of triads is that random errors in biomarker assessment are independent of those in both FFQ and 24HR measurements.[2]

Dietary assessment methods are known to have a bias towards underestimation of habitual energy intake. Therefore, we investigated the overall under-reporting bias. Compared with the cut-off value

of 1.55 that allows measurement imprecision arising from day-to-day variability, some under-reporting may have been present.[7, 8] However, the FFQ covered a four weeks period and therefore, the day-to-day variability of food intake is minimised. Moreover, the FFQ is representative of long-term habitual intake according to the cut-off value of 1.35.[7, 8]

The B-vitamin concentrations may be influenced by general characteristics like smoking or the use of a vegetarian diet.[27, 28] In our study, some women were smoking or used a vegetarian diet, but their biomarker concentrations were within the normal ranges.[31, 32] The biochemical concentrations reflect the individual vitamin status that is not only determined by the metabolism and genes, but as well by parameters affecting the food intake, such as seasonal variation and pregnancy. These effects on dietary intake are minimised because the study was performed in 53 non-pregnant women between October 2004 and January 2005.

In conclusion, these findings indicate that the FFQ adapted for folate and vitamin B12 intake is a valid method to estimate the dietary intake of energy, macronutrients and these B-vitamins in women of reproductive age. Moreover, the relative validity of the FFQ for folate and vitamin B12 intake is comparable with the validity in other studies. Therefore, this FFQ is suitable for the investigation of associations between nutrition and disease in Dutch women in future.

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Chapter 5

Dietary intake of B-vitamins in mothers born a child with a congenital heart defect

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Abstract

Background: Periconceptional use of multivitamins reduces the risk of a child with a congenital heart defect (CHD). Data on the impact of maternal diet, however, are lacking.

Aim of the study: We investigated the association between the maternal dietary intake of B-vitamins and having a child with a CHD.

Methods: A case-control study was performed in 192 mothers of a child with a CHD and 216 mothers of a healthy child. Mothers filled out food frequency questionnaires covering the current dietary intake, and general questionnaires at 17 months after the index-pregnancy. Maternal blood samples were taken to determine B-vitamin and plasma total homocysteine (tHcy) concentrations as nutritional biomarkers. Pregnant and lactating mothers and those with another diet compared with the preconceptional period were excluded for analysis. Case-mothers and controls were compared using the Mann-Whitney *U* test and logistic regression.

Results: The dietary intake of macronutrients and B-vitamins was comparable between both groups, but all mothers had a substantially lower median folate intake (cases 161 µg, controls 175 µg) than the Dutch recommended dietary allowance of 300 µg. Within the case-group, the intake of proteins and vitamin B6 and the concentrations of serum vitamin B12 and folate were significantly lower in hyperhomocysteinaemics (tHcy ≥ 14.5 µmol/L) than in normohomocysteinaemics. The maternal educational level was positively associated with B-vitamin intake, except for vitamin B12 in controls. Low educated case-mothers showed a significantly lower median vitamin B12 intake than controls (2.8 µg and 3.8 µg, $P = 0.01$). The CHD risk doubled if vitamin B12 intake in these mothers reduced by 50% (OR 2.0; 95% CI: 1.1-3.5).

Conclusions: A diet low in vitamin B12 is associated with an increased risk of a child with a CHD, especially in low educated women. A disbalance in the maternal intake of proteins and low folate intake may play a role as well, but needs further investigation. As hyperhomocysteinaemia is a strong risk factor for adult cardiovascular disease, these data may imply that the hyperhomocysteinaemic mothers and their children should be targeted for nutritional interventions.

Introduction

Worldwide, one million children per year are born with a congenital heart defect (CHD).[1] These complex malformations are responsible for a high infant mortality and morbidity rate and go together with substantial health care costs.[2, 3] Both genetic and environmental factors, such as nutrition and lifestyle, are implicated in the pathogenesis of CHDs. The mother serves as the environment of the child during embryogenesis, whereby the maternal dietary intake plays an important role.

After the Second World War epidemics of congenital malformations and miscarriages were found in all European cities that were affected by the famine.[4] Recently, low maternal intakes of nutrients, such as vegetable proteins, polysaccharides, dietary fibers, iron, and magnesium, were associated with an increased risk of a child with a spina bifida or orofacial cleft.[5, 6] B-vitamin intakes were also significantly lower in mothers of a child with an orofacial cleft than in controls.[7, 8] Several epidemiological studies demonstrated the preventive effect of periconceptional folic acid

supplementation against the development of CHDs.[9-11] Among non-vitamin using mothers, the daily intake of folic acid fortified cereals significantly reduced the risk of conotruncal heart defects by 83%.[11]

Kapusta *et al*. firstly reported of the association between a mild maternal hyperhomocysteinaemia and CHD risk, which was recently confirmed by others.[12, 13] The main cause of mild hyperhomocysteinaemia is a low intake of B-vitamins. Folate and vitamin B12 are involved in the remethylation of homocysteine and donation of one-carbon groups to proteins, lipids and nucleotides, whereas vitamin B6 is important in the transsulphuration of homocysteine.[14] Insufficient intake of B-vitamins results in biochemical derangements leading to hyperhomocysteinaemia and DNA hypomethylation that may contribute to the development of CHDs.[15] Interestingly, DNA hypomethylation is minimised at intake levels in excess of current recommended dietary folate and vitamin B12 intakes.[16]

Therefore, we hypothesise that low maternal B-vitamin intakes detrimentally affect the embryonic cardiovascular development. We investigated the maternal dietary intake in a case-control study conducted in the Netherlands.

Materials and methods

Recruitment of subjects

The Dutch HAVEN study, acronym for the study of heart anomalies and the role of genetic and nutritional factors, is an ongoing case-control study designed to identify environmental and genetic factors in the pathogenesis of CHDs. The study has been performed at the Department of Obstetrics and Gynaecology of Erasmus MC in Rotterdam in close collaboration with the Centres of Paediatric Cardiology of the same hospital and of Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam and with the child health centres of 'Thuiszorg Nieuwe Waterweg Noord' in the Rotterdam region. Eligible families are children with a CHD and healthy children with both parents living in the Western part of the Netherlands.

The paediatric cardiologists of the aforementioned Centres of Paediatric Cardiology diagnose and recruit the case-children and their parents in collaboration with the research team of the HAVEN study. The selected diagnoses comprise transposition of the great arteries, tetralogy of Fallot, atrioventricular or perimembranous ventricular septal defect, aortic valve stenosis, pulmonary valve stenosis, coarctation of the aorta, and hypoplastic left heart syndrome. Healthy control-children and both parents are enrolled in cooperation with the physicians of the child health centres. Control-children do not have congenital malformations or chromosomal defects according to the medical record and regular health checks by the physician of the child health centre. Invited case and control-children are between 11 and 18 months of age. Case and control-families are not related, and speak, read and write the Dutch language.

We obtained questionnaire and biochemical data of 247 case and 266 control-mothers that were collected during the hospital visit at Erasmus MC in Rotterdam between October 2003 and July 2005. Mothers who were pregnant, lactating, or those who reported a changed diet compared with the preconceptional period were excluded for analysis. This resulted in a dataset of 192 case and 216

control-mothers. The Central Committee of Research in Human and the Medical Ethics Committees of the participating hospitals reviewed and approved the study protocol. Prior to participation, written informed consent was obtained from both parents.

Study design

Dietary habits are rather stable and do not change except for increased needs because of breastfeeding and pregnancy, and episodes of illnesses and dieting.[17-19] In addition, most congenital malformations are detected during the first year of life. Therefore, we carried out a standardised investigation between 11 and 18 months after the index-pregnancy under the assumption that these data reflect the maternal nutritional status in the preconceptional period according to our previous studies.[5-8]

At home mothers filled out the food frequency questionnaire (FFQ) that covered the intake of the previous four weeks, and the general questionnaire. During the study visit we performed maternal anthropometry and obtained maternal blood samples as biomarkers of nutritional intake. We checked the questionnaire data for completeness and consistency.

Data collection

We estimated daily habitual energy, macronutrient, and micronutrient intakes using a modified version of the semiquantitative FFQ of Feunekes et al.[20] This FFQ has been updated twice based on data of Dutch national food consumption surveys in 1992 and 1998.[21, 22] Additionally, this FFQ has been modified for the estimation of dietary B-vitamin intakes. Food items rich in B-vitamins were added to the food list when they contributed more than 0.1% to the intake of each of the nutrients of interest according to the food consumption survey of 1998.[22] Thus, the FFQ covers the daily intake of each nutrient or food of interest for at least 90% of the population mean intake.

The FFQ consists of 121 items and is structured according to a meal pattern. Participants report the intake of foods used during the previous month. Questions about preparation methods, portion sizes and additions are also included. The average daily nutrient intake was calculated using the 2001 electronic version of the Dutch food composition table.[23] We evaluated the existence of under-reporting. The mean basal metabolic rate (BMR) was estimated according to the Schofield equations.[24] The physical activity level was calculated by the ratio of the reported energy intake (EI)/BMR.[25]

Extracted data of the general questionnaire included maternal age, time after index-pregnancy, body mass index (BMI), educational level, ethnicity, smoking, use of alcohol, oral contraceptives and vitamin supplements at the study moment. Mothers were considered smokers or alcohol drinkers when any smoking or alcohol consumption was reported. Educational level and ethnicity were classified according to the definitions of Statistics Netherlands.[26] Educational level was categorised into low (primary/lower vocational/intermediate secondary), intermediate (intermediate vocational/higher secondary) and high education (higher vocational/university). Mothers were classified as Dutch natives, Western or non-Western immigrants.

We performed standardised maternal measurements of weight (weighing scale, SECA, Hamburg, Germany) with 0.5 kg accuracy and height (anthropometric rod, SECA, Hamburg, Germany) up to 0.1 cm accuracy. Venous blood samples were drawn from all mothers to measure concentrations of red

blood cell (RBC) and serum folate, serum vitamin B12 and plasma total homocysteine (tHcy) as nutritional biomarkers as described before.[27]

Immediately after blood sampling 0.1 mL EDTA whole blood was haemolysed with 0.9 mL freshly prepared 1.0% ascorbic acid. Subsequently, the haematocrit of the EDTA whole blood was measured (ADVIA 120 Haematology Analyser, Bayer Diagnostics, Germany). Another EDTA-tube was put on ice and centrifuged immediately after blood sampling for measurement of the tHcy concentration. Blood samples were centrifuged at 4,000 x *g* for ten min at 4°C and separated within one hour after blood sampling. Folate and vitamin B12 concentrations were routinely determined by immunoelectrochemiluminescence assay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). Shortly before the folate measurement the haemolysate was centrifuged at 1,000 x *g* for five min at 18°C. The folate concentration in the haemolysate was recalculated in RBC folate using the following formula: (nM haemolysate folate*10/haematocrit) - (nM serum folate*{1-haematocrit}/haematocrit) = nM RBC folate. The tHcy concentration was routinely measured by high performance liquid chromatography with reverse phase separation and fluorescence detection.[27]

The inter-assay coefficients of variation (CV) for vitamin B12 was 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; for folate these CV were 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L and for tHcy 5.9% at 15.3 µmol/L and 3.4% at 39.3 µmol/L. Until measurement, all sera and plasma were stored at –80°C. Some biomarkers were missing due to failures in blood sampling or laboratory testing. All laboratory analyses were performed anonymously in batches within three months after collection.

Statistical analysis

Differences in the distributions of categorical variables were tested by the Chi-square test. The dietary intakes were approximately normally distributed except for vitamin B12 and folate intake. These two variables were log-transformed. The nutritional biomarkers showed skewed distributions even after transformation. Therefore, all data are presented as medians with interquartile range and differences between cases and controls were evaluated by the Mann-Whitney *U* test. We compared the data with the Dutch dietary recommended intakes (DRIs) for non-pregnant women to check the appropriateness of the dietary intakes of our study population.[28, 29] Moreover, Pearson correlation coefficients were computed to investigate the associations between the B-vitamin intakes and the corresponding biomarkers. The B-vitamin intakes were compared between cases and controls stratified for educational level using ANOVA.

The mean dietary B-vitamin intakes were adjusted for total energy intake using the residual method.[30] Shortly, the B-vitamin intakes were regressed on the total energy intake and the predicted mean B-vitamin intake was calculated for the mean total energy intake of the study population. The energy-adjusted B-vitamin intake was calculated by adding the individual residuals to the predicted mean B-vitamin intake.

We assessed the association between maternal dietary intake of B-vitamins and CHD risk for the crude and energy-adjusted data. Because of the complex pathogenesis of CHDs, it is most likely that a low dietary intake of B-vitamins might be a risk factor in a subgroup of cases only. Therefore, we created the 10th percentile of vitamin B6, vitamin B12 and folate intake based on the control-data and

estimated the CHD risk using odds ratios (OR) and 95% confidence intervals (CI) in a logistic regression model. In addition, we performed a logistic regression analysis of B-vitamin intake stratified for educational level. *P* values < 0.05 were considered statistically significant. All analyses were performed using SPSS-software package version 11.0 (SPSS Inc, Chicago, IL, USA).

Results

The median maternal age of cases was slightly higher than that of controls (*Table 1*). The distributions of ethnicity and educational level were comparable in case and control-mothers. There were no significant differences in BMI, use of vitamin supplements, alcohol and cigarettes at the standardised study moment. All CHDs were pooled because subgroup analyses did not reveal significant differences in demographics, biomarkers and dietary intakes.

	Cases (*n* = 192)	Controls (*n* = 216)
Maternal age (years)	33.3 (29.9-36.7)	32.6 (29.0-34.9) [a]
Time after index-pregnancy (months)	17.0 (15.3-20.7)	16.8 (15.2-18.6)
BMI (kg/m²)	24.1 (22.0-27.4)	24.1 (22.0-27.8)
Educational level [*n* (%)] [b]		
low	58 (30)	53 (25)
intermediate	88 (46)	108 (50)
high	46 (24)	55 (25)
Ethnicity [*n* (%)] [c]		
Dutch native	155 (81)	176 (81)
Western immigrants	14 (7)	12 (6)
Non-Western immigrants	23 (12)	28 (13)
Use of [*n* (%)]		
Alcohol	98 (51)	125 (58)
Cigarettes	39 (20)	43 (20)
Oral contraceptives	78 (41)	102 (47)
Vitamin supplements	35 (18)	41 (19)

Table 1. General characteristics of mothers of a child with a CHD and controls at the study moment. Values are median (interquartile range) or number (percentage).

[a] *P < 0.05 (Mann-Whitney U test).*

[b] *Categorised as low (primary/lower vocational/intermediate secondary education), intermediate (higher secondary/intermediate vocational education) or high (higher vocational/university education).*[26]

[c] *Classified according to the definitions of Statistics Netherlands.*[26]

As shown in *Table 2*, all mothers had a substantially lower folate intake than the Dutch DRI. The daily dietary intake of macronutrients and B-vitamins was comparable between cases and controls. The ratio EI/BMR was 1.46 for cases and 1.44 for controls. Among the biomarkers, only the median tHcy concentration was significantly higher in case-mothers (10.4 µmol/L) than in controls (10.0 µmol/L).

Daily intake of nutrients	Cases (*n* = 192)	Controls (*n* = 216)	DRI [a]
Energy (MJ)	8.5 (7.3-10.3)	8.7 (7.4-10.4)	9.7-10.2
Fats (g)	82.0 (69.5-105.3)	83.4 (68.2-104.7)	
(en%)	36.5 (33.7-40.6)	36.8 (32.5-39.4)	20-40
Proteins (g)	75.1 (62.2-85.1)	76.0 (64.4-87.0)	50-52
(en%)	14.5 (12.8-16.2)	14.5 (13.0-15.7)	9-25
Carbohydrates (g)	238 (203-290)	250 (209-293)	
(en%)	47.0 (43.5-51.0)	48.3 (44.4-51.3)	40
Vitamin B6 (mg)	1.5 (1.3-1.8)	1.6 (1.4-1.9)	1.5
Vitamin B12 (µg)	3.6 (2.5-4.5)	3.6 (2.8-4.4)	2.8
Folate (µg)	161 (122-206)	175 (135-210)	300
Biomarkers [b]			
tHcy (µmol/L)	10.4 (8.9-12.9)	10.0 (8.4-12.2) [c]	
Vitamin B12, serum (pmol/L)	272 (211-359)	247 (200-349)	
Folate, serum (nmol/L)	15.0 (12.3-18.6)	14.6 (12.1-19.4)	
Folate, RBC (nmol/L)	666 (535-793)	674 (537-855)	

Table 2. Dietary intake and biomarkers of nutritional intake of mothers of a child with a CHD and controls at the study moment. Values are medians (interquartile range).

[a] *Dutch DRI for non-pregnant women aged 19 to 50 years.*[28, 29]

[b] *Homocysteine, 151 cases and 209 controls. Serum vitamin B12 and folate, 150 cases and 210 controls. RBC folate, 149 cases and 210 controls.*

[c] *P < 0.05 (Mann-Whitney U test).*

Within the case-group, the intake of proteins and vitamin B6 and the concentrations of serum vitamin B12 and folate were significantly lower in hyperhomocysteinaemics (tHcy ≥ 14.5 µmol/L) than in normohomocysteinaemics (*Table 3*). Hyperhomocysteinaemic controls had a significantly lower intake of proteins and vitamin B12 and significantly lower biomarker concentrations. The correlation coefficient between the intake and serum vitamin B12 concentration in all mothers was 0.27 ($P < 0.001$). The correlations between dietary folate intake and the serum and RBC folate concentration were 0.12 ($P < 0.03$) and 0.17 ($P = 0.002$), respectively. Overall, a low maternal dietary intake of vitamin B12 was associated with an increased risk of a child with a CHD. The risk increased approximately two-fold (OR 1.9, 95% CI 1.03-3.4) at the 10th percentile of vitamin B12 intake (*Table 4*). The energy-adjusted vitamin B12 intake did not show a significant association nor did vitamin B6

Case-mothers			Normal tHcy (*n* = 125)	High tHcy (*n* = 26)
Energy	MJ	9.7-10.2 [a]	8.4 (7.2-9.9)	8.8 (7.4-11.1)
Fats	g	-	80.2 (70.2-98.8)	84.9 (64.4-123.1)
	en%	20-40	36.5 (33.3-39.9)	35.9 (32.4-40.5)
Proteins	g	50-52	75.3 (63.8-83.9)	68.5 (54.2-81.0)
	en%	9-25	14.8 (13.0-16.5)	12.7 (11.4-14.7) [b]
Carbohydrates	g	-	232 (202-290)	262 (221-299)
	en%	40	47.1 (43.7-51.2)	47.7 (44.1-52.7)
Vitamin B6	mg	1.5	1.5 (1.3-1.8)	1.3 (1.1-1.7) [b]
Vitamin B12	µg	2.8	3.8 (2.5-4.6)	3.4 (2.3-4.0)
Folate	µg	300	165 (122-210)	138 (120-174)
Vitamin B12, serum [c]	pmol/L	-	285 (229-372)	175 (152-273) [b]
Folate, serum [c]	nmol/L	-	15.4 (12.9-19.4)	11.1 (10.0-15.1) [b]
Folate, RBC [c]	nmol/L	-	666 (572-790)	627 (466-842)
Control-mothers			Normal tHcy (*n* = 188)	High tHcy (*n* = 21)
Energy	MJ	9.7-10.2	8.7 (7.4-10.4)	8.7 (7.3-9.5)
Fats	g	-	84.1 (67.5-105.9)	80.0 (71.9-90.5)
	en%	20-40	36.8 (32.6-39.8)	36.8 (32.5-38.0)
Proteins	g	50-52	76.1 (65.7-88.1)	65.5 (55.1-82.3) [b]
	en%	9-25	14.6 (13.2-15.9)	13.1 (12.0-14.9) [b]
Carbohydrates	g	-	246 (208-293)	267 (221-291)
	en%	40	47.8 (44.2-51.1)	49.2 (46.6-55.1)
Vitamin B6	mg	1.5	1.6 (1.4-1.9)	1.5 (1.3-1.8)
Vitamin B12	µg	2.8	3.6 (2.9-4.5)	3.1 (2.3-3.8) [b]
Folate	µg	300	176 (137-210)	156 (106-202)
Vitamin B12, serum [c]	pmol/L	-	255 (207-358)	200 (150-258) [b]
Folate, serum [c]	nmol/L	-	14.9 (12.3-19.7)	12.4 (10.6-14.9) [b]
Folate, RBC [c]	nmol/L	-	679 (547-892)	538 (467-716) [b]

Table 3. Daily nutrient intakes of the case and control-mothers after stratification for the tHcy concentration. Values are medians (interquartile range). Stratification based on the 90th percentile (i.e. 14.5 µmol/L) of tHcy concentrations in controls.

[a] Dutch DRI for non-pregnant women aged 19 to 50 years.[28, 29]

[b] Reflects the comparison of hyper- and normohomocysteinaemics within the case and within the control-group, P < 0.05 (Mann-Whitney U test).

[c] Hyperhomocysteinaemic cases: serum vitamin B12 and folate, n = 124; RBC folate, n = 123. Normo-homocysteinaemic controls: n = 187.

	Cut-off value [a]	Case/Control (*n* = 192/216)	OR (95% CI)
Vitamin B6 (mg)	< 1.1	25/21	1.4 (0.8-2.6)
Vitamin B12 (µg)	< 2.1	32/21	1.9 (1.03-3.4)
Folate (µg)	< 103.6	22/21	1.2 (0.6-2.3)
	Cut-off value [b]	Case/Control (*n* = 192/216)	OR (95% CI) [b]
Vitamin B6 (mg)	< 1.3	27/21	1.5 (0.8-2.8)
Vitamin B12 (µg)	< 2.2	28/21	1.6 (0.9-2.9)
Folate (µg)	< 117.6	26/21	1.5 (0.8-2.7)

Table 4. Risk estimates for the association between maternal dietary B-vitamin intake per day and CHDs. Data of vitamin B12 and folate intake were log-transformed.

[a] Cut-off values are based on the lowest 10th percentile of the control vitamin intake.

[b] Cut-off values and OR (95% CI) of energy-adjusted dietary intake.[30]

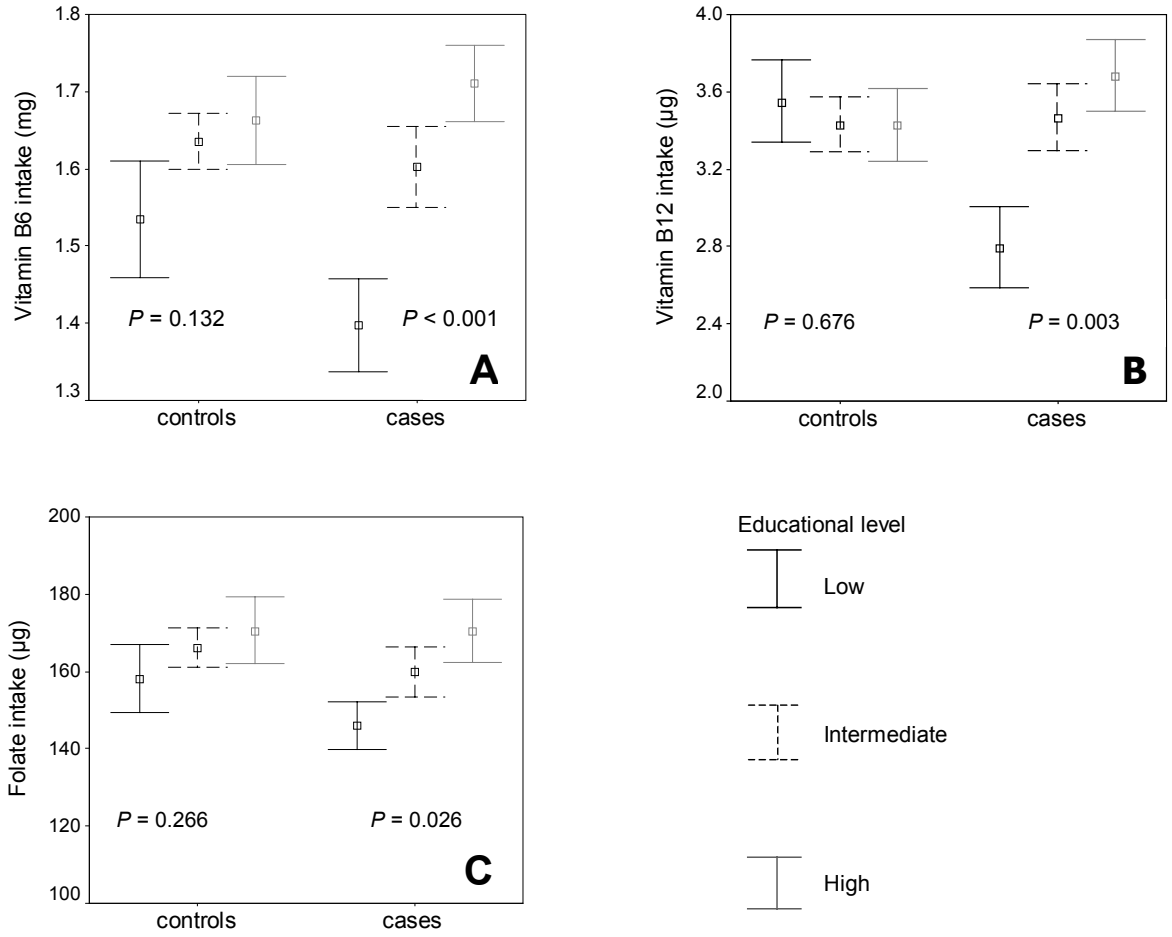

Figure 1. Vitamin B6 (A), vitamin B12 (B) and folate (C) intake in 216 mothers of children with a congenital heart defect and 192 control-mothers stratified for educational level.

Data shown are mean +/- SEM for vitamin B6 and geometric mean +/- SEM for folate and vitamin B12. ANOVA P values < 0.05 indicate a significant trend over the educational levels.

or folate intake. In both groups, educational level was positively associated with B-vitamin intake, except for vitamin B12 intake in controls (*Figure 1*). The associations were significant for cases only. Low educated case-mothers showed a significantly lower median vitamin B12 intake of 2.8 µg per day than controls (3.8 µg). The CHD risk doubled if their vitamin B12 intake reduced by 50% (OR 2.0 (1.1-3.5), P_{trend} = 0.01) (*Table 5*). Median daily intakes of vitamin B6 (1.5 mg and 1.3 mg) and folate (169 µg and 143 µg) were not significantly lower in low educated cases than in low educated controls. The corresponding risk estimates were 1.8 (0.8-3.8) per unit decrease of vitamin B6 intake and 1.5 (0.7-3.1) per 50% reduction of the folate intake.

Educational level [a]	Case/Control (*n* = 192/216)	OR (95% CI)	OR (95% CI) [b]
Low	58/53	2.0 (1.1-3.5)	1.9 (1.1-3.3)
Intermediate	88/108	1.0 (0.6-1.5)	1.0 (0.6-1.6)
High	46/55	0.7 (0.3-1.5)	0.6 (0.2-1.5)

Table 5. Risk estimates for the association between CHDs and maternal dietary vitamin B12 intake per day stratified for educational level. Data of vitamin B12 intake were log-transformed (base 2).

[a] Low (primary/lower vocational/intermediate secondary), intermediate (higher secondary/ intermediate vocational) or high education (higher vocational/university).[26]

[b] OR (95% CI) for energy-adjusted dietary intake.[30]

Discussion

In this study we demonstrate for the first time that a low maternal dietary vitamin B12 intake is associated with an approximately two-fold increased risk of a child with a CHD. So far, associations between maternal vitamin B12 intake and a child with other malformations have not been found.[5, 8] Our findings are in line with other reports showing an association between a low blood vitamin B12 concentration and an increased risk of neural tube defects[31] and orofacial clefts.[32] A low vitamin B12 intake results in low blood vitamin B12 concentrations and increased homocysteine concentrations. Therefore, our finding is consistent with previous reports that showed an association between maternal hyperhomocysteinaemia and having a child with a CHD.[12, 13] The significant correlation between dietary vitamin B12 intake and the corresponding biomarker supports this finding. Moreover, experimental studies substantiate the finding of the teratogenic effect of mild hyper-homocysteinaemia.[33]

Our data suggest that hyperhomocysteinaemia in the subgroup of case-mothers can partly be explained by the significantly lower vitamin B6 intake and low folate and vitamin B12 status. In contrast, hyperhomocysteinaemic controls did not show a lower vitamin B6 intake but their protein intake was lower than in normohomocysteinaemic controls. This illustrates the heterogeneous aetiology of hyperhomocysteinaemia.[34]

The overall diet met the Dutch DRI in both cases and controls except for the low folate intake. The

protein intake has to be in balance with the B-vitamin intakes. Dietary protein intake is the main source of methionine, which is the sole precursor of homocysteine. A high-protein diet increases tHcy concentrations throughout the day,[35] and particularly folate is an important substrate for the remethylation of homocysteine. Therefore, a disbalance in the maternal intake of proteins and folate deranges the homocysteine metabolism and easily leads to mild hyperhomocysteinaemia. The finding of low median folate intakes is supported by other Dutch studies and strengthens the recommendation to use a folic acid supplement to achieve an optimal periconceptional folate status.[5, 36]

Low educated women more often demonstrated a low dietary vitamin B12 intake that was associated with a higher risk of CHDs than women with a higher educational level. This risk was substantiated by a significant dose-response relation within these women. This supports the findings of others that people with a low education and low income are more likely to engage in poor dietary practice than their wealthier and higher educated counterparts.[37] Dietary vitamin B12 is only available from animal sources like fish and red meat. Therefore, low educated people may not have enough income for these more expensive foods or they are not correctly informed to buy and prepare healthy food.

Interestingly, hyperhomocysteinaemia is also a strong risk factor for adult cardiovascular disease.[38] Our experimental data suggest that prenatal exposure to hyperhomocysteinaemia induces the first features of atherosclerosis that are associated with cardiovascular diseases in later life.[39] Together with our current results this may imply that the hyperhomocysteinaemic mothers and their children are both at risk for the development of cardiovascular disease in adulthood. Since B-vitamin intake is an important determinant of hyperhomocysteinaemia this group should, therefore, be targeted for nutritional interventions.

We address some issues of our study design. Dietary assessment methods have a strong bias towards underestimation of habitual energy intake. Some under-reporting may equally be present in both our case and control-group according to the cut-off value of 1.55 that allows measurement imprecision arising from day-to-day variability.[25] However, the FFQ covered a four weeks period and therefore, the day-to-day variability of food intake is minimised. Moreover, the ratio was representative of long-term habitual intake according to the cut-off value of 1.35.[25] Furthermore, adjustment for energy intake or expression of macronutrient intake as percentages of total energy intake minimises the bias generated by under-reporting.[40] We excluded some mothers from analysis because of conditions that affect the nutritional intake, such as pregnancy, lactation or the use of another diet compared with the preconceptional period. Moreover, multivitamin users may be more aware of the importance of a healthy diet than non-users. However, exclusion of the multivitamin users did not significantly affect the results.

The case-control study is the usually used epidemiological study design for congenital defects due to the relatively low birth prevalence rates. Although, recall bias is not frequently present in case-control studies on congenital malformations,[41] we have chosen for one investigation at a fixed moment shortly after pregnancy to reduce the potential for recall bias. A single investigation increases the feasibility of the study and compliance of participants and decreases the potential for selection bias as well. An earlier study moment after birth would imply a significant interference of the maternal

physiology and endocrinology with the biomarkers as well as some misclassification of the cases and controls, because most malformations are detected and completely diagnosed during the first postnatal year. Moreover, this study moment is around two years after conception of the index-pregnancy and equals the season of the preconceptional and periconceptional period. Thus, the seasonal influences on food intake are comparable in both groups. Therefore, after exclusion of pregnant and breastfeeding women and those who changed their diet during the preconceptional period, a standardised investigation between 11 and 18 months after the index-pregnancy in both cases and controls is the best moment to mimic the maternal nutritional status in the preconceptional period and to minimise the risk of misclassification of CHDs. This is substantiated by the concentrations of tHcy, folate and vitamin B12 in our study that were comparable to the concentrations measured in the preconceptional period by Cikot *et al.*[19] To homogenise the CHD group, we only included mothers of patients with a type of CHD that has been associated with folic acid or other environmental factors, because periconceptional use of multivitamins containing folic acid is suggested to reduce the occurrence of cardiac outflow tract anomalies.[9]

Although, the correlation coefficients between folate and vitamin B12 intake, and their blood levels were statistically significant, they were rather low. This was not due to time delay in data collection, because the FFQ covered the four weeks before the study moment and blood sampling was performed directly thereafter. Blood samples were centrifuged and separated within one hour after blood sampling and stored at –80 ^{o}C until measurement. All laboratory measurements were performed within three months after blood sampling. Therefore, it is very unlikely that this procedure has affected the blood levels. In addition, the B-vitamin biomarkers are not only determined by intake, but also by absorption, metabolism, clearance, and genetic polymorphisms encoding enzymes in the folate and homocysteine metabolism.

In conclusion, we show that a low maternal dietary vitamin B12 intake is associated with an increased risk of a child with a CHD, especially in low educated women. A disbalance in the maternal intake of proteins and folate may play a role as well, but needs further investigation. If future studies confirm our findings, women who are planning a pregnancy should use vitamin supplements containing both folic acid and vitamin B12.

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Part II

Gene-nutrient interactions

Chapter 6

Two methylenetetrahydrofolate reductase polymorphisms, maternal intake of folate and vitamin B2 and the risk of congenital heart defects

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Abstract

It is not clear whether the methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms involved in the homocysteine pathway are associated with congenital heart defects (CHDs). We studied both polymorphisms, the maternal intake of folate and vitamin B2 via food and supplements and CHD risk. A case-control family study was conducted in a European population in the Netherlands including 230 case and 251 control-children with both parents. Approximately 17 months after the index-pregnancy, mothers filled out standardised questionnaires on the periconceptional use of folic acid supplements and a validated food frequency questionnaire on current dietary folate and vitamin B2 intake. All subjects were genotyped for the MTHFR C677T and A1298C polymorphisms. Data were analysed by logistic regression analysis using the dominant model. The MTHFR 677 CT/TT genotypes showed odds ratios (ORs) and 95% confidence intervals (CIs) of 1.2 (0.8-1.7) in mothers, 1.3 (0.9-1.8) in fathers and 1.2 (0.8-1.7) in children. However, the MTHFR 1298 AA genotype in fathers and children significantly increased CHD risk, 1.7 (1.2-2.4) and 1.5 (1.1-2.2), respectively. Furthermore, a two-fold increased CHD risk was shown in children with the MTHFR 1298 AA genotype without periconceptional exposure to folic acid supplements compared with periconceptionally exposed MTHFR 1298 AC/CC carriers (OR 2.1 (1.2-3.8)). In conclusion, the MTHFR C677T polymorphism is not an independent risk factor for CHDs. Periconceptional folic acid supplementation is beneficial, particularly in children carrying the MTHFR 1298 AA genotype.

Introduction

Congenital heart defects (CHDs) are the most frequent birth defects and account each year for over one million affected newborns worldwide.[1] Genetic risk factors for CHDs can be identified in both the mother, father and the child by a candidate gene approach based on the molecular biologic pathways implicated in the embryogenesis of the heart. Since the mother is the environment of the child in utero, maternal environmental exposures, such as the intake of vitamins, medicines and smoking, influence the development of the unborn child as well.

Synthetic folic acid has been proven to contribute to the prevention of CHDs. The protective effect seems even more distinct for outflow tract defects and ventricular septal defects.[2] Evidence from experimental studies reveals that during early embryogenesis, hyperhomocysteinaemia deranges the migration of neural crest cells from the neural tube to the tissues forming the cardiac outflow tract.[3, 4] Low folate and low vitamin B2 intakes result in a mild hyperhomocysteinaemia.[5] In addition, genetic factors are also implicated in the folate-homocysteine pathway. The methylenetetrahydrofolate reductase (MTHFR) [EC 1.5.1.20] gene is located on chromosome 1p36.3 and encodes for the enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate. The latter substrate is essential for the remethylation of homocysteine into methionine. Vitamin B2 is a cofactor for the MTHFR enzyme. The single nucleotide polymorphisms C677T and A1298C in the MTHFR gene cause amino acid changes in the MTHFR enzyme, thereby decreasing MTHFR enzyme activity and increasing the homocysteine level. The MTHFR C677T polymorphism is the most widely studied genetic polymorphism in many complex diseases.[6-8] The association between the MTHFR

C677T polymorphism and CHD risk is not clear. Several studies investigated other neural crest cell related malformations, such as the cleft lip with or without cleft palate, and found a strong interaction between the MTHFR C677T polymorphism and periconceptional folic acid supplementation, but also in combination with a low food folate intake.[9, 10] Only few studies reported on the association between the MTHFR A1298C polymorphism and CHDs.

Therefore, we hypothesize that the functional MTHFR C677T and A1298C polymorphisms in the mother and/or the child result in a higher risk of CHDs, either independently or in combination with low intake of folate and vitamin B2. Since it is not yet feasible to test this hypothesis in a preconceptional cohort study, we have chosen for the best alternative of conducting a case-control study at a fixed study moment between 11 and 18 months after the index-pregnancy. At that study moment, the nutritional status of the mother is rather comparable with the periconceptional period, because nutritional intake is stable during life except for periods of dieting, breastfeeding and extreme growth. We tested the hypothesis in an ethnically homogeneous case-control family study in the Netherlands.

Materials and methods

Study population

The HAVEN study, which is a Dutch acronym for the study of genetic and environmental factors in the aetiology and prevention of CHDs, was conducted from June 2003 onwards at four Academic Medical Centres and coordinated by the project team of the Department of Obstetrics and Gynaecology/Division of Obstetrics and Prenatal Medicine of Erasmus MC, University Medical Centre in Rotterdam, the Netherlands. The methods for this study are extensively described previously and are summarized hereafter.[11] The study population consisted of case and control-family triads (child, mother, father). For analysis, we only included case-families of which DNA was available. These families involved 230 children affected with a CHD and both parents and we refer to them as cases, case-mothers and case-fathers, respectively (*Figure 1*). The phenotypes of the CHDs comprised of tetralogy of Fallot, transposition of the great arteries, atrioventricular septal defect, perimembranous ventricular septal defect, coarctation of the aorta, aortic valve stenosis, pulmonary valve stenosis and hypoplastic left heart syndrome. The paediatric cardiologist in each centre confirmed the diagnoses by echocardiography, and/or cardiac catheterisation and/or surgery.

Non-malformed children and both parents (n = 251) served as controls (*Figure 1*). Controls were excluded if they had a congenital malformation or chromosomal abnormality ascertained by their physician at the child health centre. Exclusion criteria for both groups were age of the child below 11 months or above 18 months, participation of a relative in this study and parents that were not familiar with the Dutch language in writing and/or reading. General and food frequency questionnaires were filled out, blood samples were collected and a signed informed consent form was obtained from every parent. The study was approved by the Central Committee on Research in Human and the Medical Ethics Committees of all participating hospitals.

Measurements

At the fixed study moment of approximately 17 months after the index-pregnancy, both parents filled out a general questionnaire at home. In addition, the mother filled out a food frequency questionnaire (FFQ). All questionnaires were reviewed and checked for completeness and consistency by the researcher during the hospital visit. The general questionnaires were subdivided in parts concerning two different periods in time. The first period referred to the periconceptional period, which is defined as four weeks before until eight weeks after conception and corresponds to the recommended period of folic acid supplementation in the Netherlands. From the general questionnaires, we collected supplement intake data of both mother and father during this period. Mothers were considered users of folic acid supplements and/or multivitamins containing folic acid when they used a supplement every day in the periconceptional period. Inconsistent users or mothers who used supplements only during a part of the periconceptional period were considered as non-users. The second period was defined as the month prior to the study moment of approximately 17 months after the index-pregnancy. At this moment, we collected the general characteristics of all participants, such as age,

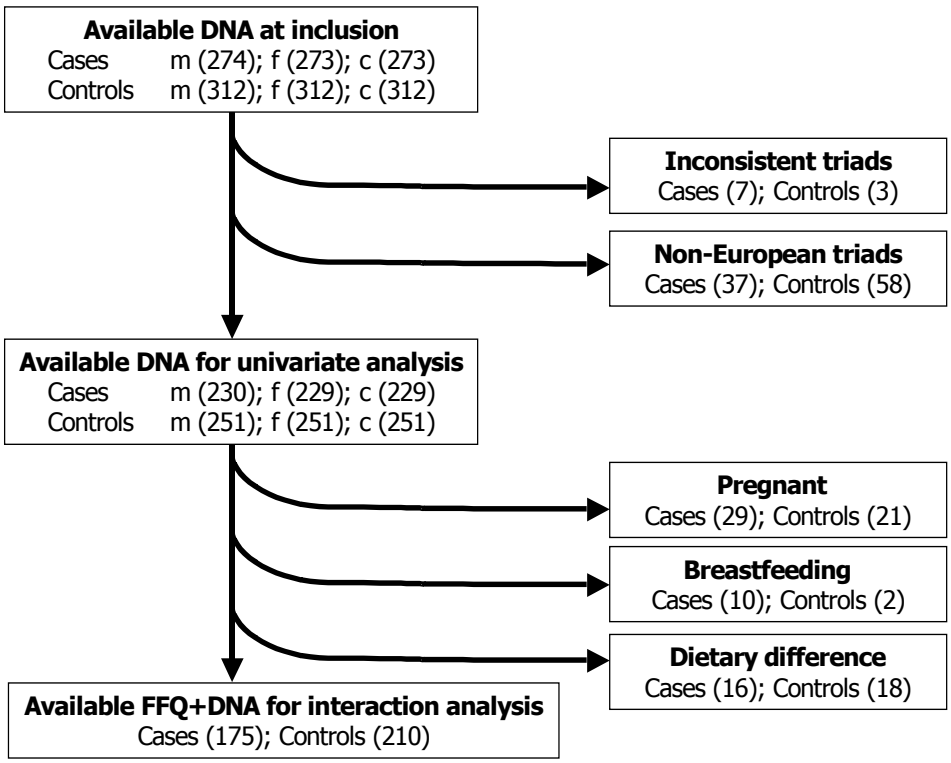

Figure 1. Flowchart of the study population.

The inconsistent and non-European triads were excluded from analysis (m, mothers; f, fathers; c, children) with available DNA at inclusion. Therefore, we used the data of 230 case and 251 control-mothers for the univariate logistic regression analysis of the genotypes. After exclusion of all pregnant and lactating women and those who had a different diet at the study moment than in the periconceptional period, we used the data of 175 case and 210 control-mothers for the univariate regression analysis of nutrients as well as for the interaction analysis.

ethnicity, educational level, family history of CHDs and the use of vitamin supplements. Educational level was categorized as low (primary/lower vocational/intermediate secondary education), intermediate (higher secondary/intermediate vocational education) or high (higher vocational/university education).[12] With regard to ethnicity, we classified our participants into three different groups: Dutch natives (*n* = 426), European others (*n* = 55) and non-Europeans (*n* = 95).[13] The latter group was significantly different from the other two groups with concern to genotype frequencies, general characteristics and nutritional intake (data not shown). Therefore, we excluded the non-European group from further analysis (*Figure 1*).

The standardised and validated FFQ comprised of questions concerning maternal dietary intake of the month before the study moment.[14] From this FFQ, the total energy intake, folate and vitamin B2 intake were extracted for analysis. During the hospital visit, maternal weight (weighing scale, SECA, Hamburg, Germany) and height (anthropometric rod, SECA, Hamburg, Germany) were measured.

Laboratory determinations

The DNA from all mothers, fathers and children was derived from either a blood sample or a buccal swab. Genomic DNA was isolated from 0.2 mL ethylenediamine tetra-acetate (EDTA) whole blood with the Total Nucleic Acid Extraction kit on a MagNA Pure LC (Roche Molecular Biochemicals, Mannheim, Germany). The DNA yields were estimated by comparison with a lambda ladder. From eight cases, two case-fathers and one control-father, the DNA isolation was performed from buccal swabs instead of blood samples due to logistic problems or failure in blood sampling. The DNA isolation was carried out using the QuickExtract DNA Extraction Solution 1.0 according to the manufacturers' instructions (Epicentre, Madison, Wisconsin, USA). We determined the C677T polymorphism in the MTHFR gene from isolated DNA using real-time polymerase chain reaction (PCR) (Taqman®, Applied Biosystems, Foster City, CA, USA) and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) with *Hinf*1 digestion.[15] The PCR-RFLP was also applied to detect the MTHFR A1298C polymorphism.[16] Three percent of the determinations served as positive controls and over two percent were negative controls. The genotyping success rate was 100%.

Data analysis

Some of the continuous variables had skewed distributions even after transformation. Therefore, all continuous variables are presented as medians with interquartile range and compared between cases and controls using the Mann-Whitney *U* test. Categorical variables were tested with the Chi-square test. Genotype data were checked for Mendelian segregation errors and inconsistent triads (*n* = 10) were excluded from analysis (*Figure 1*). Using the Chi-square test, we tested deviation of the genotype frequencies from those expected under Hardy Weinberg Equilibrium.

Allele and genotype frequencies were compared between cases and controls. Odds ratios (OR) with 95% confidence intervals (CI) were computed for the associations of case-control status and genotype using univariate logistic regression analysis. We used the dominant model for both MTHFR polymorphisms based on the functional effects of the polymorphisms. We excluded all pregnant (*n* = 50) and lactating women (*n* = 12) as well as those who had a different diet at the study moment compared with the periconceptional period (*n* = 24) in order to analyse associations between dietary

intake of folate and vitamin B2 and the risk of a CHD. For the nutritional and interaction analyses, this resulted in a dataset of 175 cases with their mothers and 210 controls with their mothers (*Figure 1*). We tested both the crude and the energy adjusted nutrient data by univariate logistic regression analysis and used the nutrient residual method to adjust for total energy intake.[17] Multiplicative interactions were tested between genotype and periconceptional supplement use and between genotype and (energy adjusted) dietary intake using a comparison between joint expected and joint observed associations.[18] For this analyses, dietary intakes of folate and vitamin B2 were dichotomised into low and high intakes. The cut-off values were based on the median intakes of the control-mothers. We used the groups with the lowest expected risk as reference categories, i.e., the MTHFR 677 CC genotype combined with periconceptional supplement use or high dietary intake of folate and vitamin B2 and the MTHFR 1298 AC or CC genotype in combination with periconceptional supplement use or a high dietary intake of folate and vitamin B2. All analyses were done with SPSS for Windows software (version 11.5; SPSS Inc., Chicago, IL, USA).

Results

General characteristics of case and control-triads both at the study moment and in the periconceptional period and maternal nutritional data are presented in *Table 1*. Case-mothers were 0.7 years older than control-mothers. However, this small difference did not confound the association between the polymorphisms and intake of folic acid supplements or dietary intake of folate and vitamin B2 and CHD risk. Other potential confounders, such as education, family history of CHDs, gender of the child and ethnicity, were not significantly different between case and control-mothers and between case and control-fathers. Although not significantly different, the prevalence of family history of CHDs was twice as high in cases as in controls.

Table 2 presents the genotype frequencies of the MTHFR polymorphisms using the dominant model. All genotype distributions of mothers, fathers and children with or without a CHD were in Hardy Weinberg Equilibrium. Univariate logistic regression analysis was performed to test for the independent effects of the MTHFR genotypes. Slightly more case-mothers, case-fathers and cases carried the MTHFR 677 CT and TT genotype compared with controls, although these numbers were not significantly different. The MTHFR A1298C polymorphism in case-mothers was not significantly associated with CHD risk. However, the MTHFR 1298 AA genotype in case-fathers showed a significant association with CHD risk (P = 0.006). The MTHFR 1298 AA genotype in cases showed a comparable significant effect (P = 0.024).

Gene - environment interaction

The periconceptional use of supplements and the dietary intakes of total energy, folate and vitamin B2 were not significantly different between case and control-mothers. In addition, energy-adjusted dietary intakes of folate and vitamin B2 did not show a significant difference between case and control-mothers (*Table 1*). We investigated the interactions between genotypes of the mother or the child and maternal periconceptional supplement use and between the genotypes and maternal (energy-adjusted) dietary intake of folate and vitamin B2. In *Table 3*, the risk estimates are shown for

	Mothers		Fathers	
Periconceptional period [a]	Cases (*n* = 230)	Controls (*n* = 251)	Cases (*n* = 230)	Controls (*n* = 251)
Supplement use [*n* (%)] [b]	125 (54)	149 (59)	32 (14)	33 (13)
Study moment				
Age (years)	33.5 (30.6-36.7)	32.8 (29.2-35.4) [c]	35.3 (32.2-38.7)	35.7 (32.8-39.0)
Body mass index (kg/m^2)	24.4 (22.0-27.7)	24.1 (22.1-27.3)	-	-
Educational level [*n* (%)] [d]				
Low	60 (26)	48 (19)	59 (26)	58 (23)
Intermediate	102 (44)	125 (50)	79 (34)	108 (43)
High	68 (30)	78 (31)	92 (40)	85 (34)
Dietary intake of [e]				
Total energy (MJ)	8.4 (7.3-10.2)	8.6 (7.3-10.3)	-	-
Folate (µg)	157 (118-200)	165 (129-198)	-	-
Folate (µg) [f]	152 (128-186)	160 (134-191)	-	-
Vitamin B2 (mg)	1.4 (1.0-1.6)	1.4 (1.1-1.6)	-	-
Vitamin B2 (mg) [f]	1.3 (1.1-1.6)	1.4 (1.1-1.5)	-	-
	Children			
Study moment	Cases (*n* = 230)	Controls (*n* = 251)		
Age (months)	16.8 (15.4-20.0)	16.7 (15.3-18.4)		
Male gender [*n* (%)]	133 (58)	142 (57)		
Family history of CHDs [*n* (%)] [g]	20 (9)	11 (4)		
Ethnicity [h]				
Dutch Natives	201 (87)	225 (90)		
European Others	29 (13)	26 (10)		

Table 1. General characteristics. Values are median (interquartile range) or number (percentage).

[a] Periconceptional period is defined as 4 weeks before until 8 weeks after conception.

[b] Supplement use is defined as daily use of folic acid containing vitamin supplements.

[c] P = 0.042 (Mann-Whitney U test).

[d] Categorized as low (primary/lower vocational/intermediate secondary), intermediate (higher secondary/intermediate vocational) or high education (higher vocational/university).[22]

[e] Cases: n = 175; Controls: n = 210.

[f] Energy-adjustment by the residual method of Willett.[27]

[g] Family members with a CHD in the first, second and third degree.

[h] Dutch Natives: Both parents and grandparents are born in the Netherlands or one of the parents is born in another country, but both grandparents are born in the Netherlands. European Others: One of the parents or grandparents is born in a European country, Indonesia, or is from European origin and living in the USA or Australia.[23]

Genotypes	Cases	Controls	OR (95% CI)
Mothers			
	(*n* = 230)	(*n* = 251)	
MTHFR 677 CT or TT	139 (60)	140 (56)	1.2 (0.8-1.7)
MTHFR 677 CC	91 (40)	111 (44)	1.0 (Reference)
	(*n* = 230)	(*n* = 251)	
MTHFR 1298 AA	104 (45)	116 (46)	1.0 (0.7-1.4)
MTHFR 1298 AC or CC	126 (55)	135 (54)	1.0 (Reference)
Fathers			
	(*n* = 229)	(*n* = 251)	
MTHFR 677 CT or TT	124 (54)	122 (49)	1.3 (0.9-1.8)
MTHFR 677 CC	105 (46)	129 (51)	1.0 (Reference)
	(*n* = 228)	(*n* = 251)	
MTHFR 1298 AA	111 (49)	91 (36)	1.7 (1.2-2.4)
MTHFR 1298 AC or CC	117 (51)	160 (64)	1.0 (Reference)
Children			
	(*n* = 229)	(*n* = 251)	
MTHFR 677 CT or TT	130 (57)	132 (53)	1.2 (0.8-1.7)
MTHFR 677 CC	99 (43)	119 (47)	1.0 (Reference)
	(*n* = 229)	(*n* = 251)	
MTHFR 1298 AA	112 (49)	97 (39)	1.5 (1.1-2.2)
MTHFR 1298 AC or CC	117 (51)	154 (61)	1.0 (Reference)

Table 2. Distribution of the MTHFR C677T and A1298C genotypes. Values are numbers (percentages).

the MTHFR A1298C polymorphism in the mother and child, which are stratified for periconceptional supplement use of the mother and adjusted for maternal age. To investigate multiplicative interaction between the MTHFR A1298C polymorphism and periconceptional supplement use, we compared the expected joint effect with the observed joint effect. The MTHFR 1298 AA genotype in the child demonstrated a non-significant OR of 1.1 (*Table 3*). The risk estimate is 1.1 if the mother did not use periconceptional supplements. The expected joint OR would be 1.1 x 1.1 = 1.2. The observed OR for the combined effect is 2.1 (P = 0.015), which is higher than the expected OR, thereby indicating interaction. Thus, children who carried the MTHFR 1298 AA genotype and who were not periconceptionally exposed to folic acid supplements, showed a more than two-fold increased risk of a CHD compared with children carrying either the MTHFR 1298 AC or CC genotype without periconceptional exposure to folic acid supplements. No significant interaction was found for the MTHFR A1298C polymorphism in the mother and periconceptional folic acid supplementation. Moreover, no interaction could be found between the MTHFR C677T polymorphism and supplement

use of the mother and between both polymorphisms and dietary intake of folate or vitamin B2 (data not shown).

Supplement use [a]	Children	Cases (*n* = 175)	Controls (*n* = 209)	OR (95% CI) [b]
No	MTHFR 1298 AA	44	33	2.1 (1.2-3.8)
	MTHFR 1298 AC+CC	38	52	1.1 (0.6-2.0)
Yes	MTHFR 1298 AA	40	51	1.1 (0.6-2.0)
	MTHFR 1298 AC+CC	52	73	1.0 (Reference)
	Mothers			
No	MTHFR 1298 AA	48	38	1.5 (0.8-2.5)
	MTHFR 1298 AC+CC	34	47	0.8 (0.5-1.4)
Yes	MTHFR 1298 AA	31	58	0.5 (0.3-1.0)
	MTHFR 1298 AC+CC	62	66	1.0 (Reference)

Table 3. Interaction between the MTHFR A1298C genotype in mothers or children and periconceptional supplement use of the mother.

[a] Supplement use in the periconceptional period is defined as daily use of folic acid containing vitamin supplements by the mother in the period of four weeks before until eight weeks after the conception.

[b] Adjusted for maternal age

Discussion

In this case-control family study in an ethnically homogeneous European population, we examined the impact of two polymorphisms in the MTHFR gene on CHD risk. The genetic polymorphisms were analysed, both independently and in combination with maternal periconceptional vitamin supplement use and dietary intake of B-vitamins. We demonstrated that the MTHFR 1298 AA genotype of the father and the child is significantly associated with an increased CHD risk. Furthermore, the MTHFR A1298C polymorphism in children and maternal periconceptional folic acid supplementation as a gene-nutrient interaction is significantly associated with CHD risk.

No significant association was observed between CHD risk and the MTHFR C677T polymorphism, neither independently nor in combination with maternal periconceptional supplement use or dietary intake of folate or vitamin B2. The meta-analysis by Verkleij-Hagoort *et al*. revealed an overall OR of 1.0 (0.8-1.3) in mothers and 1.1 (0.9-1.5) in children for the MTHFR C677T polymorphism and CHD risk, thereby supporting our finding.[19] So far, only four studies[20-23] were performed on the association between the MTHFR A1298C polymorphism and CHD risk and in only two of them a similar case-control family design was used.[21, 23] In two of those studies, no significant association was found between the MTHFR A1298C polymorphism in the mother and the risk of CHD offspring. This might have been due to smaller sample sizes of 103[23] and 25 cases[21], respectively, or ethnic heterogeneity or differences in the included phenotypes. Two case-only family studies used the Transmission Disequilibrium Test (TDT) approach, in which only the heterozygous case-parents are informative.[20, 22]

McBride *et al*. did not show a significant association between the MTHFR A1298C polymorphism in the mother and CHD risk. Hobbs *et al*., however, demonstrated a protective effect of the fetal MTHFR 1298 C-allele on CHD risk. Moreover, they confirmed the TDT results with a log-linear analysis after inclusion of the data of all available case-families. However, a parent-of-origin effect could not be demonstrated.[20] These results are in line with our finding of the significant association between the MTHFR 1298 AA genotype of the child and increased CHD risk. The significant CHD risk in association with the paternal MTHFR 1298 AA genotype is herewith in line and may suggest genetic imprinting.

The MTHFR A1298C is extensively studied in relation with other complex diseases, such as colorectal cancer and acute leukaemia. For these diseases, the MTHFR 1298 CC genotype is more consistently associated with an overall risk reduction, albeit not significantly.[7, 8] However, recent reports showed a strong and significant inverse association between the MTHFR 1298 C-allele and colon cancer.[24, 25] The cancer researchers hypothesize that the MTHFR 1298 CC genotype causes decreased activity of the MTHFR enzyme. The MTHFR enzyme determines the balance between the different forms of folate for DNA synthesis and DNA methylation.[26] Therefore, a decreased MTHFR enzyme activity inhibits the conversion of 5,10-methylenetetraydrofolate to 5-methyltetrahydrofolate, thereby producing an abundant 5,10-methylenetetrahydrofolate pool level that leads to less misincorporation of uracil during DNA synthesis. Error-free DNA synthesis is also critically important during cardiovascular development and, therefore, the proposed biological pathway may also be involved in the pathogenesis of CHDs. On the other hand, low MHTFR enzyme activity may also lead to DNA hypomethylation, thereby contributing to the development of congenital anomalies.[27] The MTHFR A1298C polymorphism has a small influence on enzyme activity compared with the C677T polymorphism.[16] This may implicate that the functional effect of the MTHFR 1298 C-allele is only a slightly lower MTHFR enzyme activity that delicately affects the balance between DNA synthesis and DNA methylation towards a protective effect in cardiovascular development. Nevertheless, one has to keep in mind that selective survival could have diluted our findings. Children with the MTHFR 1298 A-allele might be more viable due to the wide spread use of folic acid supplements.[28, 29]

Methylation reactions are important in the regulation of gene expression by chemical modification of DNA. Unlike the irreversible nature of genetic events, which introduce permanent changes in the primary DNA sequence, epigenetic modifications are reversible. Maternal nutritional status can alter the epigenetic state of the fetal genome.[30] Folic acid treatment restores DNA methylation and corrects the patterns of abnormal gene expression.[31] Herewith in line, a significant gene-nutrient interaction was demonstrated between the MTHFR A1298C polymorphism in children and maternal use of a folic acid supplement in the periconceptional period. This finding suggests that the maternal nutritional status is of major importance compared with the small and non-significant effects of maternal polymorphisms, which fits with the multifactorial aetiology of CHDs.

We have to consider the limitations of our study inherent to the case-control study design. Concerning selection bias, the main question is whether exposed subjects are more likely to be included in the study. We do not expect that individuals with a high risk genotype were more likely to be included in the study, because they did not know their genotype beforehand. Moreover, the allele frequencies in

controls were comparable with the allele frequencies in the European population. For example, Nurk *et al*. found allele frequencies in 14,449 control-mothers of 30% for the MTHFR 677 T-allele and 32% for the MTHFR 1298 C-allele.[21] In our study, allele frequencies were 35% and 33%, respectively. Secondly, non-differential misclassification might have occurred, because periconceptional supplement use was based on retrospective questionnaire data. On the other hand, differential misclassification is not likely, because vitamin B2 or folate intake via supplements and food was independent of genotype distributions within both the case and the control-families. These arguments are in line with the report of Infante-Rivard *et al*. who showed that differential misclassification is rare in case-control studies.[32] Moreover, participants were unaware of the detailed study objectives.

Our study has several strengths too. The large sample size of 230 cases and 251 controls enabled us to detect a 40% significant risk reduction with a power of 78% (risk allele frequency of 0.34, type 1 error of 0.05, CHD population risk of 0.006). Our results are based on a controlled and standardised study design, which has successfully been used for many times in the investigation of gene-environment interactions in other complex malformations.[9, 33, 34] We used a fixed study moment of approximately 17 months after the index-pregnancy, which is especially important with regard to the nutritional parameters. Several authors demonstrated reasonable correlations between FFQ data that are determined before pregnancy and two to four years later. In general, no differences occur in the dietary patterns between the periconceptional period and one year postpartum.[35, 36] Moreover, at this study moment, most of the CHD diagnoses are completed, and thereby, the risk of undiagnosed, less severe CHDs in the control group is minimised and, thereby, misclassification is reduced. Regarding nutrition, our food is not yet fortified by folic acid in contrast to other countries, which assures the reliability of the estimation of the true dietary folate intake. Furthermore, we only included Dutch natives and European families to increase the homogeneity of the study population.

In conclusion, our findings suggest that the MTHFR 1298 AA genotypes of fathers and children are independent risk factors for the investigated CHDs. Of interest is the observed interaction between the MTHFR A1298C polymorphism and periconceptional folic acid supplementation. Future studies with larger sample sizes should be performed to investigate the maternal–fetal interactions with regard to the most favourable balance between DNA synthesis and DNA methylation, thereby affecting fetal cardiovascular embryogenesis. New evidence is given to stimulate the periconceptional use of folic acid supplements in public health programs. A periconceptional high methyl diet should be considered in future studies as well.

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Chapter 7

Genetic and lifestyle risk factors related to the periconceptional vitamin B12 status and congenital heart defects: a Dutch case-control study

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Abstract

Maternal hyperhomocysteinaemia is associated with congenital heart defects (CHDs) in the offspring. A low periconceptional vitamin B12 status determined by genetic and lifestyle factors causes hyperhomocysteinaemia. We investigated the methionine synthase reductase (MTRR) and transcobalamin (TC) genes and maternal intake and serum concentrations of vitamin B12 in association with CHD risk. Seventeen months after the index-pregnancy, we studied 230 children with a CHD and 251 non-malformed children and their parents. Data were collected on current and periconceptional maternal vitamin supplement use and maternal dietary vitamin B12 intake of the month before the study moment. Blood samples were taken for the determination of MTRR A66G and TC C776G genotypes in families and serum vitamin B12 concentrations in mothers. Transmission disequilibrium tests and univariate and multivariate analyses were applied. Allele transmissions were not significantly distorted and MTRR and TC genotypes did not significantly affect CHD risk. Neither polymorphisms in mothers and/or children did reveal significant interactions nor in combination with a low vitamin B12 intake. Low maternal serum vitamin B12 combined with the maternal or child's MTRR 66 GG genotype resulted in odds ratios (ORs) of 1.4 (95% confidence interval 0.6-3.5) and 1.3 (0.5-3.4), respectively. The TC 776 GG genotype in mothers or children and a low maternal serum vitamin B12 resulted in ORs of 2.2 (0.7-7.1) and 1.9 (0.5-7.4), respectively. We conclude that larger sample sizes are required to demonstrate whether MTRR 66 GG and TC 776 GG genotypes and maternal vitamin B12 status significantly contribute to the development of CHDs.

Introduction

Congenital heart defects (CHDs) are the most common congenital malformations in newborns and account for more than one-third of infant deaths due to congenital anomalies.[1] In humans, the embryonic heart develops between the third and eighth weeks after conception.[2] Since the mother is the environment of the developing embryo and fetus, interactions between genetic and lifestyle factors in early pregnancy are assumed to be involved in the pathogenesis of complex malformations, including CHDs.

We and others demonstrated a strong association between maternal hyperhomocysteinaemia and the risk of CHD offspring.[3-5] Increased homocysteine levels are caused by a low status of one or more of the B-vitamins, which is determined by genetic polymorphisms and lifestyle factors, such as low intake of B-vitamins by food and supplements.[6] Experimental studies support the teratogenicity of moderate hyperhomocysteinaemia.[7, 8] A part of the preventive effect of periconceptional folic acid supplementation on the occurrence of CHDs and other malformations can be explained by the normalisation of decreased folate and elevated homocysteine blood levels.[9, 10] Besides the substrate folate, vitamin B12 is also an important determinant in the homocysteine pathway. A compromised vitamin B12 status leads to mild hyperhomocysteinaemia due to a reduced conversion of homocysteine into methionine by the enzyme methionine synthase, whereby vitamin B12 serves as a cofactor. Methionine synthase reductase (MTRR) regenerates the functional status of methionine synthase via the chemical reduction of vitamin B12.[11] In addition, vitamin B12 is important for the

conversion of folate monoglutamates into polyglutamates, the stored form of folate in red blood cells.[12] Vitamin B12 in blood is mainly bound to the metabolically inert protein haptocorrin. Approximately 10-25% of vitamin B12 in blood is bound to transcobalamin (TC), which is the biologically active fraction required for the cellular uptake of vitamin B12.[13]

The MTRR and TC polymorphisms result in a decreased activity of methionine synthase due to a diminished cellular uptake and decreased cellular availability of reduced vitamin B12, respectively.[11, 14] So far, few studies showed a significant effect of the MTRR A66G and TC C776G polymorphisms on the vitamin B12 and homocysteine concentrations. It was shown that MTRR 66 AA homozygotes had 36% higher vitamin B12 concentrations than individuals with the AG or GG genotypes ($P < 0.05$).[15] Others observed hyperhomocysteinaemia in TC 776 CG heterozygotes[14] and reported 15% ($P = 0.021$)[16] and 39% ($P = 0.002$)[17] higher transcobalamin concentrations for the CC genotype compared with the GG genotype.

A low maternal vitamin B12 status is associated with a higher risk of neural tube defects (NTD)[18, 19] and orofacial clefts.[20] Neural crest cells are not only involved in the embryogenesis of the neural tube, lip and palate, but in cardiovascular development as well. We have demonstrated that the migration and differentiation of neural crest cells is influenced by homocysteine.[21] Since vitamin B12 is an important determinant in the homocysteine pathway, it may thereby contribute to the embryogenesis of the heart in the first weeks after conception. In that period, the maternal and embryonic vitamin B12 status are determined by genetic polymorphisms of vitamin B12-related genes in the mother and the embryo as well as maternal lifestyle factors, such as vitamin B12 intake by food and supplements.

Prospective studies on congenital malformations are not yet feasible and, therefore, a case-control study is the best alternative. For many years, we have successfully conducted our studies on neural crest related malformations at a fixed study moment after the index-pregnancy in order to reduce recall bias when estimating the exposures of interest in the periconceptional period.[19, 20, 22] From this background, the aim of the current case-control family study was to investigate associations between CHD risk and MTRR A66G and TC C776G genetic polymorphisms, periconceptional vitamin B12 intake and biochemical determinants of the homocysteine pathway.

Materials and methods

Study population

The ongoing HAVEN study, a Dutch acronym for the study of heart anomalies and the role of genetic and nutritional factors, is a case-control family (child, mother, father) study conducted in the Western part of the Netherlands, which has previously been described.[3, 23] In summary, 274 cases were included with the following phenotypes: tetralogy of Fallot ($n = 31$), transposition of the great arteries ($n = 44$), atrioventricular septal defect ($n = 27$), perimembranous ventricular septal defect ($n = 73$), coarctation of the aorta ($n = 22$), aortic valve stenosis ($n = 8$), pulmonary valve stenosis ($n = 54$), and hypoplastic left heart syndrome ($n = 8$). Diagnoses were confirmed after birth by echocardiography and/or cardiac catheterisation and/or surgery. The 312 children who were eligible as controls, did not have a major congenital malformation or chromosomal defect according to the

medical records and regular health checks by the physician at the child health centre. Families visited the hospital at the standardised study moment of around 17 months after the index-pregnancy. The Central Committee on Research in Humans and the Medical Ethics Committees of the participating hospitals approved the study protocol and written informed consent was obtained from every parent.

Questionnaire data

At the study moment, the general characteristics, maternal food frequency data and lifestyle exposures were collected by questionnaires. The data comprised of the following information at the study moment: maternal age, time after index-pregnancy, body mass index, ethnicity, education, dietary intake of energy and vitamin B12. Maternal ethnicity was classified as Dutch natives, Europeans and non-Europeans.[24] Educational level was categorised according to the definitions of Statistics Netherlands.[25]

Importantly, we estimated the daily maternal dietary intakes of energy and vitamin B12 of the month before the study moment to reflect the regular food intake and, thereby, during the periconceptional period of the index-pregnancy as well. We used a validated semiquantitative food frequency questionnaire (FFQ).[26] In a standardised manner, the questionnaire data were checked for completeness and consistency by the researcher during the hospital visit. Standardised anthropometric measurements were performed including maternal height (anthropometric rod, SECA, Hamburg, Germany) up to 0.1 cm accuracy and weight (weighing scale, SECA, Hamburg, Germany) with 0.5 kg accuracy. The maternal lifestyle factors of interest included the use of B-vitamin supplements, tobacco and alcohol, which were evaluated both at the study moment and during the periconceptional period. The periconceptional period was defined as four weeks before until eight weeks after conception. The B-vitamin supplements contained folic acid and/or vitamin B12 and were used daily.

Biochemical measurements and analysis of the polymorphisms

Maternal blood samples were used to determine the concentrations of serum vitamin B12, plasma total homocysteine (tHcy), and serum and red blood cell (RBC) folate. Blood sampling and measurements were described before.[3, 23] Blood samples were obtained of the child, mother and father for DNA-analysis. Genomic DNA was isolated from 0.2 mL EDTA whole blood with a Total Nucleic Acid Extraction kit on a MagNA Pure LC (Roche Molecular Biochemicals, Mannheim, Germany). DNA yields were estimated by comparison with a lambda ladder. We obtained buccal swabs from three fathers and nine children because of logistic problems or failures in blood sampling. The DNA isolation from buccal swabs was performed using the QuickExtract DNA Extraction Solution 1.0 according to the manufacturers' instructions (Epicentre, Madison, Wisconsin, USA). The MTRR A66G (rs1801394) and TC C776G (rs1801198) polymorphisms were analysed by the Taqman® system, according to protocols provided by the manufacturer (Taqman, Applied Biosystems, Foster City, CA, USA). Approximately three percent of the samples were re-genotyped to check for genotype calling consistency. The genotyping success rate was more than 96%.

Statistical analysis

Maternal age, time after index-pregnancy and body mass index are presented as medians and were compared between cases and controls using the Mann-Whitney *U* test. Differences in frequencies of categorical variables were tested by the Chi-square test. The mean dietary vitamin B12 intakes were adjusted for total energy intake using the residual method.[27] The vitamin B12 intakes and biomarkers are shown as medians with interquartile range and were compared by the Kruskal-Wallis and Mann-Whitney *U* tests. Biomarkers are stratified for MTRR A66G and TC C776G genotypes. For the analyses concerning the vitamin B12 intake and biomarkers, we excluded all pregnant and lactating mothers and those with a changed diet compared with the periconceptional period to minimise confounding of these parameters. The median daily dietary intake of vitamin B12 in controls was used as a cut-off value for low (≤ 3.4 μg/day) or normal (>3.4 μg/day) vitamin B12 intake. The 15th percentile of the control serum vitamin B12 concentration was used as a cut-off value for low (≤ 175 pmol/L) or normal (>175 pmol/L) serum vitamin B12.

Hardy-Weinberg equilibrium (HWE) was examined for all genotype frequencies, separately for cases and controls. The transmission disequilibrium test (TDT) was applied to analyse transmission of the parental MTRR 60 G-allele and the TC 776 G-allele in case-families.[28] The risk of CHD was estimated for the MTRR A66G and TC C776G genotypes of all family members using a univariate logistic regression model.

We examined these polymorphisms in mothers and children and their interaction with periconceptional vitamin B12 intake by food and periconceptional B-vitamin supplement use in association with CHD risk. We used a recessive model for the genetic analyses.[11, 16, 17, 29] Odds ratios (ORs) and 95% confidence intervals (CI) were calculated in a logistic regression model with the lowest category as a reference. We performed a trend analysis across the four categories ranging from reference (wild type or heterozygous genotype in combination with normal vitamin B12 intake) to high risk (homozygous mutant genotype and low vitamin B12 intake). In multivariate logistic regression analyses, we studied interactions between the two polymorphisms in mothers and children, maternal dietary vitamin B12 intake and serum vitamin B12 with adjustment for periconceptional B-vitamin supplementation.

Stratified analyses were executed for the effects of the polymorphisms on the maternal biochemistry for all pooled data as well as for case and control-data separately. In a multivariate model, we adjusted the biochemical data for use of B-vitamin supplements, tobacco and alcohol at the study moment. A *P* value ≤ 0.05 was considered statistically significant. All analyses were performed using SPSS for Windows software (version 11.0; SPSS Inc, Chicago, Illinois, USA) with an exception for the transmission disequilibrium test.[28]

Results

In the study population of 274 case and 312 control-families, inconsistent triads (*n* = 10) were excluded from all analyses. The distributions of the three ethnic groups were comparable between cases (Dutch natives 75%, Europeans 11%, non-Europeans 14%) and controls (73%, 8% and 19%), respectively. However, the distributions of the genetic polymorphisms were significantly different in

non-Europeans compared with Dutch natives and Europeans, particularly for MTRR genotypes in control-mothers, case and control-fathers, and case-children. Therefore, non-European families (*n* = 95) were excluded from further analysis, which resulted in a dataset of 230 case and 251 control-families.

In *Table 1*, the general characteristics and lifestyle factors are presented of 230 case and 251 control-mothers. The median age of case-mothers was 0.7 years higher than of controls (*P* = 0.042). The time after the index-pregnancy, body mass index and educational level were comparable in both

Study moment	Cases (*n* = 230)	Controls (*n* = 251)
Maternal age (years)	33.5 (30.6-36.7)	32.8 (29.2-35.4) [a]
Time after index-pregnancy (months)	16.8 (14.4-20.0)	16.7 (15.3-18.4)
Body mass index (kg/m²)	24.4 (22.0-27.7)	24.1 (22.1-27.3)
Educational level, low [*n* (%)] [b]	60 (26)	48 (19)
Family history of CHD [*n* (%)] [c]	20 (9)	11 (4)
Use of [*n* (%)]		
B-vitamin supplements [d]	60 (26)	64 (26)
Tobacco	41 (18)	46 (18)
Alcohol	118 (51)	154 (61) [a]
Daily dietary intake of [e]		
Energy (MJ)	8.4 (7.3-10.2)	8.6 (7.2-10.3)
Vitamin B12, crude (µg)	3.6 (2.5-4.5)	3.4 (2.7-4.3)
Vitamin B12, energy-adjusted (µg) [f]	3.6 (2.5-4.5)	3.4 (2.7-4.3)
Periconceptional period [g]		
Use of [*n* (%)]		
B-vitamin supplements	125 (54)	149 (59)
Tobacco	46 (20)	57 (23)
Alcohol	94 (41)	97 (39)

Table 1. Maternal characteristics and lifestyle factors at the study moment and during the periconceptional period. Values are median (interquartile range) or number (percentage).

[a] P = 0.042 (Mann-Whitney U test) or P = 0.026 (Chi square test).

[b] Primary, lower vocational and intermediate secondary education.

[c] Family members with a CHD in the first, second or third degree.

[d] B-vitamin supplements contained folic acid and/or vitamin B12.

[e] Dietary intakes are based on 175 cases and 210 controls, after exclusion of pregnant and lactating mothers and those with a changed diet compared with the periconceptional period.

[f] Energy-adjustment by the residual method.[27]

[g] Defined as four weeks before until eight weeks after conception.

groups. There were no significant differences in the use of B-vitamin supplements and tobacco at the study moment. However, compared with cases, 10% more control-mothers used alcohol (P = 0.026). After exclusion of the pregnant and lactating mothers and those with a changed diet compared with the periconceptional period, the analyses of energy and vitamin B12 intake and biomarkers were based on 175 case and 210 control-mothers. The dietary intake of energy and the crude and energy-adjusted vitamin B12 intakes were comparable in both groups. At the study moment, the use of B-vitamin supplements, tobacco and alcohol was comparable between cases and controls. In the periconceptional period, there were no significant differences either concerning the use of B-vitamin supplements, tobacco or alcohol. A positive family history of CHD was associated with a CHD risk of 2.1 (95% CI 0.97-4.4).

The MTRR and TC genotype frequencies were consistent with HWE in mothers, fathers and children, both in the case-group and in the control-group. The frequencies of the MTRR 66 G-allele in cases and controls, respectively, were 55% and 56% for mothers, 61% and 57% for fathers, and 59% and 55%

Genotypes	Cases	Controls	OR (95% CI)
Mothers			
	(n = 230)	(n = 251)	
MTRR 66 AG or GG	181 (79)	201 (80)	1.0 (0.7-1.5)
MTRR 66 AA	49 (21)	50 (20)	1.0 (Reference)
	(n = 221)	(n = 238)	
TC 776 CG or GG	147 (67)	167 (70)	1.0 (0.6-1.5)
TC 776 CC	74 (33)	71 (30)	1.0 (Reference)
Fathers			
	(n = 229)	(n = 251)	
MTRR 66 AG or GG	196 (86)	206 (82)	1.3 (0.9-1.9)
MTRR 66 AA	33 (14)	45 (18)	1.0 (Reference)
	(n = 225)	(n = 242)	
TC 776 CG or GG	148 (66)	156 (64)	1.0 (0.6-1.6)
TC 776 CC	77 (34)	86 (36)	1.0 (Reference)
Children			
	(n = 229)	(n = 251)	
MTRR 66 AG or GG	191 (83)	199 (79)	1.3 (0.9-1.8)
MTRR 66 AA	38 (17)	52 (21)	1.0 (Reference)
	(n = 218)	(n = 246)	
TC 776 CG or GG	139 (64)	163 (66)	0.9 (0.6-1.5)
TC 776 CC	79 (36)	83 (34)	1.0 (Reference)

Table 2. The MTRR A66G and TC C776G genotype frequencies of the families and the association with CHD risk.

for children. The frequencies of the TC 776 G-allele in cases and controls, respectively, were 44% and 46% for mothers, 40% and 39% for fathers, and 42% and 42% for children. Allele transmissions were not significantly distorted in case-families. The MTRR 66 A-allele was transmitted 105 times and the G-allele 113 times (Chi-square 1.467, *P* = 0.23). For the TC 776 C and G-allele, these data were 107 and 90 times (Chi-square 0.294, *P* = 0.59). Logistic regression analyses did not show significant associations between the MTRR and TC genotypes in mothers, fathers or children and CHD risk (*Table 2*). The MTRR genotypes of fathers and children showed slightly higher CHD risks, albeit not significantly. The analyses of the various CHD phenotypes did not reveal significant associations for any of the genotypes.

		Case-mothers		
MTRR A66G	All (*n* = 137)	AA (*n* = 26)	AG (*n* = 68)	GG (*n* = 43)
Folate, serum (nmol/L)	15.3 (12.0-19.4)	14.2 (11.9-19.0)	15.0 (11.3-18.4)	15.5 (14.1-21.5)
Folate, RBC (nmol/L)	676 (533-802)	645 (494-880)	676 (543-813)	679 (536-777) [a]
Vitamin B12, serum (pmol/L)	276 (222-366)	284 (212-343)	274 (225-372)	272 (185-397)
Homocysteine, plasma (µmol/L)	10.6 (8.9-13.2) [b]	10.1 (8.7-12.2)	11.1 (9.5-13.3)	10.2 (8.1-13.1)
TC C776G	All (*n* = 130)	CC (*n* = 45)	CG (*n* = 58)	GG (*n* = 27)
Folate, serum (nmol/L)	15.3 (12.1-19.3)	15.7 (12.4-20.4)	15.0 (11.6-19.0)	15.3 (11.4-18.1)
Folate, RBC (nmol/L)	678 (536-798)	679 (550-821)	682 (521-836)	640 (539-770)
Vitamin B12, serum (pmol/L) [c]	278 (222-371)	285 (238-420)	299 (235-378) [c]	234 (171-272) [c]
Homocysteine, plasma (µmol/L)	10.6 (8.9-13.1)	10.4 (8.5-12.6)	10.3 (8.8-13.3)	11.4 (9.4-13.7)
		Control-mothers		
MTRR A66G	All (*n* = 203)	AA (*n* = 40)	AG (*n* = 102)	GG (*n* = 61)
Folate, serum (nmol/L)	14.3 (12.1-19.2)	16.2 (11.5-22.2)	13.4 (11.9-18.2)	14.7 (12.8-19.1)
Folate, RBC (nmol/L)	670 (533-819)	728 (565-1044)	645 (521-793)	671 (537-770)
Vitamin B12, serum (pmol/L)	249 (202-359)	241 (205-367)	266 (206-370)	245 (187-319) [d]
Homocysteine, plasma (µmol/L)	10.3 (8.4-12.1)	10.1 (8.4-11.7) [d]	10.0 (8.4-12.2)	10.7 (8.4-12.3) [c]
TC C776G	All (*n* = 193)	CC (*n* = 54)	CG (*n* = 94)	GG (*n* = 45)
Folate, serum (nmol/L)	14.1 (12.1-19.2)	14.4 (12.3-17.6)	14.0 (12.0-19.0)	16.0 (12.1-20.2)
Folate, RBC (nmol/L)	670 (533-808)	673 (530-773)	639 (531-792)	702 (543-948)
Vitamin B12, serum (pmol/L)	253 (202-364)	239 (195-334)	249 (201-341) [e]	281 (203-420)
Homocysteine, plasma (µmol/L)	10.3 (8.5-12.3)	10.5 (8.8-11.8)	10.3 (8.2-11.8) [e]	10.2 (8.5-13.3) [e]

Table 3. Maternal biochemistry at the study moment stratified for MTRR A66G and TC C776G genotypes.

[a] RBC folate, n = 42.

[b] P = 0.042 (Mann-Whitney U test) for comparison between cases and controls.

[c] P = 0.006 (Kruskal-Wallis test); P = 0.007 and P = 0.002 (Mann-Whitney U test) for comparison between CG and GG, and between CC and GG genotypes.

[d] GG vitamin B12, n = 60; AA homocysteine, n = 41; GG homocysteine, n = 60.

[e] CG vitamin B12, n = 93 ; CG homocysteine, n = 95 ; GG homocysteine, n = 44.

The maternal biochemistry in the pooled group of cases and controls did not show significant differences within the group of MTRR genotypes and within the group of TC genotypes. The same data are stratified for case/control status and presented in *Table 3*. The tHcy concentration was significantly higher in cases than in controls (P = 0.042). The MTRR genotypes in case-mothers did not significantly affect the folate, vitamin B12 and tHcy concentrations.

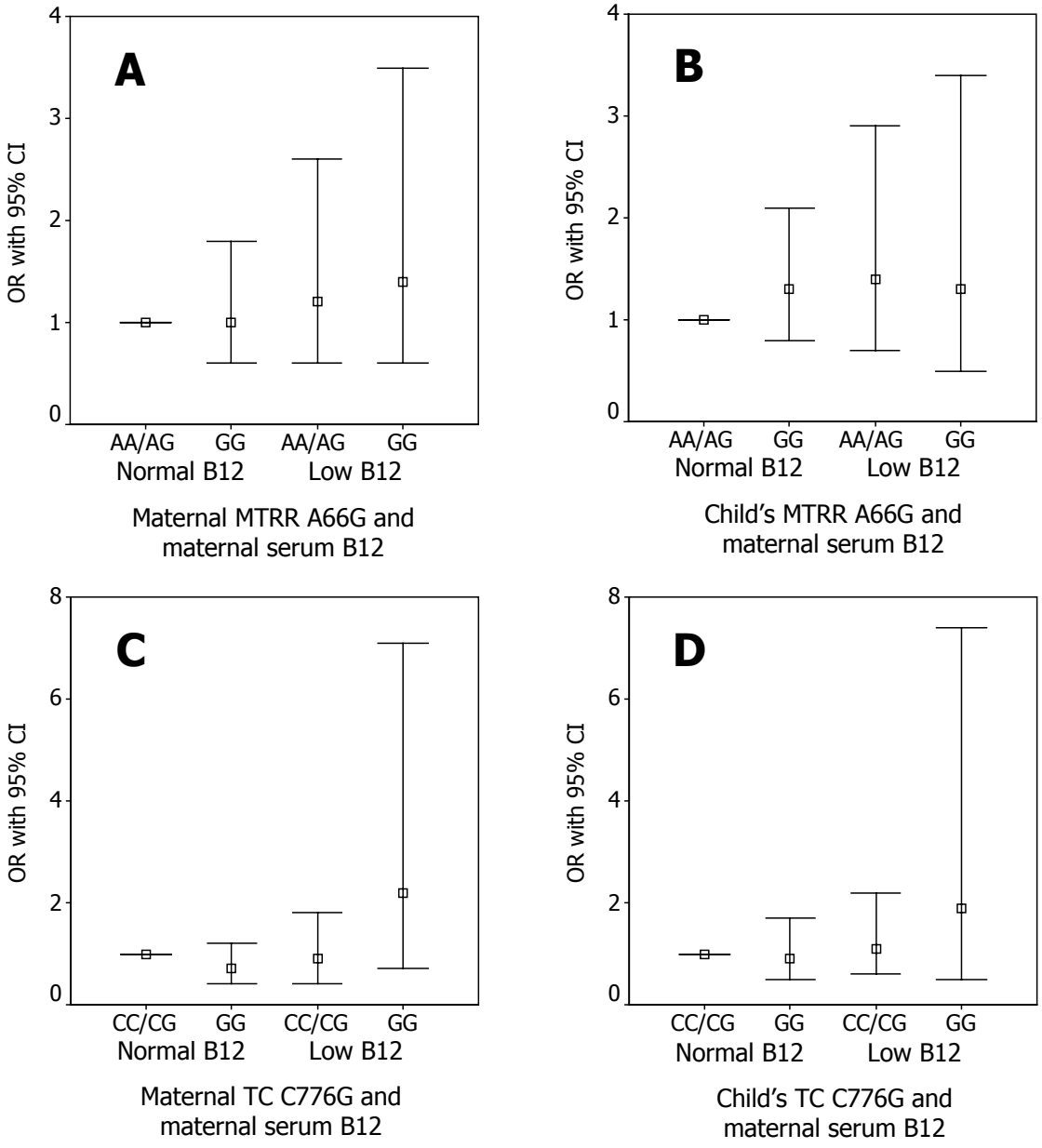

Figure 1. Interaction between maternal serum vitamin B12 and the MTRR A66G and TC C776G genotypes of mothers and children, and CHD risk. *The risk estimates are shown for the combination of maternal serum vitamin B12 concentrations and the MTRR A66G (mothers in panel A, children in panel B) and TC C776G (mothers in panel C, children in panel D) genotypes in mothers. The cut-off value for a low serum vitamin B12 concentration is the 15th percentile (175 pmol/L) of serum vitamin B12 concentrations in control-mothers.*

Compared with the CC and CG genotypes, the maternal TC 776 GG genotype in cases demonstrated significantly lower vitamin B12 concentrations (P = 0.006) and slightly higher tHcy concentrations (P = 0.50). No significant findings were observed in the control-group. In a multivariate model, we adjusted the biomarker concentrations for MTRR and TC genotypes and the use of B-vitamin supplements, tobacco and alcohol at the study moment, which did not significantly change the biochemistry results. The multivariate logistic regression analyses showed no significant interactions between the MTRR and TC genotypes, low maternal periconceptional vitamin B12 intake by food and supplements and the association with CHD risk. Risk estimates ranged between 0.5 and 1.4 in mothers and between 0.6 and 1.7 in children. *Figure 1* shows the subtle interactions between the maternal serum vitamin B12 and MTRR and TC genotypes of mothers and children. A low serum vitamin B12 in combination with the MTRR 66 GG genotype in mothers and children, respectively, demonstrated ORs of 1.4 (95% CI 0.6-3.5) and 1.3 (0.5-3.4). For the TC 776 GG genotype of mothers and children, the CHD risk estimates were 2.2 (0.7-7.1) and 1.9 (0.5-7.4), respectively.

Discussion

In this case-control study conducted in the Western part of the Netherlands, we showed that the MTRR and TC genetic polymorphisms in mothers and children are no independent CHD risk factors. The maternal TC genotype significantly influenced serum vitamin B12 in case-mothers only, which was not due to confounding by the use of B-vitamins at the study moment. However, a low maternal serum vitamin B12 and the TC 776GG genotype in mothers or children may increase CHD risk. This risk estimate was higher than the risk estimate for the combination of a low maternal vitamin B12 concentration with the MTRR 66GG genotype. Both results, however, should be very carefully interpreted, because the associations are weak and not significant. These associations have to be further investigated in larger sample sizes.

We could not demonstrate a significant association between the MTRR and TC polymorphisms in families and CHD risk. Only one smaller study than ours investigated the MTRR genotypes and CHD risk before without significant results.[30] Studies investigating MTRR and TC polymorphisms in related malformations, such as NTD and orofacial clefting, show inconclusive results as well.[11, 17, 29, 31-36]

The maternal biomarker analyses stratified for maternal TC genotypes showed a significantly lower serum vitamin B12 in case-mothers with the GG genotype compared with the CC or CG genotype. This is in line with studies of others.[14, 16, 17] Moreover, Afman *et al*. reported in mothers of NTD offspring that the proportion of vitamin B12 bound to TC was significantly lower in women with the GG genotype than in women with the CC genotype.[17] Since the serum vitamin B12 concentration represents the total of holo-haptocorrin and holo-TC concentrations, this may imply that in our study the lower serum vitamin B12 concentrations in case-mothers with the GG genotype coincide with lower holo-TC concentrations. This is substantiated by von Castel-Dunwoody *et al*., who reported lower holo-TC concentrations in TC 776 GG homozygotes than in CC homozygotes.[16]

The weak trend for hyperhomocysteinaemia across the TC genotypes confirm the data of other studies.[14, 17] The functional consequences of this TC polymorphism are a lower cellular vitamin B12

availability, either due to reduced transcription or conformational changes in the protein, thereby affecting its affinity for the receptor or the binding of vitamin B12 to TC.[14, 17] Thus, this TC polymorphism may affect cellular vitamin B12 availability, thereby increasing tHcy concentrations, particularly in individuals with a low vitamin B12 status. This substantiates our previous finding and that of others that maternal hyperhomocysteinaemia is associated with increased CHD risk.[3-5]

We found a significant effect of the TC genotypes on the vitamin B12 concentration in the case-group only, despite the reversed non-significant effect on vitamin B12 concentrations in controls. Overall comparisons of the MTRR genotypes and the biochemistry in cases and controls did not reveal significant differences, except for the tHcy concentration. Therefore, it is very likely that other factors are involved as well, such as differences in other vitamin B12-related polymorphisms and lifestyle factors. We could not show significant interactions between the MTRR and TC polymorphisms, maternal dietary intake of vitamin B12 and CHD risk. As serum vitamin B12 is a stronger indicator of vitamin B12 status than vitamin B12 intake, we investigated interactions between a low maternal serum vitamin B12 and the MTRR and TC polymorphisms in both mothers and children. The risk estimates may suggest a weak trend only towards a higher risk across the categories of the TC genotype in mothers and children. For the MTRR genotype in mothers and children, this is less clear. Thus, in addition to the MTRR and TC genotypes, the maternal vitamin B12 status is suggested to be important for the cardiovascular development of the child in utero.

A high plasma methylmalonic acid concentration is a sensitive indicator of a low vitamin B12 status. Van Beynum *et al*. described that the maternal MTRR 66 GG genotype in combination with a high plasma methylmalonic acid concentration was associated with a three-fold increased CHD risk, albeit not significantly.[30] Studies on NTD risk support the observed interaction between both polymorphisms and vitamin B12 status in our study, but the sample sizes of these NTD studies are much smaller.[11, 29] In addition, the ethnic background was not clear for 25% of the study population[11] or not described at all.[29] This is important information, because differences in allele frequencies of genetic polymorphisms are striking between and within continents.[24] With concern to confounders, Wilson *et al*. excluded vitamin supplement users.[11] Van der Linden *et al*. adjusted for significant differences in maternal age, but did not consider use of vitamin supplements at the study moment.[29]

One of the strengths of our study is that we standardised the data collection at a fixed study moment relatively soon after pregnancy for both cases and controls. This is in contrast to the designs of others, in which subjects were investigated at a mean time interval of 10 to 11 years[11] or even at different time intervals in controls and cases (8 and 16 years after pregnancy).[29] We previously showed the importance of considering the time-interval between delivery and the post partum study moment.[37] A standardised study moment relatively soon after the index-pregnancy is important with regard to nutritional and biochemical parameters. For many years, we have shown the value of a fixed study moment between 11 and 18 months after the index-pregnancy to estimate the nutritional status of the periconceptional period, and to reduce recall bias.[3, 19, 20, 22, 23] An earlier study moment after birth would significantly interfere with the maternal physiology and biochemistry and it might cause

misclassification of cases and controls, because most malformations are detected and completely diagnosed in the first year of life.

Other strengths of the HAVEN study are the recruitment of patients with a CHD phenotype that has been associated with maternal hyperhomocysteinaemia and the inclusion of only Dutch native as well as European families in the analyses in order to increase the homogeneity of the study population. In addition, we performed all analyses separately for CHD phenotypes. These analyses showed comparable results, thereby substantiating the homogeneity of our study population. However, we have to consider the limitations of our study as well. With regard to laboratory issues, it has been suggested that MMA or holo-TC concentrations are better predictors of vitamin B12 status than serum vitamin B12, but the determination of MMA concentrations is quite complex and expensive. Holo-TC is a sensitive marker for vitamin B12 deficiency and has a reasonable specificity as well and might be a better metabolic indicator of vitamin B12 status than serum vitamin B12.[38] Moreover, measurement of both holo-TC and total vitamin B12 concentrations may be a better predictor of the vitamin B12 status than either assay alone.[39] Finally, we only showed weak associations, which may be due to our sample size. Calculations revealed that the sample size of both the case and the control-group should be 416 for the MTRR polymorphism and 495 for the TC polymorphism to find a significant OR of 2.5 for the gene-serum vitamin B12 interaction with a power of 70%.

In conclusion, our findings suggest that interactions between TC 776 GG and MTRR 66 GG genotypes, and low periconceptional vitamin B12 status may be involved in the pathogenesis of CHDs with very small effects on the risk estimates. This suggestion is substantiated by our previous report that a maternal diet low in vitamin B12 is associated with an increased CHD risk in the offspring.[23] Therefore, it might be favourable to advise women to use a diet rich in vitamin B12 and eventually a vitamin B12 supplement in addition to a folic acid supplement in the periconceptional period to achieve an optimal vitamin B12 status. Future research may focus on other polymorphisms in the MTRR and TC genes, their functional and biochemical effects and the implications of lifestyle factors in order to gain insight into the role of vitamin B12 in the pathogenesis of CHDs.

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Chapter 8

General Discussion

Introduction

The overall aim of this thesis was to investigate associations between some biomarkers of the homocysteine metabolism, nutritional factors, polymorphisms in B-vitamin related genes and the risk of congenital heart defects (CHDs) in man. Moreover, gene-nutrient interactions were studied between the biomarkers, dietary intake and polymorphisms in the methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR) and transcobalamin (TC) genes and CHD risk.

In this thesis, we demonstrate that maternal hyperhomocysteinaemia, defined as a plasma total homocysteine (tHcy) concentration above 14.3 µmol/L, nearly three-fold increases the risk of CHD offspring. This is substantiated by the establishment of higher risk estimates when tHcy concentrations rise. Maternal hyperhomocysteinaemia shows to be partially due to low folate and low vitamin B12 status. Moreover, a low dietary intake of vitamin B12 is associated with a significantly higher CHD risk, particularly in low educated women. The dietary intakes are estimated with an adapted food frequency questionnaire (FFQ), which was validated in the HAVEN study (i.e., a Dutch acronym for the study of heart anomalies and the role of genetic and nutritional factors) for the estimation of the dietary intake folate and vitamin B12. The adapted FFQ shows to be a reliable tool to estimate the dietary intake of energy, macronutrients, folate and vitamin B12 in women of reproductive age. The investigation of folate-related genes reveals that the MTHFR A1298C polymorphism in children and fathers is independently associated with a lower CHD risk. Moreover, the interaction between the MTHFR 1298 AA genotype in children and no periconceptional use of folic acid supplements is significantly associated with a two-fold increased CHD risk. The MTRR A66G and TC C776G polymorphisms are not associated with CHD risk, but the TC 776 GG genotype in case-mothers significantly decreases the serum vitamin B12 concentrations. In addition, subtle interactions between serum vitamin B12 and TC 776 GG genotypes in mothers and children produce risk estimates of 2.2 (0.7-7.1) and 1.9 (0.5-7.4), respectively, and interactions between serum vitamin B12 and MTRR 66 GG genotypes in mothers and children show CHD risks of 1.4 (95% confidence interval 0.6-3.5) and 1.3 (0.5-3.4). These interactions may therefore be involved in the complex pathogenesis of CHDs.

We have to consider several methodological issues of the studies that are described in this thesis and put our results into the perspective of known and relevant literature. Consecutively, we can make inferences about the identified nutritional risk factors, e.g., maternal hyperhomocysteinaemia, a low vitamin B12 diet, the contribution of the MTHFR A1298C polymorphism in children and fathers to the development of CHDs, and the gene-nutrient interactions between the polymorphisms in the MTRR A66G and TC C776G genes and serum vitamin B12. At the end of this chapter, we recommend future research and discuss the implications regarding clinical practice and public health care.

Methodological issues

Ascertainment of the cases

Case-families consist of a mother, father and child and are recruited in collaboration with the

Departments of Paediatric Cardiology of Erasmus MC in Rotterdam, Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam. The two paediatric cardiologists who cooperate in the study recruit the families in collaboration with the project team and have diagnosed the case-children with tetralogy of Fallot, transposition of the great arteries, atrioventricular septal defect, perimembranous ventricular septal defect, coarctation of the aorta, aortic valve stenosis, pulmonary valve stenosis, and hypoplastic left heart syndrome. Both paediatric cardiologists are trained in Paediatric Cardiology at Leiden University Medical Centre through which the uniformity of the diagnoses is enhanced. We select these specific CHDs, because their association with folate or other environmental factors has previously been shown.[1, 2] Diagnoses are confirmed after birth by echocardiography and/or cardiac catheterisation and/or surgery. The paediatric cardiologists again verify the diagnoses before the families are invited for the HAVEN study. Most CHDs are detected and completely diagnosed during the first postnatal year. Although, still some misclassification of less severe CHDs might occur, the study moment of approximately 17 months after the index-pregnancy is, therefore, an appropriate moment for the inclusion of cases. Male gender is present in a higher proportion of cases than female and this predominance has been noted in several cardiovascular malformations, especially in transposition of the great arteries and left-sided obstructions.[3] Compared with controls, the rate of close-related family members with a CHD is higher in the case-group (9% and 4%, respectively, P = 0.06). These data further substantiate the validity of our case-group.

Ascertainment of the controls

Control-families consist of healthy control-children and both parents. They are enrolled in collaboration with a physician of the child health centres in the surroundings of Rotterdam. Children are eligible as controls if they do not have a major congenital malformation or chromosomal defects according to the medical record and regular health checks by the physician of the child health centre. Control-families are not related to case-families.

Comparability of cases and controls

In January 2005, 274 cases and 312 controls were enrolled in the ongoing HAVEN study. The response rates are 45% for cases and 17% for controls. Most of the invited families refuse to participate due to emotional or medical reasons and practical problems, such as the distance to the hospital and difficulties to take a day off for this reason. The differences in response rates between cases and controls can partially be explained by different motivations to participate in the HAVEN study. Case-parents often want to know more about the aetiology of CHDs and the possibilities of prevention if they want to become pregnant again. The motivation of control-families to participate is either because somebody in their family or neighbourhood has a child with a CHD, they want a check-up of their health status or they want to contribute to the prevention of CHDs.

At the start of this study, we aimed to recruit children with an indication for grommet insertion and their parents as controls via the Departments of Otorhinolaryngology of the participating hospitals

through which both cases and controls would be derived from the same domain population. However, the Medical Ethics Committees of some of these hospitals did not give permission to recruit control-families in this way. Therefore, we enrol the controls via the child health centres in the surroundings of Rotterdam and thereby, we increase the compliance rates. Thus, case and control-families are derived from the Western part of the Netherlands.

All general characteristics are comparable between cases and controls, except for maternal age. Compared with controls, the median age of case-mothers is significantly, though only slightly higher than in controls (33.5 and 32.8 years, respectively). One might expect that there would be an overrepresentation of non-Western immigrants in the case-group due to motivation and mode of recruitment. However, the distributions of the ethnic groups (Dutch natives, Western and non-Western immigrants)[4] are comparable for cases and controls. Moreover, these distributions are corresponding to the numbers for the Western part of the Netherlands (75%, 10% and 15% in 2006).[5] Several reports have described socioeconomic trends of higher risks in lower social classes for CHDs in general, for septal defects and for some specific cardiac defects.[6, 7] Besides employment, neighbourhood, occupation and rental occupancy, education is a measure of socioeconomic status. One might assume that parents with a higher education are more motivated to participate in comparison with low educated people. On the other hand, the low educated parents might have more time to participate than the high educated counterparts. However, in our study the distributions of the maternal and paternal educational level are comparable between cases and controls. This is the likely explanation that we do not demonstrate a significant association between parental educational level and CHD risk. With concern to the nutritional parameters, the overall dietary intakes are comparable between cases and controls. However, a maternal diet low in vitamin B12 is associated with a higher risk of a child with a CHD, especially in low educated women. It is known that people with a low education and low income are more likely to engage in poor dietary practice than their wealthier and higher educated counterparts.[8] Therefore, these findings subscribe that educational level is a determinant of nutritional status and lifestyle factors. Nevertheless, the parental educational levels do not differ between case and control-families and therefore, adjustment of the risk estimates for educational level would have led to overadjustment. In conclusion, we assume that selection bias is not an important issue, because of the comparability of both cases and controls with regard to the general characteristics.

Our main hypothesis is that the environmental factors involved in CHD pathogenesis are mainly derived from the mother and are considered to be much stronger determinants than the studied genetic polymorphisms. The latter determinants should be more regarded as subtle risk modifiers. In the studies concerning environmental factors, such as biochemistry and dietary intake, we have based the classification of ethnicity on the definitions of Statistics Netherlands.[4] We thereby categorise case and control-families by the assumed lifestyle and nutrition of the mother. Population admixture is a major confounder in investigating associations between genetic markers and disease. Therefore, we have used another classification for ethnicity in our studies concerning the genetic polymorphisms of MTHFR, TC and MTRR. This classification recognizes continental population structure and continental

genetic ancestry.[9] It is only slightly different from the classification used by Statistics Netherlands[4] as it incorporates the genetic background of the grandparents of the index-child as well. Using this genetic classification of ethnicity, participating families are categorised into three different groups: Dutch natives, European others and non-Europeans. However, the non-European group is significantly different from the other two groups concerning genotype frequencies, general characteristics and nutritional intake and is, therefore, excluded from the genetic analyses. In addition, all genotype data are checked for Mendelian segregation errors and inconsistent triads are excluded from analysis. Therefore, the study population is rather homogeneous with regard to the genetic backgrounds and, thereby, confounding due to population admixture is minimised.

Comparability of information

The potential for information bias is an issue inherent to the case-control design of studies investigating congenital malformations. Therefore, we have to consider recall bias and subsequent misclassification of exposures in the periconceptional period. Parents of affected children may have a better recall, because they actively search for an explanation of the disease of their child or have an assumption about its underlying cause. We have chosen for one fixed study moment relatively soon after pregnancy in both cases and controls compared with other studies to minimise the recall bias. Periconceptional exposures, e.g., the use of vitamin supplements, tobacco and alcohol, are equally distributed in cases and controls. Thus, it is not likely that our findings are distorted by these exposures. Moreover, differential misclassification is not likely, because participants are not aware of the specific hypotheses of this study. Although, random misclassification might have occurred, it may have attenuated our findings. These arguments are in line with the study of Infante-Rivard *et al.*, who demonstrated that in case-control studies inaccuracies are frequent but recall bias is rare and is found only for very specific exposures, such as when an association is published or when the exposure is socially undesirable.[10]

As we investigate current dietary intake, recall bias is not an important issue. However, dietary assessment methods have a strong bias towards underestimation of habitual energy intake and, therefore, we have evaluated the existence of reporting bias. Some under-reporting may equally be present in both cases and controls, but the dietary intakes are representative of long-term habitual intake (*Chapter 4 and 5*). Furthermore, adjustment for energy intake or expression of macronutrient intake as percentages of total energy intake minimises the bias generated by under-reporting.[11]

Accuracy

Considering dietary intake, we assume that the maternal nutritional status around 17 months after the index-pregnancy is the best estimate of the nutritional status in the periconceptional period, which is the crucial period of embryogenesis and cardiovascular development. This hypothesis is supported by previous research[12, 13] substantiating the assumption that there are no strong differences in the preconceptional dietary habits and those of one year post partum.[13-15] In addition, it takes approximately 12 months after pregnancy and breastfeeding to return to the preconceptional

hormonal, metabolic and nutritional status. Moreover, the season of the pre- and periconceptional period and the study moment, i.e., approximately two years after conception, is the same.

All blood samples are analysed anonymously within three months after collection. The samples are analysed in batches and, thereby, possible measurement errors are random. Moreover, all blood samples are separated within one hour after blood sampling to reduce the risk of haemolytic samples. One EDTA-tube is put on ice and centrifuged immediately after blood sampling. This is extremely important for accurate measurement of the fasting tHcy concentration.[16] The between-run coefficients of variation (CVs) are all below 10%: for vitamin B12, e.g., 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; for folate these CVs are 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L and for tHcy 5.9% at 15.3 µmol/L and 3.4% at 39.3 µmol/L. Importantly, the concentrations of tHcy, folate and vitamin B12 in our study are comparable with the concentrations that are measured in the preconceptional period as described by Cikot *et al.*[17] Hereby, we substantiate the value of our fixed and standardised study moment.

The questionnaire data have been checked for completeness and consistency by the researcher during the hospital visit. The dietary intakes of macronutrients and B-vitamins are in line with the Dutch dietary reference intakes[18, 19] and the data of the Dutch national food consumption survey of 2003.[20] Although, random measurement errors may still have occurred, the estimated dietary intakes are adjusted for nutrient loss due to processing and cooking of the food. Therefore, our FFQ produces valid data of dietary intake in women of reproductive age, which has been proven by our validation study as well.[21]

With regard to the DNA analysis, all genotype frequencies in controls are in Hardy Weinberg Equilibrium and they are in line with other European studies that have investigated MTHFR,[22, 23] TC[24-26] and MTRR[27, 28] genetic polymorphisms. More than three percent of the samples were re-genotyped to check for genotype calling consistency. The genotyping success rate is more than 96%. Laboratory errors are not likely because the genotyping has been done using Taqman® techniques according to protocols provided by the manufacturer (Taqman, Applied Biosystems, Foster City, CA, USA). We have restricted the analyses to complete and consistent triads only, thereby increasing the accuracy of our genotype data and minimising the risk of including non-biological fathers.

Power

In all power calculations, we have used a population CHD risk of six per 1000 live births and a type I error of 0.05. Calculations reveal that we have identified maternal hyperhomocysteinaemia as a risk factor for CHD in the offspring with a power of 77% (control prevalence of 10%, cases *n* = 98 , OR 2.9) and a low dietary intake of vitamin B12 with a power of 57% (control prevalence 10%, cases *n* = 192, OR 1.9). The sample size of 229 cases and the MTHFR 1298 A allele frequency of 0.34 enables us to detect a 40% significant risk reduction with a power of 78%. The risk allele frequencies are 0.46 for the MTRR polymorphism and 0.56 for the TC polymorphism, respectively. We would be able to find a significant OR of 2.5 for the interaction between these genetic polymorphisms and serum vitamin B12 with a power of 70% if the sample size of both the case and the control-group would have been

416 for the MTRR polymorphism and 495 for the TC polymorphism, respectively. The ongoing recruitment for the HAVEN study will solve this problem to investigate interactions between polymorphisms and environmental factors as risk or preventive factors of CHD offspring.

Inferences of the main findings

Biomarkers and dietary intake

Previous epidemiological studies show that periconceptional use of multivitamins containing folic acid reduces the risk of having a child with CHD.[1] However, it is unknown which ingredient(s) in multivitamins are responsible for this risk reduction. We do not show an independent and significant association between periconceptional use of B-vitamin supplements and CHD risk, but we demonstrate that a high maternal tHcy concentration is associated with an increased risk of a child with CHD. If mothers did not use a folic acid containing vitamin supplement in the periconceptional period, the risk estimate is even higher (Chapter 3). These findings are in line with the reported preventive effect of multivitamin use, because a compromised folate and/or vitamin B12 status results in mild hyperhomocysteinaemia. Hyperhomocysteinaemic case-mothers indeed show significantly lower serum folate and vitamin B12 concentrations than normohomocysteinaemic case-mothers. Moreover, they have a lower dietary intake of proteins and vitamin B6. Overall, a diet low in vitamin B12 is associated with an increased risk of a child with a CHD (Chapter 5). Thus, we show that derangements of several parameters in the homocysteine metabolism are associated with maternal hyperhomocysteinaemia (Figure 1).

Dietary protein intake is the main source of methionine, which is the sole precursor of homocysteine. A high-protein diet increases tHcy concentrations throughout the day[29] and particularly folate is an important substrate for the remethylation of homocysteine. Therefore, a disbalance in the maternal intake of proteins and folate deranges the homocysteine metabolism and easily leads to mild hyperhomocysteinaemia. Our study results concerning biomarkers and dietary intake suggest that several nutrients or maybe a specific dietary pattern are important and not just a single nutrient. This is in line with the various B-vitamins that are often correlated and present in the same foods.[30] This hypothesis is substantiated by the work of Gerber, who reported that the effect of diet does not occur through the addition of single nutrients. Each food combines many nutrients that allow for a synergistic action when present in a certain balance. Variety in daily food intake will avoid the repetitious intake of unfavourable food and provide the largest array of protective nutrients.[31] Our data suggest that an adequate intake of B-vitamins is necessary as well as a delicate balance between the intake of macronutrients and micronutrients. Future studies should precisely unravel which nutrients in the diet provide beneficial or teratogenic effects on cardiovascular development.

Derangements in the DNA methylation patterns can lead to developmental malformations.[32] Folate plays a critical role in the prevention of chromosome breakage and hypomethylation of DNA. These processes are compromised when the vitamin B12 concentration is low resulting in the folate trap, reduced methionine synthase activity and low levels of S-adenosylmethionine that particularly leads to hypomethylation of proteins, lipids and DNA. The DNA hypomethylation can be prevented by intake

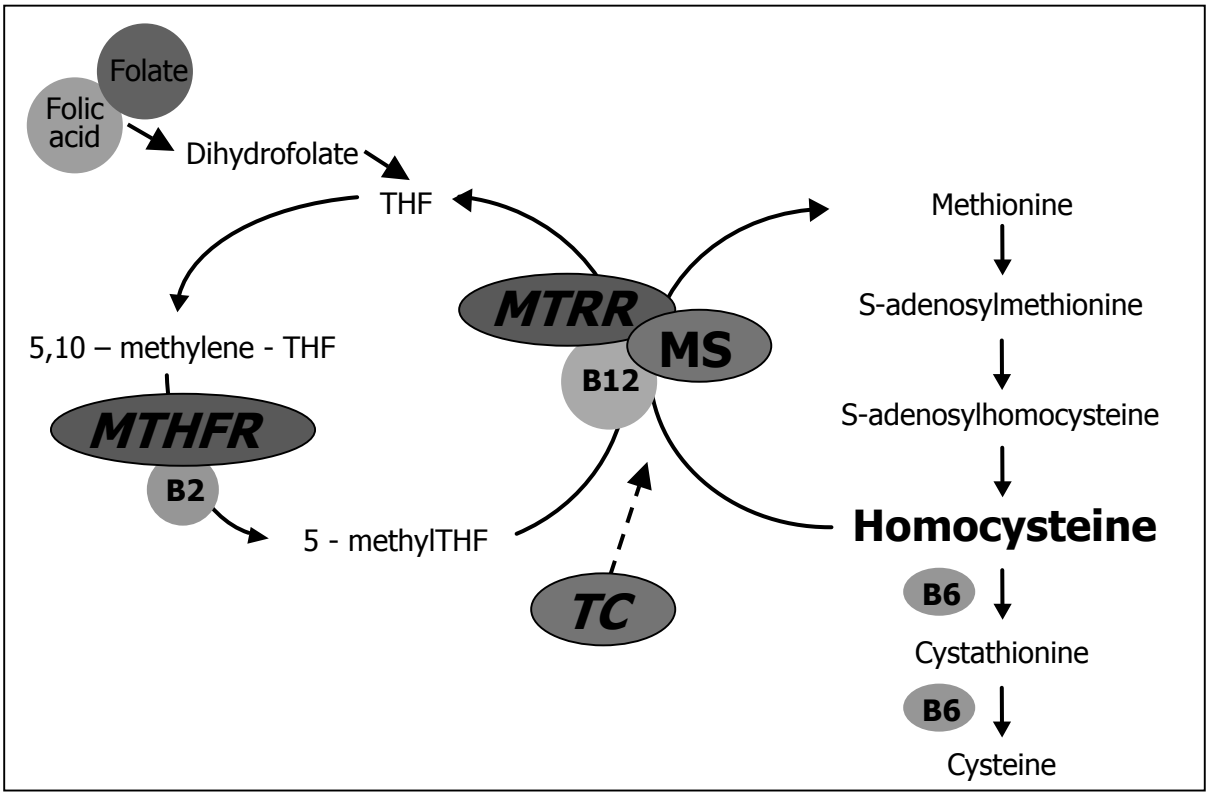

Figure 1. B-vitamin dependent homocysteine metabolism.

levels of more than 300 microgram folate per day and more than 2 microgram vitamin B12 per day.[33] These dietary intakes above the current dietary reference intakes may be important, particularly in those people with derangements in the metabolism of these vitamins and homocysteine, such as the case-mothers in our studies.

People with a low education and low income are more likely to engage in poor dietary practice than their wealthier and higher educated counterparts.[8] In our study, low educated women more often demonstrate a low dietary vitamin B12 intake that is associated with a higher risk of CHDs than women with a higher educational level. Educational level is also positively associated with the intake of vitamin B6 and folate. Therefore, low educated people may not have enough income to buy these generally more expensive foods or do not have the knowledge about the importance of nutrition on health. Moreover, others show that maternal obesity is also associated with CHD risk.[34, 35] These reports subscribe the importance of a varied and healthy diet, because a diet low in energy density coincides with a more favourable body weight. A dietary profile with a low energy density includes low fat intake and high intake of fresh fruit and vegetables being rich sources of particularly folate.[36]

We have also measured the concentrations of folate, vitamin B12 and tHcy in children. Serum and red blood cell folate concentrations are significantly higher in case-children than in controls, which is partially explained by formula feeding (*Chapter 3*). Moreover, randomly taken blood samples, alterations in the binding of folate to transport proteins and receptors, differences in the prevalence of genetic polymorphisms encoding these proteins, changed concentrations of cofactors, variations in the activity of liver enzymes and renal function affecting folate clearance may play a role as well. However, it is most likely that the folate status determined in the blood of children at the age of

around 17 months is not comparable to the folate status in the tissues forming the cardiovascular system during embryogenesis.

Folate and vitamin B12 related genes and gene-nutrient interactions

We showed that subtle genetic factors are involved in the pathogenesis of CHDs, especially the MTHFR A1298C genetic polymorphisms (*Chapter 6 and 7*). The almost significant association between a positive family history of CHD and a two-fold increased risk substantiates the involvement of genetic polymorphisms. Much stronger genetic factors for CHDs are chromosomal abnormalities, such as trisomy 21 and the 22q11 deletion.[37] This might explain why the MTHFR C677T, MTRR A66G and TC C776G genotypes do not affect CHD risk independently. As CHDs are comprised of complex malformations, both genetic and environmental factors, such as nutrition and lifestyle, are implicated in the pathogenesis and, thereby, possibly in prevention as well. Therefore, we focus on gene-nutrient interactions.

The studied polymorphisms in the MTHFR, MTRR and TC genes slightly affect the metabolic availability of folate and vitamin B12, respectively.[38-41] It is likely, therefore, that food intake and lifestyle factors, such as the use of vitamin supplements, tobacco and alcohol, interact with the genetic polymorphisms leading to altered biochemical concentrations of folate and vitamin B12. Significant interactions are not demonstrated between the genotypes and dietary intake of vitamin B2, folate and vitamin B12, and the association with CHD risk. However, the gene-nutrient interaction concerning the child's MTHFR A1298C polymorphism and maternal use of a folic acid containing vitamin supplement proves to be significantly associated with CHD risk. The bioavailability of natural folates in foods is lower compared with the synthetic folic acid form. Apart from the much lower bioavailability of food folates, the poor stability of folates in foods due to heating during cooking can substantially reduce the amount of vitamins ingested and, thereby, it can be an additional factor limiting the bioavailability of food folates to improve folate status.[42] For vitamin B12, as for many nutrients, absorption is dependent on available transport mechanisms and the load put on the body. Estimates of vitamin B12 absorption from food indicate that with normal gastric function, approximately 50% of dietary vitamin B12 intake is absorbed.[43] These arguments may explain the differences in significance levels for the investigated gene-nutrient interactions. With regard to vitamin B12, the interactions between the MTRR and TC genotypes and the use of B-vitamin supplements are not significant, which might be explained by the content of the supplements that contain not only vitamin B12 but also other multivitamins or solely folic acid. We have reanalysed the data using serum vitamin B12 as the environmental factor, because it is a stronger metabolic indicator of the vitamin B12 status than vitamin B12 intake. A possible trend towards a higher risk is revealed for the interaction of serum vitamin B12 with the maternal and child's TC genotype, that is less clear for the maternal and child's MTRR genotype. However, the *P* values for trend are not significant, which might be caused by a power problem as biochemical measurements are available in only 137 case-mothers and 203 controls. Nevertheless, these studies suggest that respectively strong and subtle gene-

nutrient interactions concerning the maternal folate and vitamin B12 status are involved in the pathogenesis of CHDs.

Future research

Our results clearly demonstrate that derangements in homocysteine metabolism are involved in the pathogenesis of CHDs. However, it is not yet known whether hyperhomocysteinaemia disturbs the normal cardiogenesis by a direct teratogenic effect or by indirect effects via a low folate and/or low vitamin B12 status. Hyperhomocysteinaemia is associated with decreased concentrations of B-vitamins, which may suggest that the nutrient status in the embryonic tissues is decreased as well. Moreover, homocysteine evokes oxidative stress through the production of reactive oxygen species, produces homocysteinylated proteins, and leads to the accumulation of its precursor, S-adenosylhomocysteine, a potent inhibitor of biological transmethylations.[44] With regard to oxidative stress, the developing embryo may be exposed to potentially teratogenic reactive oxygen species. Depending on the timing and duration of exposure, the functional consequences of oxidative injury may induce embryopathy and dysmorphogenesis.[45] However, disturbances in the DNA methylation patterns are also associated with developmental malformations.[32] An adequate supply of both folate and vitamin B12 is essential in preventing DNA hypomethylation. Interestingly, Fenech postulated that DNA hypomethylation can be prevented at intake levels in excess of current dietary reference intakes.[33] Therefore, the data that are presented in this thesis justify additional research into the role of hyperhomocysteinaemia and its underlying determinants in the pathogenesis of CHDs. Further investigations have to be performed on the critical balance between antioxidant activity and prooxidant exposures that are modifiable by genetic and/or environmental factors. Moreover, the beneficial and teratogenic effects of both excessive and deficient intakes of nutrients should be explored as well.

Both human and animal studies are necessary to investigate the effects of hyperhomocysteinaemia. A prospective cohort study is the best study design for investigation of the nutritional status in the periconceptional period and, thus, during embryogenesis. Moreover, in the prospective study design, recall bias is not a problem. However, a large prospective cohort study is not feasible, because it requires far more study subjects due to the low prevalence of CHDs, it is highly time-consuming and expensive, and it cannot be as cost-effective as the case-control study design.[46] The study design of randomised clinical trials has to deal with the same problems as the cohort study. Furthermore, it is immoral to refrain women in the reproductive age from the use of folic acid supplements, for example, because of the protective effect with regard to risk of congenital malformations in the offspring. Therefore, case-control studies in high risk populations are the best alternative.

In the past decade remarkable progress has been made within the development of genetic analyses and the unveiling of the genetic aetiologies of human disease. Nowadays, it is possible to identify disease genes by linkage analyses, positional cloning and analysis of candidate genes.[47, 48] These techniques give the opportunity to study genetic determinants in the homocysteine and related pathways. Because of the multifactorial aetiology of CHDs, gene-environmental interactions with a

biological rationale may have a greater impact on cardiovascular development than independent effects of genetic polymorphisms. Moreover, gene-environmental interactions are amendable by environmental factors and, thereby, the investigation of these interactions may provide clues for primary prevention of CHDs by the adjustment of lifestyle and nutritional habits. Nevertheless, to detect relevant gene-environmental interactions and to be able to investigate these interactions in the CHD subgroups, sample sizes have to be increased. As the HAVEN study is an ongoing study, sample sizes are currently being increased to be able to study the more subtle risk estimates and interactions. Moreover, multicentre collaborations between all Departments of Paediatric Cardiology in the Netherlands should be encouraged for this goal.

Our case-control study demonstrates a significant association between hyperhomocysteinaemia and CHD risk. However, it remains to be elucidated whether the association is causative or not. Therefore, experimental studies at the time of embryogenesis are needed. Our group has already reported that the migration and differentiation of neural crest cells can be influenced by homocysteine and folic acid.[49] Besides the development of the neural tube, lip and palate, neural crest cells are also involved in the embryogenesis of the cardiac outflow tract. Moreover, studies in chicken embryos have shown 83% subarterial ventricular septal defects after injection of homocysteine into the neural tube lumen at the time point of neural crest cell migration.[49, 50] The chicken embryo shares morphological features of the human embryo during early cardiac development and it is also very accessible for experiments. Therefore, this chicken embryo model makes it possible to mimic the human cardiovascular development and to perform interventions in vivo. Thereby, the complex homocysteine metabolism can be studied during cardiogenesis to explore the induction of oxidative stress, DNA hypomethylation and other processes induced by hyperhomocysteinaemia.

In conclusion, the reports of the association between hyperhomocysteinaemia and CHD risk provide a lot of arguments for further research in several homocysteine-related pathways. Moreover, the influence of gene-nutrient interactions in cardiovascular development suggest a wide spectrum of nutritional and lifestyle interventions to prevent CHDs in future.

Implications for public health and clinical practice

This thesis demonstrates that maternal hyperhomocysteinaemia is associated with an increased CHD risk. This risk is even higher if the mothers do not use a folic acid containing vitamin supplement in the periconceptional period. Moreover, all mothers have a substantially lower folate intake than the Dutch recommended dietary allowance of 300 µg. These findings substantiate the recommendation to use a folic acid supplement in the periconceptional period not only to prevent neural tube defects, but CHDs as well. In our study population, 54% of the case-mothers and 59% of the control-mothers have used folic acid in the advised period of four weeks before until eight weeks after conception. This is higher than the percentage of 36% in 2002 that is reported in a Dutch study, but compliance to proper use is quite poor, especially in low educated women.[51, 52] Therefore, these data show again that all women who are planning a pregnancy should be urged to use a folic acid supplement. Recently, the Dutch government has initiated some projects to stimulate the use of folic acid.[53] Firstly,

pharmacies in the Netherlands are informing non-pregnant women in the reproductive age about the proper use of folic acid in the campaign 'Zorg voor Foliumzuur'. Secondly, within a pilot-study midwives are investigating how to stimulate the periconceptional use of folic acid, particularly in immigrants and low educated women. Moreover, in 2006 ERFO-centre has developed new brochures on folic acid and a website too (www.slikeerstfoliumzuur.nl). The rather low use of folic acid raises the question whether foods should be fortified with folic acid. Besides the beneficial effects, folic acid use can mask vitamin B12 deficiency, but it may also enhance tumour growth in laboratory animals and humans and have negative effects on cancer through genetic selection.[54, 55] Therefore, at this moment, overall food fortification is still not present in the Netherlands but it is an issue of continuous discussion.[56]

The association between a low maternal dietary vitamin B12 intake and CHD risk supports the hypothesis that other nutrients may play a role as well. However, our data do not show enough evidence for a tailored advice concerning the optimal overall nutritional intake in the periconceptional period to every woman. Nevertheless, we demonstrate the beneficial effect of periconceptional exposure to folic acid supplements, particularly in children carrying the MTHFR 1298 AA genotype. Furthermore, our findings suggest that subtle interactions between TC 776 GG and MTRR 66 GG genotypes and low periconceptional vitamin B12 status might be involved in the pathogenesis of CHDs as well. Therefore, for the time being, it might be favourable to advise women to use a diet rich in vitamin B12 and maybe a vitamin B12 supplement in high risk groups in addition to the daily folic acid supplement intake in the periconceptional period. The use of B-vitamin supplements along with a healthy diet is a rational approach to prevent CHDs because it is simple and inexpensive, and it is supported by our current data.

Hyperhomocysteinaemia is not only associated with CHD risk, but it is a strong risk factor for adult cardiovascular diseases as well.[57] Since our experimental data suggest that prenatal exposure to hyperhomocysteinaemia induces the first features of atherosclerosis that are associated with cardiovascular diseases in later life,[58] this may imply that the hyperhomocysteinaemic mothers and their children should be targeted for nutritional interventions. However, results of randomised controlled studies aimed at reducing the prevalence of hyperhomocysteinaemia and related morbidity must be available before strategies based on food interventions can be recommended. The vast majority of the evidence of lowering tHcy by folate intake is related to secondary prevention trials.[59] It is still the question whether primary prevention is possible.

The results of the studies described in this thesis can be implemented in the preconceptional care. Nowadays, women that are at risk for having a child with a CHD should be screened for hyperhomocysteinaemia. Moreover, the dietary intakes of these women should be evaluated, especially the intakes of folate and vitamin B12. In addition, special attention should be paid to low educated women, because they are a high risk group, partly due to malnutrition and a low use of folic acid supplements. This preconception screening is important for future risk assessment and preconceptional counselling of parents to be. In general, hyperhomocysteinaemia can easily be treated with a folic acid and/or vitamin B12 supplement and, therefore, this risk factor can be

eliminated before conception. Moreover, our findings can be implemented via the website www.zwangerwijzer.nl, which is a joint initiative of the Erasmus MC, University Medical Centre in Rotterdam and the Dutch ERFO-Centre. Using the website questionnaire, the parental risk factors can be identified and used in the preconceptional care, and thereby, the counselling can more effectively be pointed at the identified risk factors. Since August 2006, Rotterdam is the first city in the Netherlands with institutions that give preconception care to parents to be with a normal population risk for pregnancy complications. In future, this may reduce the prevalence of congenital malformations and other pregnancy related disorders. Therefore, if any preventive approach could decrease the number of children born with a CHD or the number of chronic diseases in later life, not only the anguish in many families could be avoided, it would mean an economical gain as well.

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Summary

Introduction

Congenital heart defects (CHDs) belong to the most common group of major congenital malformations in newborns. At present, birth defects are the leading cause of infant mortality, with CHDs accounting for one in every three birth defect-related infant deaths. A substantial part of the surviving children has to undergo surgery and complex medical treatments and they often suffer from serious physical and psychological problems. In addition, the estimated average lifetime cost of the most clinically important CHDs is over $300,000 in the United States of America. Therefore, prevention of CHDs can substantially reduce the paediatric morbidity and mortality and related health care costs as well.

With regard to aetiology, only approximately 15% of the CHDs can be attributed to a known cause including genetic and chromosomal conditions with environmental exposures. Most CHDs, however, are considered complex diseases with a multifactorial aetiology, which are thought to result from interactions between both genetic and environmental factors. Several studies described that the maternal use of folic acid supplements in the periconceptional period reduces the risk of having a child with a CHD. Folic acid and its dietary form folate are important substrates of the homocysteine metabolism. Animal studies have indicated the involvement of homocysteine in heart development by stimulating the proliferation and migration of neural crest cells and inhibiting their differentiation. Mild to moderate hyperhomocysteinaemia results from inadequate intake of B-vitamins as well as from functional polymorphisms in key enzymes involved in the homocysteine metabolism or transport of B-vitamins. The mother is the environment of the developing child in utero and, therefore, the maternal nutritional status will influence the homocysteine metabolism in the embryonic tissues as well. Moreover, besides functional polymorphisms in B-vitamin related genes derived from both parents, the interactions between the maternal nutritional status and polymorphisms in B-vitamin related genes in mothers and children might be important in heart development.

In *Chapter 2*, a meta-analysis is presented of published studies investigating the association between maternal hyperhomocysteinenaemia, 5,10-methylenetetrahydrofolate reductase (MHTFR) polymorphisms in mothers and children, and the risk of a child with a CHD or cleft lip with or without cleft palate (CLP). This meta-analysis demonstrates that maternal hyperhomocysteinaemia is a risk factor for CHDs. The MTHFR C677T and A1298C polymorphisms in both mothers and children are not independently associated with CLP or CHDs. Future studies should be performed to investigate the interactions between maternal hyperhomocysteinaemia, B-vitamin intake, related polymorphisms and the risk of CLP and CHDs.

Thereafter, this thesis presents the results of the ongoing HAVEN study, which is a Dutch acronym for the study of congenital heart anomalies and the role of genetic and nutritional factors. This case-control study has been conducted from June 2003 onwards at the Department of Obstetrics and Gynaecology/Division of Obstetrics and Prenatal Medicine of Erasmus MC, University Medical Centre, Rotterdam, the Netherlands. Children with a CHD and both parents were recruited in collaboration with the Departments of Paediatric Cardiology of the same hospital and of Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam. Healthy control-children and both parents were enrolled in collaboration with the child health centres of

'Thuiszorg Nieuwe Waterweg Noord' in the surroundings of Rotterdam.

Part I

The first part of this thesis describes the maternal nutritional status of B-vitamins in association with the risk of having a child with a CHD. The nutritional status is partly determined by the metabolism and intake of nutrients. To study the B-vitamin status, we have measured the concentrations of serum vitamin B12, serum and red blood cell (RBC) folate, and plasma total homocysteine (tHcy) both in mothers and children, because these biomarkers partially reflect the effects of the nutritional intake on the homocysteine metabolism (*Chapter 3*). We demonstrate that maternal hyperhomocysteinaemia, defined as a tHcy concentration above 14.3 µmol/L, nearly three-fold increases the risk of CHD in the offspring. This is substantiated by the establishment of higher risk estimates when tHcy concentrations rise. Moreover, the risk estimates are even higher if mothers do not use a folic acid supplement in the periconceptional period. Maternal hyperhomocysteinaemia shows to be partially caused by a low folate and low vitamin B12 status. Serum and RBC folate concentrations are significantly higher in case-children than in control-children, which is partially explained by formula feeding. The folate status of children warrants further investigation.

We have performed a validation study for the estimation of the maternal dietary intake of folate and vitamin B12 with a food frequency questionnaire (FFQ) that is adapted by the Division of Human Nutrition of Wageningen University in Wageningen, the Netherlands (*Chapter 4*). The correlation and validity coefficients between the FFQ, the 24-hour recalls and the biomarkers are reasonably good. Furthermore, the estimated intakes of folate and vitamin B12 are comparable with the results of the most recent Dutch food consumption survey as well as the intake of energy and macronutrients. Therefore, the adapted FFQ is a reliable tool to estimate the dietary intake of energy, macronutrients, folate and vitamin B12 in women of reproductive age and it is suitable for the investigation of nutrient-disease associations in Dutch women.

Using this FFQ, we have assessed the maternal intake of macronutrients and B-vitamins in case and control-mothers in relation to CHDs in their offspring (*Chapter 5*). The dietary intake of macronutrients and B-vitamins is comparable between 192 case-mothers and 216 control-mothers, but they all had a substantially lower median folate intake than the Dutch recommended dietary allowance of 300 µg. The intake of proteins and vitamin B6 and the concentrations of serum vitamin B12 and folate are significantly lower in hyperhomocysteinaemic case-mothers than in normohomocysteinaemic case-mothers. A maternal dietary intake of vitamin B12 below 2.1 µg/day is associated with an approximately two-fold CHD risk, especially in low educated women. As hyperhomocysteinaemia is a strong risk factor for adult cardiovascular disease, these data may imply that the hyperhomocysteinaemic mothers and their children should be targeted for nutritional interventions.

Part II

In the second part of this thesis gene-nutrient interactions are described regarding folate and vitamins B2 and B12. In *Chapter 6*, we present the data of the interactions between nutritional and lifestyle

factors and two polymorphisms in the MTHFR gene. The MTHFR C677T polymorphisms are not significant associated with CHD risk. However, the MTHFR 1298 AA genotype in fathers and children show significantly increased CHD risks. Furthermore, a two-fold increased CHD risk is shown in children with the MTHFR 1298 AA genotype who are periconceptionally not exposed to folic acid supplements compared with MTHFR 1298 AC/CC carriers who are periconceptionally exposed. No significant interactions are detected between the MTHFR genotypes and dietary intake of folate and vitamin B2.

Chapter 7 shows analyses of polymorphisms in the methionine synthase reductase (MTRR) and transcobalamin (TC) genes in combination with vitamin B12 related environmental factors. The MTRR A66G and TC C776G polymorphisms do not affect CHD risk. No significant interactions are observed between these polymorphisms in mothers or children, low vitamin B12 intake and CHD risk. The maternal TC genotype significantly influences serum vitamin B12 in case-mothers, which is not due to a confounding effect of the current use of B-vitamins. Moreover, the interactions between a low serum vitamin B12 and the TC 776 GG and maybe MTRR 66 GG genotypes are suggested to be associated with an increased CHD risk. However, these interactions are not statistically significant. This might be due to our sample size, but the ongoing recruitment for the HAVEN study will solve this problem in future.

In the general discussion, the results of these case-control studies are discussed and our objectives are evaluated (*Chapter 8*). Furthermore, we recommend future research and discuss the implications regarding clinical practice and public health care. Maternal hyperhomocysteinaemia and a low dietary intake of vitamin B12 contribute to CHD risk as well as the interaction between the MTHFR A1298C polymorphism and folate status, and possibly the interaction between the TC C776G and MTRR C776G polymorphisms and vitamin B12 status. These findings indicate that the maternal nutritional status and gene-nutrient interactions are involved in the pathogenesis of CHDs. Therefore, it might be favourable to advise women in high risk groups to use a diet rich in vitamin B12 and maybe a vitamin B12 supplement in addition to the daily folic acid supplement intake in the periconceptional period. The use of B-vitamin supplements along with a healthy diet is a rational approach to prevent CHDs because it is simple and inexpensive, and it is supported by our current data.

The results of the studies described in this thesis can be directly implemented in the preconception care. Nowadays, women who are at risk for having a child with a CHD should be screened for hyperhomocysteinaemia and their dietary intakes should be evaluated, especially the intake of folate and vitamin B12. In addition, special attention should be paid to low educated women, because they are a high risk group, partly due to malnutrition and a low use of folic acid supplements. Preconceptional screening is important for future risk assessment and counselling of parents to be and may cause a reduction in the prevalence of CHDs in future.

Samenvatting

Introductie

Aangeboren hartafwijkingen (CHD) behoren tot de meest voorkomende aangeboren afwijkingen bij pasgeborenen. Tegenwoordig zijn aangeboren afwijkingen de voornaamste oorzaak van sterfte onder zuigelingen en een derde van deze kindersterfte wordt veroorzaakt door CHD. Een groot aantal kinderen dat overleeft, moet chirurgie en complexe medische therapie ondergaan en heeft vaak ernstige fysieke en psychologische problemen. Daarnaast zijn de gemiddelde kosten voor de klinisch meest belangrijke CHD in de Verenigde Staten geschat op $ 300.000 per mensenleven. Daarom kan preventie van CHD zowel de morbiditeit en mortaliteit onder kinderen als gerelateerde kosten voor de gezondheidszorg reduceren.

Slechts bij 15% van de CHD kan een bekende oorzaak worden aangetoond, zoals genetische of chromosomale afwijkingen en omgevingsinvloeden. Echter, de meeste CHD worden beschouwd als complexe ziekten met een multifactoriële ontstaanswijze, die de resultante zijn van interacties tussen genetische en omgevingsfactoren. Diverse studies hebben beschreven dat gebruik van foliumzuursupplementen door de moeder in de periconceptionele periode de kans op het krijgen van een kind met een CHD reduceert. Foliumzuur en het in voeding voorkomende folaat zijn belangrijke substraten van de homocysteine stofwisseling. Dierstudies wijzen op de betrokkenheid van homocysteine in de hartontwikkeling doordat homocysteine de proliferatie en migratie van neurale lijst cellen stimuleert en hun differentiatie remt. Milde tot matige hyperhomocysteinemie ontstaat door onvoldoende inname van B-vitamines en functionele variaties in sleutelenzymen die betrokken zijn in de homocysteine stofwisseling of in het transport van B-vitamines. Daar de moeder de omgeving is van het kind dat zich in haar baarmoeder ontwikkelt, zal de maternale voedingsstatus ook de homocysteine stofwisseling in de embryonale weefsels beïnvloeden. Naast de functionele variaties in de B-vitamine gerelateerde genen afkomstig van beide ouders, kunnen bovendien interacties tussen de maternale voedingsstatus en variaties in de B-vitamine gerelateerde genen in moeders en kinderen van belang zijn in de hartontwikkeling.

In *Hoofdstuk 2* wordt een meta-analyse behandeld van gepubliceerde studies betreffende de associatie tussen maternale hyperhomocysteinemie, 5,10-methyleentetrahydrofolaat reductase (MHTFR) genvariaties in moeders en kinderen en de kans op een kind met een CHD of met schisis. Deze meta-analyse toont aan dat maternale hyperhomocysteinemie een risicofactor is voor CHD. De MTHFR C677T en A1298C variaties in zowel moeders als kinderen zijn niet geassocieerd met CHD of schisis. Toekomstige onderzoeken zullen verricht moeten worden om de interacties te bestuderen tussen maternale hyperhomocysteinemie, inname van B-vitamines, gerelateerde genvariaties en de kans op het krijgen van een kind met een CHD of schisis.

Vervolgens beschrijven wij in dit proefschrift de resultaten van de nog lopende HAVEN studie, wat een acroniem is voor het onderzoek naar CHD en de rol van genetische en voedingsfactoren. Deze case-controle studie wordt sinds juni 2003 uitgevoerd door de afdeling Verloskunde en Vrouwenziekten, subafdeling Verloskunde en Prenatale Geneeskunde van het Erasmus MC te Rotterdam. Kinderen met een CHD en beide ouders worden gerecruteerd in samenwerking met de kindercardiologie afdelingen van hetzelfde ziekenhuis, het Leids Universitair Medisch Centrum, het VU Medisch Centrum en het

Academisch Medisch Centrum in Amsterdam. De werving van gezonde controle kinderen en beide ouders vindt plaats in samenwerking met de consultatiebureaus van 'Thuiszorg Nieuwe Waterweg Noord' in de omgeving van Rotterdam.

Deel I

Het eerste deel van dit proefschrift beschrijft de maternale voedingsstatus van B-vitamines en de associatie met de kans op een kind met een CHD. De voedingsstatus wordt deels bepaald door de stofwisseling en de inname van voedingsstoffen. Om de B-vitamine status in moeders en kinderen te bestuderen, zijn de concentraties gemeten van serum vitamine B12, folaat in het serum en in de rode bloed cel (RBC) en plasma totaal homocysteine (tHcy), omdat deze biomarkers gedeeltelijk de effecten van de voedingsinname op de homocysteine stofwisseling reflecteren (*Hoofdstuk 3*). Wij tonen aan dat maternale hyperhomocysteinemie, gedefinieerd als een tHcy concentratie hoger dan 14,3 µmol/L, de kans op een kind met een CHD bijna driemaal verhoogt. Dit wordt bevestigd door de hogere risico's bij een toenemende tHcy concentratie. Bovendien is het risico nog hoger als moeders geen foliumzuursupplement gebruiken in de periconceptionele periode. Maternale hyperhomocysteinemie wordt deels veroorzaakt door een lage folaat en vitamine B12 status. Serum en RBC folaat concentraties zijn significant hoger in patiënten dan in controle kinderen, wat gedeeltelijk verklaard wordt door flesvoeding. Deze folaat status van de kinderen rechtvaardigt dan ook verder onderzoek.

Wij hebben een validatie studie uitgevoerd voor de schatting van de maternale voedingsinname van folaat en vitamine B12 met een voedsel frequentie vragenlijst (FFQ) die aangepast is door de Divisie Humane Voeding van de Wageningen Universiteit in Wageningen (*Hoofdstuk 4*). De correlatie en validatie coefficienten tussen de FFQ, de 24 uur recalls en de biomarkers zijn redelijk goed. Bovendien zijn de geschatte innames van folaat en vitamine B12, maar ook die van energie en macronutrienten, vergelijkbaar met de resultaten van het meest recente voedselconsumptieonderzoek. De aangepaste FFQ is dus een betrouwbaar instrument om de voedingsinname van folaat, vitamine B12, energie en macronutrienten te schatten voor vrouwen in de reproductieve leeftijdscategorie en het is geschikt om associaties tussen voeding en ziekte in Nederlandse vrouwen te onderzoeken.

Met deze FFQ hebben we de voedingsinname van macronutrienten en B-vitamines vastgesteld bij moeders in relatie tot een CHD bij hun kind (*Hoofdstuk 5*). De voedingsinname van macronutrienten en B-vitamines is vergelijkbaar tussen 192 moeders van patienten en 216 moeders van een gezond kind, maar alle moeders hebben een aanzienlijk lagere mediane folaat inname dan de Nederlandse aanbevolen dagelijkse hoeveelheid van 300 µg. De inname van eiwitten en vitamine B6 en de concentraties van serum vitamine B12 en folaat zijn significant lager in hyperhomocysteinemische dan in normohomocysteinemische moeders van patiënten. Een maternale voedingsinname van vitamine B12 lager dan 2,1 µg/day is geassocieerd met een circa tweemaal verhoogde kans op een kind met een CHD, met name in laag opgeleide vrouwen. Aangezien hyperhomocysteinemie een sterke risicofactor is voor hart- en vaatziekten bij volwassenen, zouden deze gegevens kunnen impliceren dat de hyperhomocysteinemische moeders en hun kinderen een doelgroep zijn voor voedingsinterventies.

Deel II

In het tweede deel van dit proefschrift worden gen-voeding interacties beschreven met betrekking tot folaat, vitamine B2 en B12. In *Hoofdstuk 6* presenteren wij de resultaten van de interacties tussen voeding, leefstijlfactoren en twee variaties in het MTHFR gen. De MTHFR C677T variaties zijn niet significant geassocieerd met de kans op CHD. Echter, de MTHFR 1298 AA genotypes in vaders en kinderen zijn significant geassocieerd met een verhoogd risico. Bovendien wordt een tweemaal verhoogd risico van CHD gedemonstreerd in kinderen met het MTHFR 1298 AA genotype die periconceptioneel niet zijn blootgesteld aan foliumzuursupplementen vergeleken met MTHFR 1298 AC/CC dragers die periconceptioneel wel zijn blootgesteld. Er worden geen significante interacties aangetoond tussen de MTHFR genotypes en de voedingsinname van folaat en vitamine B2.

Hoofdstuk 7 geeft de analyses weer van de variaties in de methionine synthase reductase (MTRR) en transcobalamine (TC) genen in combinatie met vitamine B12 gerelateerde omgevingsfactoren. De MTRR A66G en TC C776G variaties beïnvloeden de kans op CHD niet. Er worden geen significante interacties aangetoond tussen deze variaties in moeders en kinderen, een lage inname van vitamine B12 en de kans op CHD. Het maternale TC genotype beïnvloedt de serum vitamine B12 concentratie significant in moeders van patiënten, wat overigens niet verstoord wordt door het huidige gebruik van B-vitamine supplementen. Bovendien suggereren de interacties tussen een lage serum vitamine B12 concentratie en het TC 776 GG genotype en wellicht het MTRR 66 GG genotype, dat er mogelijk een associatie bestaat met een verhoogd risico van CHD. Echter, deze interacties zijn statistisch niet significant. Dit kan het gevolg zijn van de huidige groepsgrootte, maar de doorgaande werving voor de HAVEN studie zal dit probleem in de toekomst oplossen.

In de algemene discussie worden de resultaten van de case-controle studies besproken en de doelstellingen geëvalueerd (*Hoofdstuk 8*). We bevelen toekomstig onderzoek aan en bediscussiëren de implicaties voor de kliniek en publieke gezondheidszorg. Maternale hyperhomocysteinemie en een lage voedingsinname van vitamine B12 dragen bij aan de kans op CHD, evenals de interactie tussen de MTHFR A1298C genvariaties en folaat status en mogelijk ook de interactie tussen de TC C776G en MTRR C776G genvariaties en vitamine B12 status. Deze resultaten geven aan dat de maternale voedingsstatus en gen-voeding interacties betrokken zijn in de ontstaanswijze van CHD. Daarom zou het gunstig kunnen zijn om vrouwen in hoog risico groepen een dieet rijk aan vitamine B12 te adviseren in de periconceptionele periode en wellicht ook een vitamine B12 supplement, naast het dagelijkse foliumzuursupplement. Het gebruik van B-vitamine supplementen naast een gezond dieet is een rationele aanpak om CHD te voorkomen, omdat het eenvoudig en goedkoop is en het ondersteund wordt door onze huidige data. De resultaten van dit proefschrift kunnen direct worden toegepast in de preconceptiezorg. Vrouwen met een verhoogd risico zouden gescreened moeten worden op hyperhomocysteinemie en hun voedingsinnames zouden geëvalueerd moeten worden, vooral voor folaat en vitamine B12. Laag opgeleide vrouwen verdienen extra aandacht, omdat zij tot een hoog risico groep behoren, ondermeer door slechte voeding en weinig gebruik van foliumzuursupplementen. Preconceptionele screening is belangrijk voor risicoschatting en advisering van aanstaande ouders en kan bijdragen aan een lagere prevalentie van CHD in de toekomst.

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Curriculum Vitae

Anneke Verkleij-Hagoort was born in Papekop on October 3rd, 1976. She passed secondary school at Driestar college in Gouda where she graduated in 1995. In this year, she attended Medical School at University Centre Antwerp in Belgium and passed the first year's exams. Thereafter, she attended Medical School at University of Utrecht from which she graduated in October 2002. In the period of November 2002 to June 2003, she worked as a junior resident at the Department of Obstetrics and Gynaecology of Erasmus MC, University Medical Centre in Rotterdam. From June 2003 until November 2006, she worked as a researcher at the Department of Obstetrics and Gynaecology, Division of Obstetrics and Prenatal Medicine of Erasmus MC in Rotterdam where she performed the studies that are described in this thesis. In November 2006, she started her training in Obstetrics and Gynaecology at Albert Schweitzer Hospital in Dordrecht.

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