METHYLATION, LIFESTYLE AND GENES IN THE PATHOGENESIS AND PREVENTION OF HUMAN CONGENITAL HEART DISEASES

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en preventie van aangeboren hartafwijkingen bij mensen

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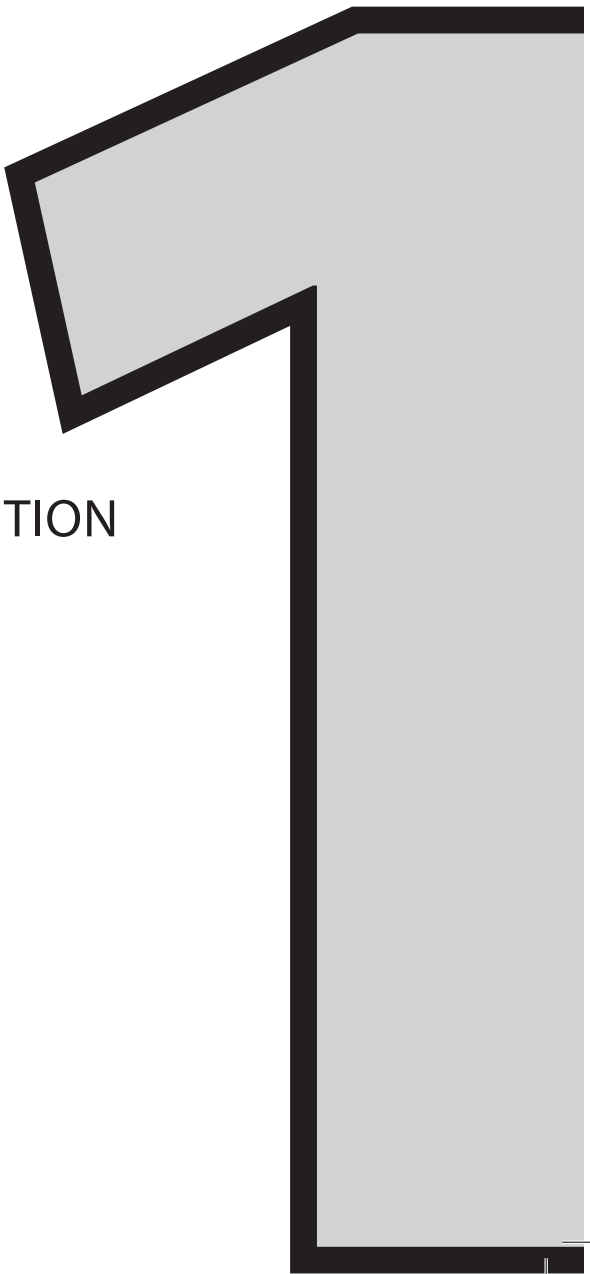


GENERAL INTRODUCTION

HISTORICAL OVERVIEW

As early as circa 30,000 years ago, the heart was known for its life-driving force. The Aurignacian man painted a heart-shaped spot in the middle of a mammoths body: generally assumed to be one of the first pictures of the heart, considered as the place a hunter needed to aim for (*Figure 1*) (1). The earliest written records of often obvious and monstrous human congenital malformations are derived from Babylonian clay tablets that are suggested to date back to 2000 BC (2). These tablets recorded sixty-two deformities, each with a corresponding consequence for the kingdom. However, less obvious malformations, such as congenital heart defects, were less likely to be noted. The superstitious perspective of congenital malformations in general shifted to a more empirico-rational view during the Greek philosophical movement of the Classical Period, pioneered by Hippocrates (460-375 BC). This 'father of medicine' was the first to describe the 'clubbing fingers' phenomenon in humans; an important sign for the diagnosis of cyanotic congenital heart defects. Around this period, human dissection was permitted, allowing the study of the human body to reach its full development. However, some decades later, due to the pressure of public opinion dominated by religious values, dissection of human bodies ended. This was one of the reasons that Claudius Galenus (AD 129-201) sometimes mistakenly applied to the human body what he saw from animal dissections.

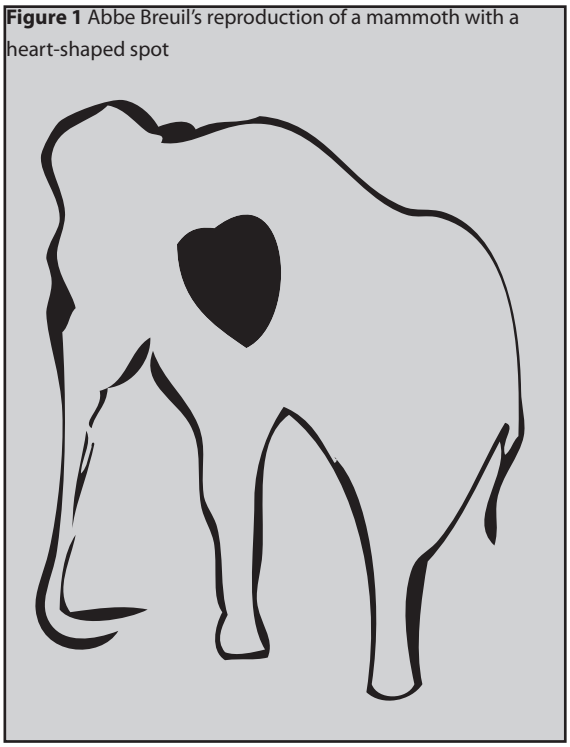


Figure 1 Abbe Breuil's reproduction of a mammoth with a heart-shaped spot

Galenus' views were to hold sway for several centuries until the birth of modern science during the Italian Renaissance in the 15th and 16th centuries. The famous artist and scientist Leonardo da Vinci (1452-1519) described and drew a 'perforating channel' in the inter-atrial septum, which was the first reference to a cardiac malformation now known as the atrial septal defect. The 17th and 18th centuries had many outstanding anatomists, who showed renewed interest in congenital heart diseases (CHD). The Danish anatomist and geologist Niels Stenson (aka Nicolaus Steno, 1638-1686) was the first to describe a cardiac lesion in a stillborn foetus, which is now recognized as tetralogy of Fallot (3). Edwardo Sandifort (1742-1814), for the first time, described the clinical symptoms. At least 40 descriptions and more than 100 years later, in 1888, the anatomic lesion was named for Etienne-Louis Fallot (1850-1911). Around the same period, the era of premortem diagnosis began with the development of the stethoscope by Rene Laennec (1781-1826). Thomas Peacock (1812-1882) published a beautifully illustrated book of many congenital malformations in 1858 (4). This book was considered the first comprehensive study covering the whole field of CHD.

Finally, two remarkable women have to be mentioned: the pathologist Maude Abbott (1869-1940) and the pediatric cardiologist Helen Taussig (1898-1986). They are the founders of modern classification of congenital heart defects and clinical pediatric cardiology.

EPIDEMIOLOGY

During earlier centuries, CHD was believed to be a rare curiosity. In the late 1950s, Mitchell *et al*. were one of the first to carry out a population-based study, in which the birth prevalence rates of CHD were determined. They proposed a new definition of CHD that is still used today: "a gross structural abnormality of the heart or intrathoracic great vessels that is actually or potentially of functional significance" (5). Within this definition, various types of CHD can be distinguished, ranging from small, isolated and often innocent defects to severe, complex defects that are often part of a syndrome. For example, small muscular ventricular septal defects can be found in 2-5% of all newborns and often close spontaneously in the first year of life (6). Tetralogy of Fallot,

on the other hand, is a less common and more complex form of CHD, which needs extensive medical treatment and follow-up throughout life. Atrioventricular septal defect, for example, can often been seen in children with Down syndrome (7).

Because of this wide range of CHD phenotypes, it is difficult to estimate the precise birth rate prevalence (8). That is why the worldwide estimated prevalence varies from 4 to 50 per 1,000 live births depending on which phenotypes, at what moment, and alive or stillborn are included (9). This makes CHD the most common type of birth defect, with worldwide approximately 1 million children born with a CHD each year (10). In the Netherlands, the prevalence is 6 per 1,000, which can be translated into a number of ≈1,300 children born with a CHD each year (11).

At present, birth defects are the leading cause of infant death with CHD accounting for one-third of these deaths. In the year 2002, worldwide, approximately 260,000 people died from CHD of which 6,400 in the United States of America and almost 200 in the Netherlands (12, 13). However, population-based studies in the United States of America have shown that mortality from CHD declined almost 40% from 1979 through 1997 (14). Moreover, just over a third of all deaths were newborns, whereas by the early 1990s, death from CHD was most common in adults aged 20 years and over (12). Thus, the decrease in mortality and increase in age at the moment of death are mutually consistent and suggest a real increase in survival. Inherent to this increasing survival, demand for the health care of adults living with CHD is growing (15, 16).

Currently, the large majority of children with CHD are submitted to a hospital for surgery or other complex medical treatments (17) and often suffer from serious physical and psychological problems during their lives (18, 19). The disease often not only diminishes the quality of life of the affected children, but also of their parents; they experience more distress and hopelessness and report more financial problems than parents of healthy children (20, 21).

Besides the enormous impact on the affected children and their families, the estimated average total costs are also immense; for example 79.6 million euro in the Netherlands (2005) (22) and around 1,258 million dollars in the United States of America (1992) for the four most clinically important CHD (23).

Thus, because CHD have a high birth prevalence rate, a high morbidity and mortality, a great impact on both the affected children and their families, and high related health care costs, prevention is the key term. Primary prevention, such as good preconception health advices to future parents, is the main focus. However, before this can be achieved, knowledge about the cardiovascular pathogenesis is essential. To be able to understand its pathogenesis, we first have to know the normal development of the embryonic heart.

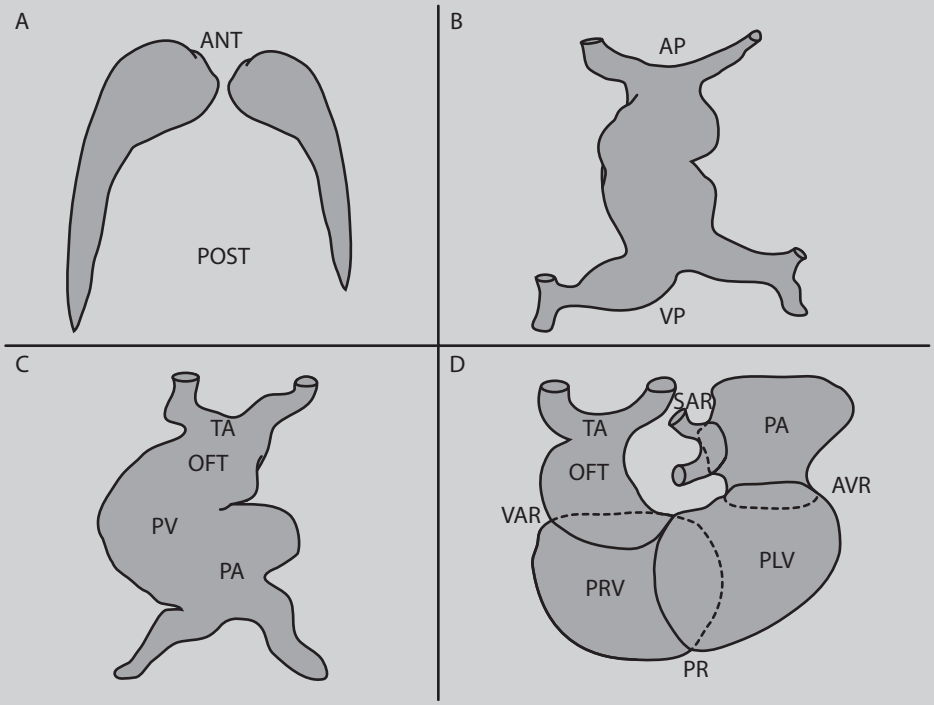
EMBRYONIC HEART DEVELOPMENT

In the first two weeks after fertilization, the developing embryo is supplied by low oxygen and nutrients via diffusion to all of its cells and waste products are efficiently removed (24). The early development of the heart and circulatory system, which starts in the third week after conception, is an embryonic adaptation that permits the rapid growth of the embryo by providing an efficient means for the distribution of nutrients. The blood flow starts very shortly after the first peristaltic contractions, which can be seen at day 22. After around 8 weeks, the embryonic heart development is essentially completed (25). This is at a moment that most pregnant women have their first antenatal visit and therefore, most detailed information on early heart development originates from animal studies.

Currently, early heart development is thought to start by the bilateral formation of two cardiogenic plates derived from the mesoderm, which is also called the primary heart field (*Figure 2A*). Because of the lateral folding of the embryo, these plates fuse in the midline to form the primitive heart tube with an arterial and a venous pole (*Figure 2B*). Thereafter, the primitive tube starts its rightward looping (*Figure 2C*). During this looping process, the heart differentiates into cardiac chambers and transitional zones (*Figure 2D*). These zones will become part of, amongst others, the septa, valves and conduction system.

After differentiation, the actual septation takes place (26). The atrium becomes separated from the ventricle by the formation of thickened atrioventricular endocardial cushions. The endocardial cells transform into mesenchyme and form the basis for the early formation of the major heart valves: the tricuspid and mitral valve. The atrioventricular cushions grow into the canal, they meet and form the final separation of the atria from the ventricles. While the atrioventricular septation takes place, an interatrial septum primum growths down from the cephalic wall of the common atrium and merges with the atrioventricular cushions. This divides the common atrium into left and right chambers. The ventricles are separated in a similar matter: a muscular interventricular septum grows upwards from the apex of the common ventricle fusing with the atrioventricular cushions.

Figure 2 Schematic representation of (A) the bilateral formation of the cardiogenic plates, (B) the primitive heart tube, (C) rightward looping, and (D) looped heart tube with differentiation in cardiac chambers and the transitional zones.



ANT, anterior; POST, posterior; AP, arterial pole; VP, venous pole; TA, truncus arteriosus; OFT, outflow tract; PV, primitive ventricle; PA, primitive atrium; PRV, primitive right ventricle; PLV, primitive left ventricle; VAR, ventriculoarterial ring; PR, primary ring; AVR, atrioventricular ring; SAR, sinoatrial ring.

For normal septation of the outflow tract of the heart into a pulmonary trunk and an aorta, the extracardiac contribution of neural crest cells plays an essential role (27). These cells migrate from the neural crest into the cardiac outflow tract, where they contribute to two endocardial outflow tract cushions. The cushions fuse from distal to proximal and form the semilunar valves and the muscular outflow tract septum. Finally, the aorta is continuous with the left ventricle and the pulmonary trunk with the right ventricle. Recently, evidence was obtained for the existence of a so-called secondary heart field (28, 29). Later in development, myocardial cells from this heart field, positioned in the pharyngeal mesoderm, are added to the outflow tract and right ventricle (30). This concept, however, is still under debate, because the question is whether there are indeed two (or multiple) sources of myocardial cells or if there is just one cardiogenic field with spatial patterning of cells (31).

The correct formation of the heart and vascular system is a delicate balance of migration, differentiation, proliferation, signaling and apoptosis. A poor balance in each and every phase can lead to various cardiac malformations.

ETIOLOGY

As described before, in ancient times, birth defects were believed to result from the action of supernatural forces. Until the experimental teratology started in the twentieth century, various other causes of birth defects were considered: hybridization with animals, maternal impressions, such as thought and sight, mechanical forces or 'wrong' semen. Etienne Geoffroy de Saint-Hilaire (1772-1844) was the first to use animal experiments to study the influence of environmental agents on embryonic development. Almost 100 years later, several articles were published on birth defects in mammals, induced by nutritional deficiencies or exposure to chemicals (32). Sir Norman McAllister Gregg (1892-1968) defined the rubella virus as the first recognized human teratogen: infants exposed to the rubella virus in utero had increased cataract, deafness and CHD (33).

In 1962, there was a major breakthrough when McBride published his findings on the suspected teratogenic effect of the commonly used drug thalidomide in the Lancet (34). Nowadays, known maternal teratogenics include maternal illnesses (pregestational diabetes mellitus, phenylketonuria, febrile illness), therapeutic drug use (anticonvulsants, folate antagonists), non therapeutic drug use (marijuana, high amounts of vitamin A), and environmental exposures (pesticides, organic solvents) (35).

From ancient years there have been theories about heredity. These theories developed more and more in the eighteenth century when knowledge on animal and plant diversity increased, resulting in the application of statistics to heredity by Mendel in 1865. Since the discovery of DNA, genetic research increasingly developed (36). The past decade has witnessed an explosion of knowledge about genes and genetic mutations resulting in cardiac malformations. Some examples of genetic mutations that have been identified to contribute to cardiac malformations are TBX5, PTPN11, NKX2.5, GATA4, and 22q11 deletion (37).

Up until now, only approximately 15% of the CHD can be attributed to a known cause. The remaining 85% is thought to result from interactions between genetic predispositions and periconception environmental exposures (38). Recently, interesting studies indicated that derangements in the one-carbon metabolism might be involved in the etiology of congenital malformations, such as neural tube defects, orofacial clefts and CHD (39-41).

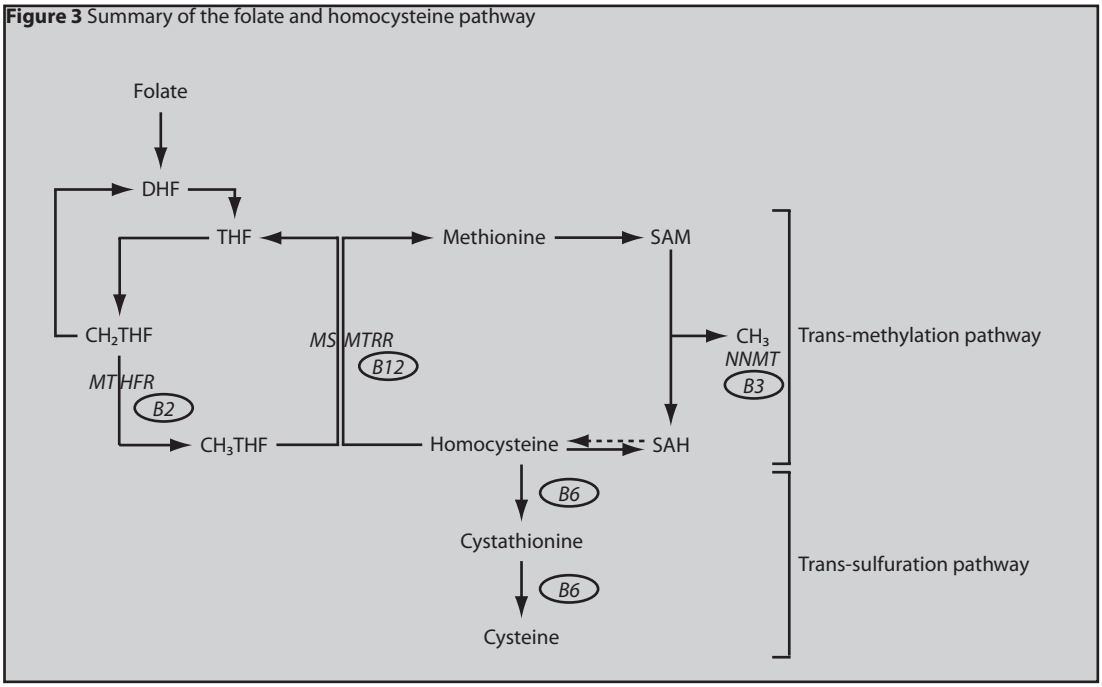
ONE-CARBON METABOLISM

From the early 1950s, animal studies showed that B vitamins might be involved in the pathogenesis of congenital malformations. Especially folate seemed of interest in relation to neural tube defects, including spina bifida and outflow tract defects of the heart; the development of both involves neural crest cells. Several epidemiological studies reported that maternal use of folic acid containing supplements reduces the risk of CHD in the offspring (42, 43). Also experimental studies have been conducted to unravel the mechanism behind this association (44, 45).

Folate is the generic term for chemically similar compounds that can be found in food, blood and tissues. It can not be synthesized in the body and must therefore be ingested. Sources of natural folate are mainly liver, leafy green vegetables and citrus fruit, but also bread and dairy products. Its bioavailability is 80%, while the synthetic form, i.e. folic acid, has a 90 to 100% bioavailability and is used in vitamin supplements and fortified foods (46). The metabolic function of folate is to supply and transfer one-carbon units, i.e. methyl groups, for several important reactions, including the synthesis and methylation of proteins, lipids and DNA (47).

The folate pathway is interrelated with the homocysteine pathway (*Figure 3*). Methyltetrahydrofolate,

Figure 3 Summary of the folate and homocysteine pathway



the most prevalent plasma form of folate, is converted by vitamin B12-dependent methionine synthase (MS) to the biologically more useful tetrahydrofolate (THF). This MS-dependent conversion is involved in the remethylation of homocysteine into methionine. Homocysteine transsulphuration involves the vitamin B6-dependent conversion into cystathionine and cysteine. Methionine is not only the remethylation product of homocysteine, but also an essential amino acid that needs to be ingested in sufficient amounts via animal and dairy products. Moreover, methionine functions as a one-carbon donor, like folate, and is converted to S-adenosylmethionine (SAM). By donating its methyl group, SAM becomes S-adenosylhomocysteine (SAH). SAH is hydrolyzed to homocysteine via a reversible reaction that favours the synthesis of SAH. The accumulation of SAH and homocysteine inhibit the activity of methyltransferases. The methyl group is transferred from SAM to acceptor substrates, such as DNA or proteins, by numerous methyltransferase enzymes, such as nicotinamide N-methyltransferase (NNMT).

All these compounds closely work together and the slightest change in one of the determinants might influence the balance in one-carbon metabolism. For example, several polymorphisms have been detected in genes encoding for enzymes that play a role in these pathways. A well known example is the MTHFR C677T polymorphism. The homozygous mutant genotype with an average frequency of around 11% results in a lower enzyme activity and is associated with higher total plasma homocysteine concentrations (48). This polymorphism is also considered to be associated with CHD, although the evidence is still not completely clear (40). In addition, deficiencies of B vitamins also increase the homocysteine concentrations (49-52), decrease the methylation capacity (53, 54) and increase the risk of several neural crest related birth defects (55, 56). However, it is unknown whether the increased homocysteine concentration is a primary cause or an epiphenomenon of another underlying mechanism, such as a methylation deficiency.

It is clear that environmental factors interact with genetic predispositions in the association with disease. These interactions are also thought to play a major role in at least the 85% of CHD with an unknown etiology. Genetic predispositions of the mother and/or child might interact with environmental exposures in utero from the mother, since the mother is the environment of the developing embryo and foetus (38).

HYPOTHESIS AND OBJECTIVES

Environmental exposures, such as the maternal nutritional status or use of medicines, might influence the early heart development. Maternal and/or embryonic functional polymorphisms of genes involved in one-carbon metabolism might also play a role. Therefore, we hypothesize that derangements in one-carbon metabolism due to gene-environment interactions are associated with maldevelopment of the primitive cardiovascular system resulting in CHD.

The main objectives of this thesis are:

To validate the study moment of approximately 1 year after delivery (part I)

To identify determinants of the biomarkers of the cellular methylation state in women of reproductive age (part I)

To investigate associations between biomarkers of the cellular methylation state in mothers and children and CHD (part II)

To study lifestyle and genetic risk factors of CHD and their interactions (part III)

OUTLINE OF THE THESIS

This thesis presents mainly the results of the ongoing HAVEN study. The HAVEN study was designed as a case-control triad study of children and both of their parents with a focus on nutrition, lifestyle and genes in the pathogenesis and prevention of CHD. This study is conducted from June 2003 at the department of Obstetrics and Gynecology/Division of Prenatal Medicine of Erasmus MC, University Medical Center in Rotterdam, the Netherlands. Case children with both parents were invited to participate in collaboration with the Departments of Pediatric Cardiology of Erasmus MC, Leiden University Medical Center in Leiden, VU University Medical Center and Academic Medical Center in Amsterdam. The control children with both parents were invited in collaboration with the child health centers of 'Thuiszorg Nieuwe Waterweg Noord' in the surroundings of Rotterdam. The HAVEN study is funded by the Netherlands Heart Foundation (grant 2002.B027) and the Bo Hjelt Foundation (grant 2005).

The first part of this thesis presents two methodological studies. The study that is presented in *Chapter 2*

was performed within the FOLFO study, which is a prospective periconception study focusing on the role of nutrition and lifestyles on fertilization, implantation and embryo quality in collaboration with the Department of Obstetrics and Gynecology/Division of Reproductive Medicine and Division of Obstetrics and Prenatal Medicine of Erasmus MC, University Medical Center in Rotterdam, the Netherlands. In this chapter, we compared the maternal nutritional status before pregnancy with 1 year after delivery. This study was designed to validate the standardized study moment that is used in the HAVEN study. The maternal nutritional status was assessed by biochemical measurements in blood (folate, vitamin B12, vitamin B6 and plasma total homocysteine (tHcy)) and a food frequency questionnaire. This existing food frequency questionnaire was adapted by the Division of Human Nutrition of Wageningen University in Wageningen, the Netherlands to estimate the intake of energy, nutrients and B vitamins (57, 58). In *Chapter 3*, we present the study in which we investigated lifestyles, nutritional and genetic determinants of the biomarkers of the cellular methylation state, namely tHcy, SAM, and SAH.

The second part of this thesis describes associations between the biomarkers of the cellular methylation state and CHD. We evaluated the concentrations of tHcy, SAM and SAH in the blood of case mothers and control mothers in relation to CHD in their child (*Chapter 4*). The same biomarkers were evaluated in the case children and control children at the age of about 17 months (*Chapter 5*).

In Part III, genetic and environmental interactions are described. In *Chapter 6*, we present data of the interactions between two MTHFR polymorphisms and maternal B vitamin intake in relation to CHD. *Chapter 7* presents genetic and lifestyle factors related to the periconception vitamin B12 status and CHD. In *Chapter 8,* three specific factors associated with CHD, namely the NNMT polymorphism, medicine use and nicotinamide (vitamin B3) are described. In the general discussion, the results of this case-control study are discussed and the objectives are evaluated (*Chapter 9*). Furthermore, we discuss the implications and recommend future research.

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Methodolo

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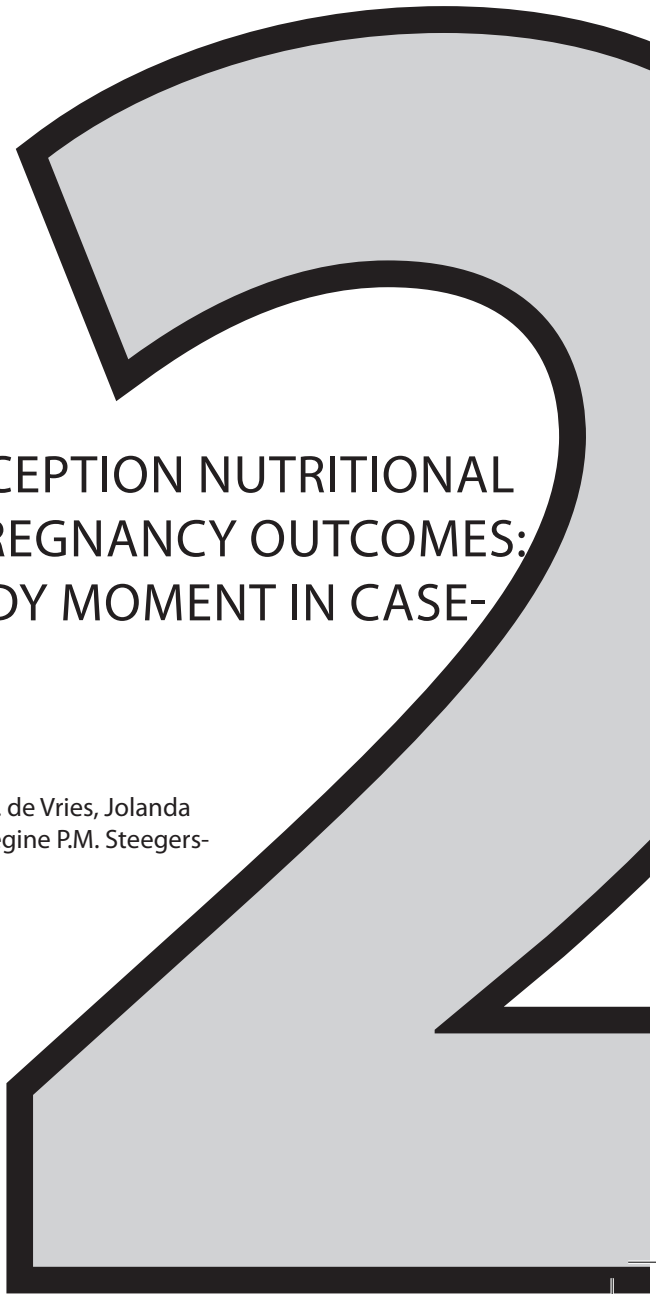
Chapter



THE MATERNAL PRECONCEPTION NUTRITIONAL STATUS AND ADVERSE PREGNANCY OUTCOMES: VALIDATION OF THE STUDY MOMENT IN CASE-CONTROL STUDIES

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ABSTRACT

Background: A poor maternal nutritional status in the preconception period is associated with adverse pregnancy outcomes. Most studies on this association are carried out during or after pregnancy. However, validation of a standardized study-moment after pregnancy reflecting the preconception nutritional status, is missing.

Objective: To validate the study moment at around 1 year after delivery reflecting the preconception study moment for the assessment of the maternal nutritional status.

Design: In a prospective study, we assessed the nutritional status of 30 women by estimating the nutrient intakes from a food frequency questionnaire and from biomarkers related to the homocysteine pathway in blood. We compared the nutritional status at two study moments, i.e., preconceptionally and 1 year after delivery. We used a linear mixed model and adjusted for possible confounders, such as body mass index and folic acid supplement use.

Results: The energy adjusted nutrient intakes were not significantly different between the two study moments, except for higher retinol, alcohol and vitamin B2 and lower carbohydrate intakes at around 1 year after delivery. The intraclass correlation coefficients of the nutrients ranged from 0.3 to 0.7. After adjustment, not any of the biomarkers was significantly different between the two study moments. The intraclass correlation coefficients of the biomarkers were all ≥0.5.

Conclusions: The assumption that the study moment at around 1 year after delivery reflects the preconception study moment for the assessment of the maternal nutritional status seems valid. In future case-control studies, we recommend this standardized moment when investigating associations between the maternal nutritional status and pregnancy outcomes.

INTRODUCTION

Maternal nutrition plays a significant role in the pathogenesis of adverse pregnancy outcomes and may even predispose to chronic diseases in later life (1). It is therefore worrisome that malnutrition during pregnancy is an increasing problem in both deprived and rich countries.

At the time of conception, the maternal nutritional status is a particular important determinant of embryonic and fetal growth (2). The growth of the placenta and fetus is most vulnerable to the maternal nutritional status during the preimplantation period and the period of rapid placentation, which takes place during the first weeks and typically before pregnancy has been confirmed (3). Most organs develop 3-7 weeks after the last menstrual period and any teratogenic effect may have been effective by this time. The nutritional status of the mother is influenced by numerous variables, including genetics, environment, lifestyles, illnesses, physiological factors, and drug-toxicant exposures (4).

The preconception period is the best window to study the role of maternal nutrition in association with adverse pregnancy outcomes. Moreover, it is also the best moment for nutritional interventions, i.e., preconception care. In most studies, however, the maternal nutritional status is assessed by food frequency questionnaires (FFQ) at various moments during and after pregnancy to determine past habitual nutritional intakes (5-8). At different study moments, varying from a few days until 24 months after delivery, mothers have been asked in retrospect to report their nutrient intake of the preconception period. The main disadvantage of this approach is its sensitivity to recall bias. A prospective pregnancy study, in which the nutritional intake is assessed and blood samples are taken for measurement of biomarkers preconceptionally and at several moments during and after pregnancy, would be the first choice. However, it is very difficult to enroll women preconceptionally. Moreover, when studying rare reproductive outcomes, such as congenital malformations and preeclampsia, a prospective study is hardly feasible with regard to sample size and costs.

Therefore, for more than ten years, our group uses a case-control design with a standardized study moment at around 15 months after the index-pregnancy to investigate associations between maternal nutritional intakes by FFQ and adverse pregnancy outcomes, in particular spina bifida, orofacial clefts and congenital heart diseases (9-13). We strongly assume that the maternal nutritional status at around 1 year after delivery reflects the preconception maternal nutritional status. This assumption is based on studies by Willett and others, who stated that, in general, the individual dietary pattern is rather constant and is influenced only by episodes of temporary dieting, illnesses, nausea and increased needs due to excessive growth, such as during pregnancy and breast-feeding (14, 15). The use of the study moment of around 1 year after delivery as a reflection of the preconception moment, however, has never been validated. Therefore, in the current prospective study, we examined whether the maternal nutritional status in the preconception period is comparable with that at around 1 year after delivery. We used FFQs and a selection of biomarkers related to the homocysteine pathway to assess the nutritional status. The results of this study will support the use of a feasible and standardized study moment in case-control studies to investigate associations between the maternal preconception nutritional status and adverse pregnancy outcomes.

MATERIALS AND METHODS

Subjects

This study was embedded in the FOLFO study, which is an ongoing prospective study focusing on the role of the homocysteine pathway in fertilization, implantation and embryo quality (16). From November 2004 until November 2006, 266 women started with the procedure of in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) at the Department of Obstetrics and Gynecology, Division of Reproductive Medicine at the Erasmus MC, University Medical Center in Rotterdam, the Netherlands. Of these women, 68 got pregnant, of which 2 women were diagnosed with an extra uterine gravidity and 14 with a spontaneous miscarriage. Four women were excluded because they gave birth to twins and 7 women were lost to follow-up. Therefore, at the second study moment, which was at around 1 year after delivery of the index pregnancy, 41 women were illegible to participate in the second round of the study. However, of these 41 women, we had to exclude the women who were pregnant again (n=3), who were still breastfeeding (n=1), or who reported to have a substantially different diet than in the preconception period (n=3). Four women were lost to follow-up, which resulted in the evaluation of the nutritional status of 30 women at both study moments (*Figure 1*). The study protocol was approved by the Central Committee for Human Research (CCMO) in The Hague, the Netherlands and the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center in

Rotterdam, the Netherlands. All participants gave their written informed consent.

Study design

At the first study moment, mothers were asked to fill out a general questionnaire and a validated FFQ during the IVF or ICSI procedure. These questionnaires were collected at the day of the embryo transfer, then checked by two experienced researchers and, if necessary, incomplete data was replenished after a telephone interview. The FFQ covered the intake of the previous 4 weeks. In addition, women were invited to visit the hospital at their first week of the menstrual cycle for blood sampling. The blood samples were thus taken before the women started on the hormonal therapy of the IVF/ICSI treatment. The following biomarkers were then measured: plasma total homocysteine (tHcy), serum and red blood cell (RBC) folate, whole blood vitamin B6, and serum vitamin B12. We selected these biomarkers, because they are all involved in the homocysteine pathway and the design of the FOLFO-study aimed to investigate the role of the homocysteine pathway in fertilization, implantation and embryo quality.

At the second study moment of around 1 year after delivery, the same women filled in the same questionnaires at home. They were then invited to visit the hospital at the first week of the menstrual cycle for blood sampling. The data from the questionnaires and biomarker concentrations in blood was compared with the data available from the preconception period. We made an effort to keep the conditions for the blood sampling the same for each woman in both periods, which resulted in 23 matching samples (21 non-fasting and 2 fasting) and 7 non-matching samples of which the fasting state was not clear in one of the periods (17).

Data collection

The data from the general questionnaire comprised age, height, weight, ethnicity, education level, smoking, and the use of medication, recreational drugs and folic acid containing vitamin supplements in the previous four weeks. Body mass index (BMI) was calculated as weight divided by the square of height. Ethnicity was categorized as follows: Dutch natives: both parents and grandparents were born in the Netherlands, or one of the parents was born in another country, but both grandparents were born in the Netherlands; European others: one of the parents or grandparents was born in a European country, or was from European origin and living in the USA, Indonesia or Australia; non-European: all others (18). Education level was categorized into low (primary/lower vocational/intermediate secondary education), intermediate (higher secondary/intermediate vocational education) or high (higher vocational/university education), according to Statistics Netherlands (19). Smoking was categorized in four groups: none, 1-10 cigarettes per day, 10-25 cigarettes per day and > 25 cigarettes per day. Medication use was defined as prescribed daily use and recreational drug use was defined as any use in the previous 4 weeks. The use of folic acid containing supplements comprised both folic acid supplements and multivitamin supplements containing folic acid.

The modified version of the semiquantitative FFQ validated by Feunekes *et al*. (20) was used to estimate daily habitual intake of energy, macronutrients, and micronutrients. Based on data of the Dutch national food consumption surveys in 1992 and 1998, the FFQ has been updated twice (21, 22). The FFQ has also been modified for the estimation of B vitamin intakes (23). After modification, the FFQ covered the daily intake of each nutrient or food of interest for at least 90% of the population mean intake. The FFQ has been structured according to a meal pattern and consists of 166 food items. For most items, the women report the intake, preparation methods, portion sizes and additions of the previous 4 weeks. The average daily energy and nutrient intake was calculated using the 2001 electronic version of the Dutch food composition table (24).

Venous blood samples were drawn from each participant at both hospital visits. We measured the concentrations of plasma total homocysteine (tHcy), serum and red blood cell (RBC) folate, whole blood vitamin B6, and serum vitamin B12 (16). For the determination of serum folate and vitamin B12, venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2,000 x g, serum was collected before being assayed. Serum folate and vitamin B12 concentrations were routinely determined by immunoelectrochemoluminescence immunoassay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). Ethylenediamine tetra-acetate (EDTA) containing vacutainer tubes were used for the determination of plasma tHcy and RBC folate. Lithium heparin containing vacutainers were used for determining vitamin B6. Immediately after blood sampling, 1 EDTA-tube was centrifuged at 2,000 x g for 10 minutes and the blood was separated. Vitamin B6 (pyridoxal'5-phosphate) and tHcy concentration were determined during routine laboratory procedures using high performance liquid chromatography with reserved phase separation and fluorescence detection (25). For the determination of RBC folate, 100 µL blood

TABLE 1 Comparison of the characteristics of the women in the preconception period and 1 year after delivery

Characteristics	Preconception *n* = 30	1 year after delivery *n* = 30	*p*-value[b]
Age (y)	33.4 (28.5 – 43.7)	35.4 (30.3 – 45.4)	<0.001
BMI (kg/m^2)	23.1 (18.2 – 34.3)	23.2 (18.2 – 35.1)	0.028
Smoking[a]			0.517
None	23 (77)	24 (80)	
1 - 10 cigarettes per day	6 (20)	4 (13)	
10 - 25 cigarettes per day	0 (0)	2 (7)	
> 25 cigarettes per day	0 (0)	0 (0)	
Recreational drug use	3 (10)	0 (0)	0.088
Medication use	3 (10)	6 (20)	0.216
Use of folic acid containing supplements	25 (83)	3 (10)	<0.001

Results are presented as number (percentage) or median (range).

[a] Smoking, *n* = 29.

[b] The *p*-value is calculated with a repeated measurements linear mixed model – variance components.

TABLE 2 Energy adjusted[a] dietary nutrient intakes in the preconception period and 1 year after delivery

Nutrient	Preconception	1 year after delivery	Mean difference (SD)[b]	*p*-value[c]	Adjusted *p*-value[d]
Energy (MJ)	8.0 (4.1-29.8)	8.8 (3.7-16.3)	-0.07 (3.2)	0.944	0.759
Total FA (g)	80.8 (53.3-104.8)	83.1 (66.2-107.8)	1.7 (8.6)	0.295	0.535
Saturated FA (g)	30.1 (23.4-39.6)	30.4 (21.6-42.7)	0.3 (5.5)	0.743	0.219
Total Unsaturated FA (g)	41.6 (32.2-59.8)	43.2 (24.8-67.3)	1.4 (8.0)	0.372	0.111
- MUFA (g)	26.2 (19.4-36.4)	25.8 (19.3-42.7)	1.4 (5.0)	0.972	0.238
- PUFA (g)	16.0 (11.4-24.9)	17.0 (19.3-41.8)	0.06 (4.0)	0.158	0.089
Linoleic acid (g)	13.3 (8.6-20.1)	13.3 (2.8-25.2)	0.9 (4.2)	0.270	0.113
ALA (mg)	1214 (500-2660)	1101 (4700-2180)	92 (423)	0.230	0.101
EPA (mg)	39 (0-180)	44 (0-810)	17 (131)	0.553	0.950
DHA (mg)	64 (0-270)	69 (0-1050)	17 (165)	0.673	0.967
Cholesterol (mg)	164.9 (106.7-280.8)	166.8 (87.9-273.1)	-3.8 (60.3)	0.587	0.064
Proteins (g)	75.3 (55.4-91.7)	74.0 (43.8-104.2)	0.5 (11.2)	0.854	0.101
Carbohydrates (g)	257.4 (214.6-306.2)	248.1 (185.5-299.0)	-8.5 (21.3)	0.044	0.142
Dietary fibers (g)	22.3 (13.9-30.9)	22.0 (14.2-35.2)	0.3 (4.1)	0.687	0.369
Vitamin B1 (mg)	1.3 (0.8-2.1)	1.2 (0.7-2.0)	-0.05 (0.3)	0.406	0.150
Vitamin B2 (mg)	1.5 (0.8-1.9)	1.5 (0.7-2.6)	0.1 (0.3)	0.029	0.361
Vitamin B3 (mg)	15.3 (9.2-20.1)	15.2 (7.9-23.3)	0.1 (3.0)	0.908	0.010
Vitamin B6 (mg)	1.6 (1.1-2.3)	1.7 (0.8-2.3)	0.03 (0.3)	0.637	0.692
Folate (µg)	190.8 (118.9-302.3)	190.7 (118.6-338.7)	4.5 (43.9)	0.735	0.573
Vitamin B12 (µg)	3.5 (1.3-7.9)	3.7 (2.1-11.6)	0.8 (1.8)	0.078	0.904
Alcohol (mg)	2211 (0-16430)	5429 (204-31440)	4839 (6133)	<0.001	0.047
Zinc (mg)	8.5 (5.8-10.9)	8.7 (5.1-12.7)	0.4 (1.2)	0.137	0.022
Retinol (µg)	474.5 (192.3-1368.6)	557.1 (268.5-2335.8)	178.9 (402.6)	0.027	0.988
Vitamin C (mg)	109.0 (32.1-231.4)	100.7 (32.0-173.5)	-12.8 (41.2)	0.083	0.403
Vitamin E (mg)	13.2 (7.3-19.0)	12.7 (6.0-22.2)	0.5 (3.3)	0.429	0.945

out of one EDTA tube was hemolyzed with 2 ml freshly prepared ascorbic acid directly after blood sampling. Subsequently, the hematocrit of the EDTA-blood was determined on a Sysmex XE-2100 (Groffin Meyvis, Etten-Leur, the Netherlands). The folate concentration in the hemolysate was recalculated in RBC folate using the following formula: (nmol hemolysate folate x 21) – (nmol/L serum folate x {1 – hematocrit}) / hematocrit = nmol/L RBC folate. The between-run coefficients of variation for serum total folate were 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L, for tHcy 3.3% at 14.55 µmol/L and 2.3% at 34.23 µmol/L, for vitamin B6 1.8% at 40 nmol/L and 1.3% at 115 nmol/L and for vitamin B12 5.1% at 125 pmol/L and 2.9% at 753 pmol/L.

Statistical analysis

The characteristics of the women are presented as medians with ranges or as a number with percentage. We compared the time-dependent characteristics of the preconception period with those at 1 year after delivery. Differences between both periods were tested with the repeated measurements linear mixed model. Biochemical data and dietary energy and nutrient intakes are also presented as medians with ranges. To eliminate confounding due to variation in the amount of food consumed, we used the residual method to adjust the mean nutrient intakes for total energy intake (26). This method also controls for possible under-reporting or over-reporting of food consumption. In short, the nutrient intakes were regressed on the total energy intake and the predicted mean nutrient intake of the total group. The energy adjusted nutrient intake was then calculated by adding the individual residuals to the predicted mean nutrient intake.

We conducted a multivariable analysis to examine the independent absolute differences of the biomarkers and dietary nutrient intakes between the two periods as repeated measurements using a mixed linear regression model. Within this model, the fixed-in-time and time-varying confounders are taken into account. Another advantage of the mixed linear regression model is that it handles data with missing measurements. In the first model, the biomarkers and dietary nutrient intakes were put into the model as dependent variable, with the time-factor as categorical predictor. This model computes a *p*-value for the time-factor with values <0.05 indicating a significant difference between the two measurements. In the second model, we additionally included possible confounders based on their biologically plausible association with nutritional status and not solely on their significance. The fixed-in-time confounders were education and ethnicity, which were assessed at the preconception period only. The time-varying confounders were age, BMI, smoking, and use of recreational drugs, medication and folic acid containing supplements, which were assessed at both time periods. The continuous variables BMI and age were put into the model as covariates and the categorical variables as factors.

Intraclass correlation coefficients (ICC) of the biomarkers and nutritional intakes were calculated to evaluate the linear associations of the biomarkers and nutritional intakes over the two periods (27). This method is more preferable than the Pearson correlation coefficient, because it takes the within-person variation into account. The ICC indicates whether the ranking of the data in the preconception period is comparable with those around 1 year after delivery. The ICC was computed by the following formula with measurements from the repeated measurements linear mixed model: estimated intercept variance / (estimated residual variance + estimated intercept variance).

We performed an additional method to evaluate possible under-reporting by estimating the mean basal metabolic rate (BMR) using the new Oxford equation for women aged 30-60 years: BMR (MJ/day) = 0.0407 x weight (kg) + 2.90 (28). The physical activity level was then calculated by dividing the mean reported energy intake (EI) by the mean BMR (29). The cut-off point of EI/BMR 1.35 was used to evaluate under-reporting. *p*-values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS software package version 16.0 (SPSS Inc, Chicago, IL).

RESULTS

The first study moment was before pregnancy and the second study moment was at a median of 12.9 months (range 9.5 – 18.9) after delivery of the index-pregnancy; thus, approximately 21 months in between. The population comprised 70% Dutch natives, 10% European others and 20% non-Europeans. The majority of women had an intermediate (57%), 10% a low and 30% a high education level. *Table 1* shows the characteristics of the women in the preconception period and at approximately 1 year after delivery. BMI was slightly, though significantly lower preconceptionally than at 1 year after delivery. Smoking, use of recreational drugs and medication were comparable between both periods. Use of folic acid containing supplements was significantly higher in the preconception period than after delivery.

In *table 2*, the daily energy-adjusted dietary nutrient intakes are shown. The intake of vitamin B2, alcohol

TABLE 2 Energy adjusted[a] dietary nutrient intakes in the preconception period and 1 year after delivery

MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids; ALA, alpha linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

[a] Nutrient intake adjusted for energy intake according to the residual method of Willett *et al.*, except for total energy.

[b] Difference = 1 year after delivery – preconception

[c] *p*-value is computed with a repeated measurements linear mixed model.

[d] *p*-value is computed with a repeated measurements linear mixed model, adjusted for education, ethnicity, age, BMI, use of supplements, medication, tobacco and drugs.

TABLE 3 Intraclass correlation coefficients for dietary nutrient intakes comparing the preconception study moment with around 1 year after delivery.

Nutrient	Unadjusted ρ[a]	Adjusted ρ[b]
Energy	0.606	0.418
Total FA	0.667	0.710
Saturated FA	0.317	0.341
Total Unsaturated FA	0.557	0.673
- MUFA	0.672	0.693
- PUFA	0.351	0.550
Linoleic acid	0.322	0.549
ALA	0.340	0.447
EPA	0.314	0.918
DHA	0.356	0.873
Cholesterol	-	-
Proteins	0.412	0.525
Carbohydrates	0.667	0.705
Fibers	0.636	0.708
Vitamin B1	0.445	0.577
Vitamin B2	0.741	0.747
Vitamin B3	0.420	0.644
Vitamin B6	0.695	0.672
Folate	0.552	0.559
Vitamin B12	0.348	0.781
Alcohol	0.270	0.136
Zinc (mg)	0.639	0.686
Retinol	0.318	0.387
Vitamin C	0.483	0.551
Vitamin E	0.465	0.385

MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids; ALA, alpha linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

[a] The ρ (rho) was computed by the following formula with measurements from the repeated measurements linear mixed model: estimated intercept variance / (estimated residual variance + estimated intercept variance).

[b] Adjusted for education, ethnicity, age, BMI, use of supplements, medication, tobacco and drugs.

and retinol was significantly higher after delivery than in the preconception period. The energy-adjusted intake of carbohydrates was significantly lower. All differences became non significant after adjustment, except for alcohol. Interestingly, after adjustment, intake of vitamin B3 and zinc was significantly different. The daily energy-adjusted dietary intake of all other nutrients was comparable between the two study moments.

The ICC for the energy-adjusted nutrient intakes are shown in *table 3*, ranging from 0.3 to 0.7.

The mean BMR was calculated for the preconception period and at 1 year after delivery with the Oxford equation. BMR (preconception period) = 0.0407 x 67 (weight) + 2.90 = 5627 MJ/day. BMR (after delivery) = 0.0407 x 68 (weight) + 2.90 = 5668 MJ/day. The estimation of physical activity level in both periods was calculated as mean reported EI / mean BMR, which was 8697 / 5627 = 1.55 in the preconception period and 8694 / 5668 = 1.53 after delivery.

Serum and RBC folate concentrations were significantly higher preconceptionally than after delivery. However, serum folate was not significantly different after adjustment for education, ethnicity, age, BMI, use of folic acid containing supplements, medication, tobacco and drugs. The other biomarkers were comparable between the two study moments (*Table 4*).

As shown in *table 5*, the ICC of all biomarkers were significant, ranging from 0.5 to 0.99.

DISCUSSION

The aim of this study was to validate the study moment of around 1 year after delivery reflecting the preconception study moment for the assessment of the maternal nutritional status. The nutritional status was assessed by FFQ to estimate the dietary energy and nutrient intakes and by measurement of a selection of biomarkers in blood related to the homocysteine pathway. To our knowledge, this is the first study on this subject with an unique prospective design.

The first part of the results showed that daily dietary intake of energy and the energy-adjusted nutrients was comparable between the two study moments. The only exceptions were vitamin B2, alcohol and retinol intake, which were significantly lower, while the intake of carbohydrates was significantly higher preconceptionally compared with the study moment after delivery. After adjustment for possible confounders, none of the differences was significant, except for alcohol. The ICC for the intake of energy and the energy-adjusted nutrients ranged from 0.3 to 0.7.

These results confirm previous assumptions that pregnancy planning does not significantly affect the maternal nutritional intakes (30) and that women return to their nutritional habits of the preconception period (14). There are, however, a few aspects to consider.

The first point is the significantly different intake of vitamin B2, alcohol, retinol and carbohydrates. After adjustment for the included confounding factors, only alcohol intake remained significantly different. Moreover, after adjustment for multiple comparisons, again, only alcohol intake remains significantly different (data not shown). This may be not surprising, since women who want to become pregnant are advised not to consume alcoholic beverages before and during pregnancy. The mean difference of energy-adjusted alcohol intake between both periods was 4839 mg/day, which might be explained by the fact that these women were highly motivated to get pregnant and/or that they gave a socially desirable answer to this specific question.

Another aspect related to epidemiological studies, which needs to be considered, is the ICC, which ranged from 0.3 to 0.7 for the nutrient intakes. The lowest ICC was for alcohol (ρ=0.270), which was not surprising considering the aforementioned explanation. Interestingly, the ICC for vitamin B2 (ρ=0.741) and carbohydrates (ρ=0.667) were relatively high, in contrast to the significant absolute differences. In other words, it might be that in the preconception period, overall, women equally eat more food items with vitamin B2, for example dairy products, and less carbohydrates, such as soft drinks.

The results of this study are difficult to compare with those from other reproducibility studies, because of differences in study populations, number of days of dietary recording, intervals between the dietary assessments, and different correlation coefficients that were used. Some studies have reported higher ranges of correlation coefficients than in our study (from 0.5 to 0.7), but these studies used intervals of 1 month to a maximum of 1 year (31-33). Overall, the mean unadjusted ICC was 0.483 and the mean adjusted ICC was 0.593, which are comparable to the mean correlation coefficients of the aforementioned studies. We calculated the ICC also after exclusion of the 8 women (27%) who responded positively to the following question, "Has your diet changed since the preconception period?". Excluding this group of women did not change the results (data not shown). This was previously reported by Ajani *et al*. (31) and it indicates that this question is not very important. Nevertheless, we did exclude the women who reported to have changed their diet substantially, for example a specific low-fat or low-caloric diet to lose weight (*Figure 1*).

TABLE 4 Concentrations of biomarkers in blood collected in the preconception period and around 1 year after delivery

Biomarkers	Preconception *n* = 30[a]	1 year after delivery *n* = 26[b]	Mean difference (SD)	*p*-value[c]	Adjusted *p*-value[d]
Homocysteine (µmol/L)	9.4 (6.6 – 21.8)	9.3 (5.9 – 19.2)	-0.28 (2.2)	0.530	0.200
Folate, serum (nmol/L)	30.8 (7.5 – 908.0)	16.0 (8.2 – 79.1)	-14.4 (22.6)	0.003	0.682
Folate, RBC (nmol/L)	1111 (459 – 2768)	690 (320 – 1848)	-540 (404)	<0.001	0.017
Vitamin B6 (µmol/L)	79.0 (43.0 – 310.0)	72.5 (45.0 – 310.0)	-3.4 (58.5)	0.811	0.661
Vitamin B12 (pmol/L)	312 (155 – 775)	354 (22 – 717)	7 (156)	0.887	0.455

Results are presented as median (range) or as indicated otherwise. RBC, red blood cell.

[a] RBC folate, *n* = 24; serum folate, *n* = 29; homocysteine, *n* = 29.

[b] RBC folate, *n* = 25.

[c] *p*-value calculated with a repeated measurements linear mixed model.

[d] *p*-value calculated with a repeated measurements linear mixed model, adjusted for education, ethnicity, age, BMI, use of supplements, medication, tobacco and drugs.

TABLE 5 Intraclass correlation coefficients for biomarkers comparing the preconception study moment with around 1 year after delivery.

Biomarkers	Unadjusted ρ [a]	Adjusted ρ [b]
Homocysteine (µmol/L)	0.741	0.679
Folate, serum (nmol/L)	0.990	0.989
Folate, RBC (nmol/L)	0.601	0.647
Vitamin B6 (µmol/L)	0.606	0.747
Vitamin B12 (pmol/L)	0.529	0.318

RBC, red blood cell

[a] The ρ (rho) was computed by the following formula with measurements from the repeated measurements linear mixed model: estimated intercept variance / (estimated residual variance + estimated intercept variance).

[b] Adjusted for education, ethnicity, age, BMI, use of supplements, medication, tobacco and drugs.

The second part of the results shows that all biomarkers were comparable between the two periods. The ICC of all biomarkers were highly significant, ranging from 0.5 to 0.99. It is difficult to compare these ICC to those reported in other studies, since most studies comprise a maximum study period of 12 months, they mostly use different statistical measurements, different populations and laboratory methods. There are only a few studies on the within-person variability of these biomarker concentrations (34-36), which are mainly on tHcy and folate and comparable to the ICC we found. To our knowledge, there are no studies on the long-term inter-person variability of serum vitamin B12 or vitamin B6.

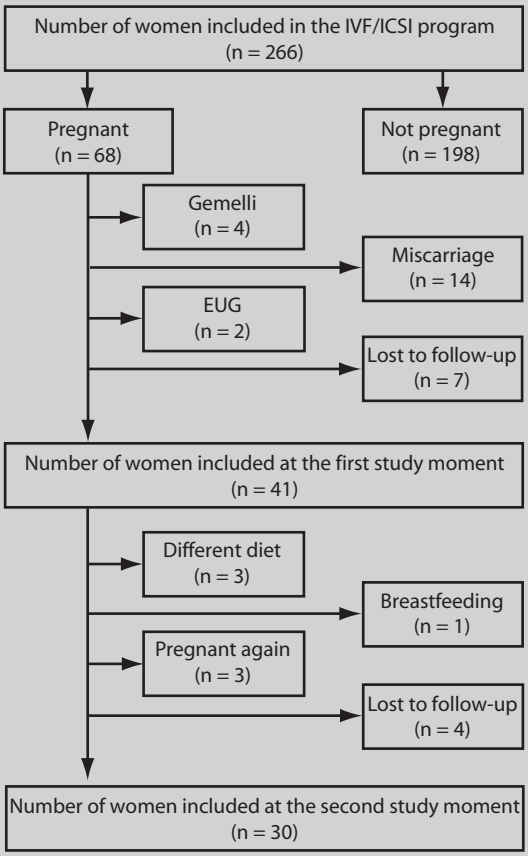
Even with a single measurement and with a period of two years in between, these results indicate an acceptable reproducibility of tHcy, vitamin B6 and vitamin B12 concentrations. Previous studies have shown that these concentrations are rather constant over time (34) with no seasonal variation (37). From this study and from the study of McKinley *et al*., we may conclude that a single measurement reflects the individual average biomarkers concentrations in blood. However, there are two things that have to be considered. First, in our study, it is not just the two measurements over a certain period: the first measurement was taken before pregnancy and the second measurement was taken around 1 year after the delivery. The question is whether the pregnancy state, which influences metabolic and hormonal processes, may have irreversibly affected the biomarkers measured at 1 year after delivery. At least two studies have shown that biomarkers change during pregnancy (38, 39). However, as soon as 6 weeks after delivery, the concentrations returned back to the preconceptional values (38), except for those women who were breastfeeding (39). Therefore, in the current study, we took these confounding effects into account by excluding women who were pregnant again, breastfeeding, or had an ectopic pregnancy in the previous 4 months. Thus, a single measurement of these biomarkers around 1 year after delivery seems useful for studying the relationship between the biomarkers and birth defects or adverse pregnancy outcome.

The results show that folate in RBC and serum, on the other hand, varied significantly over a two-year period. However, after adjustment for the preconception use of folic acid containing supplements, serum folate was not significantly different anymore, and the *p*-value for RBC folate changed from <0.001 to 0.017. In an additional analysis, excluding all supplement users, both serum folate (*p*-value = 0.735) and RBC folate (*p*-value = 0.227) were comparable between the two study moments. This indicates that the variation in serum and RBC folate was mainly due to the significantly higher use of folic acid containing supplements in the preconception period than after delivery. The high percentage of women using folic acid containing supplements in the preconception period does not reflect the 37% use in the Dutch population (40). This difference is probably due to the fact that women participating in an IVF/ICSI program are more motivated to follow the recommendation to use folic acid containing supplements. When taking these supplements into account, at least the serum and probably also the RBC folate concentrations at 1 year after delivery can be used as preconception values. Moreover, in epidemiological studies, the classification of women is of greater importance than the absolute values. In this case, the ICC is more meaningful than the absolute difference. We have shown that the ICC of serum and RBC folate were indeed very high (0.990 and 0.601, respectively), indicating that these biomarkers are also useful for studying the relationship with birth defects or adverse pregnancy outcome.

Since folate concentrations are correlated with tHcy concentrations (37), we would have expected an accompanying significant change in tHcy concentration. However, tHcy was not significantly different between both periods, despite the significantly higher use of folic acid supplements in the preconception period. Therefore, we performed an additional analysis to test the correlation between the biomarkers in both periods and in the supplement users and non-supplement users separately. In all analyses neither serum nor RBC folate was correlated with tHcy (data not shown). It is known that the response of tHcy on folic acid supplementation depends on 1) the baseline concentration of tHcy; there is a threshold of tHcy in terms of ability to respond to folic acid (41, 42), and 2) the time effect and dosage effect of the supplementation; only dosages >0.8 mg/day will further reduce tHcy concentrations and chronic use of supplements has more lowering effects on tHcy concentrations than acute use (41), whereas acute use has a greater impact on serum folate. Apparently, the women in our study already had an optimal tHcy status. Moreover, of the women who reported to have used folic acid containing supplements in the previous 4 weeks, we do not know whether they used the supplements for more than 4 weeks, which would have had a greater impact on the tHcy concentration.

Although we may conclude that both the nutrient intakes and the biomarkers are useful in studies on the relationship with adverse pregnancy outcomes, some other important aspects have to be considered. It is known that people tend to change their dietary habits after being diagnosed with a disease (31, 43). This might also apply to women who gave birth to a child with a serious birth defect. Moreover, it is very important to keep in mind the upcoming preconception counselling and recommendations for a healthy maternal nutritional status. This could imply changes towards a substantial healthier diet, while it is questionable whether these

FIGURE 1 Inclusion flowchart



EUG, extra uterine gravidity.

women would adhere to the healthier diet after delivery.

We also have to consider the strengths and weaknesses of the study. The main strength of this study is the unique prospective design. However, the sample size is rather small. Therefore, studies with larger sample sizes are recommended to confirm our results. Dietary assessment methods are known to have a bias towards underestimating habitual energy intake. Therefore, we investigated the overall under-reporting bias by determination of the physical activity level (PAL), which was 1.55 in the preconception period and 1.53 at 1 year after delivery. These PAL are very similar and when comparing these levels with the cut-off value of 1.35, under-reporting was not likely to play a role in our study (29). Moreover, the FFQ covered a 4 weeks period and therefore, the day-to-day variability of food intake is minimised. The findings of this study do not address validity, since this FFQ has shown to be a valid method to estimate the included dietary energy and nutrient intakes in Dutch women (20, 23).

In summary, we showed that the determination of the maternal nutritional status at around 1 year after delivery largely reflects the preconceptional nutritional status. In addition, when using this standardized study moment in future case-control studies to investigate associations between the maternal nutritional status and adverse pregnancy outcomes, lifestyle factors such as the use of alcohol and folic acid containing supplements should be taken into account.

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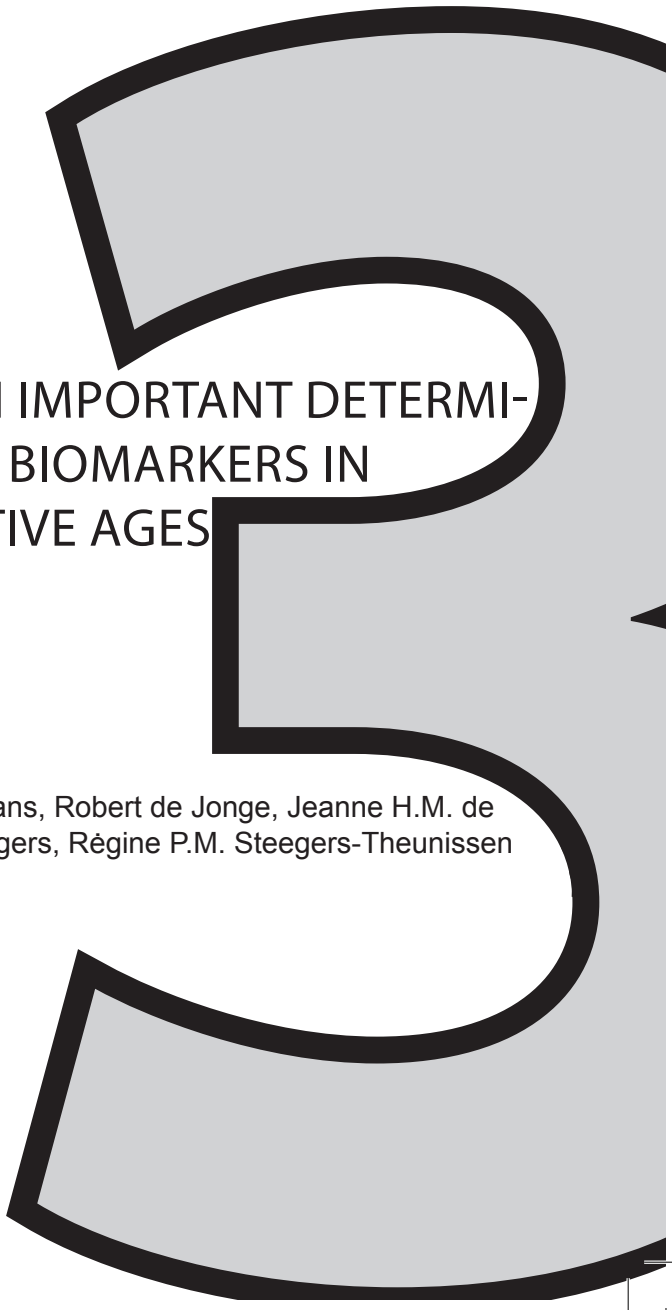
Chapter



BODY MASS INDEX IS AN IMPORTANT DETERMINANT OF METHYLATION BIOMARKERS IN WOMEN OF REPRODUCTIVE AGES

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ABSTRACT

Background: B vitamin deficiencies lead to moderate hyperhomocysteinemia, which has been associated with health and disease. However, concomitant derangements in cellular methylation, reflected by altered S-adenosylmethionine (SAM) or S-adenosylhomocysteine (SAH) concentrations, may be the primary cause.

Objective: To identify determinants of homocysteine, SAM and SAH as part of a large study focusing on risk factors for reproductive disorders.

Design: Blood was obtained from 336 women, aged 20-48 years, to determine plasma SAM, SAH and total homocysteine (tHcy), serum vitamin B12 and folate, red blood cell (RBC) folate, and the related single nucleotide polymorphisms 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C>T and 1298A>C, methionine synthase reductase (MTRR) 66A>G, and nicotinamide N-methyltransferase (NNMT) IVS1-151G>A. Questionnaires provided information on demographics, lifestyles and nutrient intakes. Correlation coefficients were calculated and multivariable associations were assessed with a general linear model.

Results: The results showed that serum folate was positively correlated with SAM (r=0.159). Folate and vitamin B12 were not correlated with SAH and the SAM/SAH ratio, but inversely correlated with tHcy (serum folate r=-0.324; RBC folate r=-0.294; vitamin B12 r=-0.307, all *p*-value<0.01). From the multivariable analysis, body mass index (BMI) was the strongest determinant for SAM (standardized β=19.145, *p*-value<0.001) and SAH (standardized β=3.241, *p*-value=0.010). MTHFR 677TT (standardized β=0.195, *p*-value=0.001), B vitamin supplement use (standardized β=-0.156, *p*-value<0.001) and dietary protein intake (standardized β=-0.011, *p*-value<0.001) appeared the strongest determinants for tHcy.

Conclusions: Thus, the determinants of SAM and SAH are different from those of tHcy. Studies on cellular methylation should at least include BMI.

INTRODUCTION

Nutritional factors, in particular B vitamin deficiencies, derange homocysteine metabolism resulting in mild to moderate hyperhomocysteinemia. Hyperhomocysteinemia has been associated with cardiovascular disease, osteoporosis, Alzheimer's disease and adverse pregnancy outcomes (1). It is not known whether these relationships are causal or whether they mark derangements in pathways other than the homocysteine pathway.

Homocysteine is formed after transmethylation of methionine into S-adenosylmethionine (SAM) and subsequently S-adenosylhomocysteine (SAH). Under physiologic conditions, SAH is hydrolyzed to homocysteine and adenosine. This reaction is reversible, with a dynamic equilibrium that strongly favors SAH synthesis rather than hydrolysis. Homocysteine and SAH inhibit SAM-dependent methyltransferases that donate methyl groups to a variety of acceptor molecules, such as proteins and lipids. These methyl groups are also used for DNA methylation, which is an epigenetic mechanism to regulate gene expression. DNA methylation also programs embryonic growth and development (2). So far, SAM and SAH concentrations in blood and its ratio are the closest biomarkers of cellular methylation (3). This is important with respect to epidemiological studies investigating associations between cellular methylation and outcomes, because in those studies the access to organ tissues is often the limiting factor.

In view of the rising epidemiological and biological interest in DNA methylation, it is important to identify the covariates of cellular methylation. Therefore, we investigated associations between lifestyle, dietary and genetic factors and SAM, SAH, SAM/SAH ratio and total homocysteine (tHcy) concentrations in blood from women of reproductive age. These women were chosen from a large study focusing on the identification of gene-environment interactions and its underlying mechanisms in the pathogenesis and prevention of reproductive disorders (4).

MATERIALS AND METHODS

Subjects

Between October 2003 and January 2007, we randomly selected 336 women who had at least one healthy child of approximately 16 months old, via child health centers in the western part of the Netherlands. These child health centers are part of the Dutch Health Care system where all newborns are regularly checked in a standardized manner for health, growth, and development by physicians specialized in child health care. These women participated as controls in the previously described HAVEN study. The response rate was 55%. The study protocol was approved by the Central Committee on Research involving Human Subjects and the Institutional Review Boards (Medical Ethics Committees) of all participating hospitals and all participants gave their written informed consent.

Data collection

We investigated associations between SAM, SAH, SAM/SAH ratio and tHcy concentration and divided the determinants into 1) demographics and lifestyles, 2) dietary intakes, and 3) genetic polymorphisms.

Questionnaires were filled out at home, checked for completeness and consistency at the hospital visit and provided information on lifestyles. We extracted data on age, educational level, ethnicity and the use of coffee, alcohol, tobacco, prescribed medication, oral contraceptives and B vitamin supplements during the previous 4 weeks. Educational level was classified according to the definitions of Statistics Netherlands (5). Women were classified as Dutch Natives, European Others and Non Europeans (6). Coffee users were those who reported drinking coffee at least once a day. Alcohol use included all alcoholic drinks and use of tobacco comprised any cigarettes and/or cigars and/or pipes smoking. Medication use was defined as the use of any prescribed medication. Hormonal contraceptives included oral contraceptives and hormonal intra uterine devices. B vitamin supplement use was defined as any use of a B vitamin containing supplement. Miscarriage was defined as spontaneous miscarriage during the first 16 weeks of any prior pregnancy. Stillbirth was defined as fetal death after 16 weeks of any prior pregnancy. Pregnant or lactating women at study entry were excluded, because both conditions influence the biomarkers, some demographics and dietary intakes.

Height (anthropometric rod; SECA, Germany) was measured up to 0.1-cm accuracy and body weight (weighing scale; SECA, Germany) up to 0.5-kg accuracy. Body mass index (BMI) was defined as weight divided by the square of height. Systolic and diastolic blood pressures were manually recorded in sitting position on the

right arm up to 2 mm/Hg accuracy (Maxi Stabil 3; Speidel and Keller, Jungingen, Germany). Age, height, weight, BMI, and blood pressure were used as continuous variables. All other demographic and lifestyle factors were used as categorical variables.

Information on daily dietary intake covering the previous 4 weeks was obtained by a validated food frequency questionnaire. This FFQ was modified from the semi-quantitative FFQ of Feunekes et al. (7), and has been updated twice based on data of Dutch National food consumption surveys (8, 9) and additionally modified and validated for the estimation of dietary B vitamin intakes (10). We calculated the mean daily intake of total energy, proteins, folate, vitamin B2, vitamin B3, vitamin B6 and vitamin B12 using the 2001 electronic version of the Dutch food composition table (11).

The polymorphisms evaluated comprised 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C>T (dbSNP rs1801133; 222A>V) and 1298A>C (dbSNP rs1801133; 429E>A), methionine synthase reductase (MTRR) 66A>G (enzyme: EC1.16.1.8; 22I>M), and nicotinamide N-methyltransferase (NNMT) IVS1 -151G>A (dbSNP rs694539). The NNMT enzyme catalyzes the N-methylation of vitamin B3 and other pyridines by which it uses a methyl group that is generated during the conversion of SAM to SAH. This polymorphism was selected because it was recently identified as a new candidate gene for hyperhomocysteinemia (12). The precise effect of the polymorphism on protein activity is not yet known.

Fasting EDTA blood and serum were taken for the determination of SAM, SAH, tHcy and the polymorphisms. The standardization of the blood sampling and measurements of all biomarkers have been described before (4, 13). In short, after withdrawal the blood sample was kept on ice and centrifuged at 4˚C within one hour. Plasma aliquots were stored at -80˚C until analysis. SAM and SAH were determined using isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS; Waters Acquity UPLC Premier XE, Milford, MA, USA) by an adapted method (14). tHcy was routinely measured by LC-MS/MS (15). The concentrations of red blood cell (RBC) and serum folate, and serum vitamin B12 were routinely determined with immunoelectro-chemoluminescence assay on the Roche Modular E170 (Roche Diagnostics GmbH, Germany). The coefficients of variation for the biomarkers were as follows: SAM = 4.7%; SAH = 4.5%; tHcy 2.9% at 16.2 µmol/L and 1.2% at 46.1 µmol/L; vitamin B12 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; folate 5.9% at 5.7 nmol/L and 2.8% at 23.4 nmol/L.

Determination of the MTHFR, MTRR and NNMT polymorphisms was described before (16-18). In short, genomic DNA was isolated from 0.2 mL 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. The DNA isolation was carried out using the Quick Extract DNA Extraction Solution 1.0 according to the manufacturers' instructions (Epicentre; Madison, Wisconsin, USA). We determined the MTRR A66G and MTHFR C677T and the A1298C polymorphisms from isolated DNA using real-time polymerase chain reaction (PCR) (Taqman®, Applied Biosystems; Foster City, CA, USA). NNMT genotyping was also performed using a Taqman assays-on-demand (Taqman®, Applied Biosystems, Foster City, CA, USA) allelic discrimination assay according to the manufacturers' instructions. For every 90 genotyped individuals, we used 3 individuals as controls since their genotype was already known and proven by sequencing; the individuals' genotypes were in 100% concordance with the sequencing data.

Statistical analysis

The distributions of SAH, tHcy, vitamin B12 and serum and RBC folate were positively skewed even after log transformation and therefore presented as medians with ranges. For the same reason, the continuous variables maternal age, BMI, systolic and diastolic blood pressure, and dietary intakes are given in medians and ranges. All other results on lifestyles are presented in numbers and percentages. The Chi-square test was used to test deviation of the genotype frequencies from those expected under Hardy Weinberg Equilibrium.

All parameters were divided into 2 blocks; block 1 consisted of the biochemical variables SAM, SAH, tHcy. Block 2 comprised RBC and serum folate, serum vitamin B12, lifestyle factors, dietary intakes and genetic polymorphisms. We calculated the within-block correlations and the between-block correlations. Associations between two normally distributed continuous variables were computed with the Pearson correlation coefficient and all other correlations with the Spearman correlation coefficient. Probability values of p-value<0.01 were considered statistically significant.

In addition, multivariable associations were assessed with a general linear model (GLM) with the block 1 variables as 'dependent' variables and the block 2 variables as 'independent' variables. The measures of association in multivariate models, such as the GLM, provide information about the strength of the relationship between the determinant or the independent variable and the dependent variable. This relationship is

TABLE 1 Demographic and lifestyle factors in women

Demographics and lifestyles	***n* = 336**	**References [f]**
Multigravidae [a]	175 (52)	-
Miscarriage [b]	77 (23)	-
Stillbirths [c]	11 (3)	-
Age (y)	32.7 (20.4 - 47.6)	15 - 45
BMI (kg/m^2)	24.4 (17.1 - 45.1)	23.8
Systolic blood pressure (mmHg)	115 (90 - 155)	-
Diastolic blood pressure (mmHg)	75 (50 - 104)	-
Educational level [d]		
Low	80 (24)	76%
Intermediate	171 (51)	
High	85 (25)	24%
Ethnicity [e]		
Dutch Native	264 (79)	92%
European Others	13 (4)	5%
Non European	59 (17)	3%
Use of		
Coffee	223 (66)	-
Alcohol	193 (57)	81%
Tobacco	68 (20)	32%
Medication	70 (21)	40%
Hormonal contraceptives	167 (50)	45%
B vitamin supplements	72 (21)	13%

Values are number (percentage) or median (range)

[a] Women with more than 1 pregnancy in their obstetrical history

[b] Women with 1 or more miscarriage in their obstetrical history

[c] Women with 1 or more stillbirth in their obstetrical history

[d] Categorised as low (primary/lower vocational/intermediate secondary education), intermediate (higher secondary/intermediate vocational education) or high (higher vocational/university education) (5)

[e] 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, or is from European origin and living in the USA, Australia or Indonesia. Non European: all others (6).

[f] Statistics Netherlands, percentages from total population women aged 15-45 in the Netherlands (19).

independent of any interrelationship among the dependent variables in contrast with the univariate analyses, such as the Pearson correlation coefficient. In the GLM analyses, we had to separate SAM and SAH from the SAM/SAH ratio, because the ratio is computed from SAM and SAH. Moreover, it is not possible to put serum vitamin B12 and folate, and RBC folate in the GLM model as independent variables together with the other block 2 variables, such as nutrient intake. Therefore, we conducted a separate model including these three variables together with the genetic polymorphisms as independent variables, separately and with interaction terms between the vitamins and genetic polymorphisms. Thus, four different models were computed: 1) SAM, SAH and tHcy as independent variables, excluding the other biomarkers; 2) SAM/SAH ratio and tHcy, excluding the other biomarkers; 3) SAM, SAH and tHcy including the other biomarkers as independent variables, and 4) SAM/SAH ratio and tHcy including the other biomarkers. Standardized regression coefficients were computed to make the effects of variables comparable on the same scale. Moreover, we computed a standard eta-squared for each statistically significant variable indicating what percentage of the model is explained by the variable. All analyses were adjusted for multiple testing with the method of Bonferroni and probability values of p-value<0.05 were considered statistically significant after this adjustment. All analyses were performed with SPSS for Windows software (version 16.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Table 1 presents demographics and lifestyles. For external validity, we used national reference data of women between 15 and 45 years of age from Statistics Netherlands (19). Our population comprised more non Europeans, but BMI and educational level were comparable. Furthermore, fewer women used alcohol, tobacco and medication, but B vitamin supplement use was higher. The methylation biomarkers were all within the laboratory reference ranges, which are used in the Clinical Chemistry laboratory at Erasmus University Medical Center, Rotterdam, the Netherlands (*Table 2*) (20). The median dietary intakes were all higher than the reference values except for proteins (*Table 2*). All genetic polymorphisms were in Hardy Weinberg Equilibrium (*Table 3*).

SAM, SAH, SAH/SAH ratio and tHcy were all correlated with each other (*Figure 1A*). Serum folate was positively correlated with SAM. Vitamin B12 and serum and RBC folate were positively correlated with each other, but negatively correlated with tHcy. No correlations existed between these vitamins and SAH or SAM/SAH ratio. BMI, blood pressure and tobacco use were correlated (*Figure 1B*). Education was correlated with the use of tobacco and alcohol. Age was correlated with miscarriage, multigravidity, and the use of hormonal contraceptives, coffee and alcohol. Only BMI was positively correlated with SAM and SAH (*Figure 1C*). B vitamin supplement use was positively correlated with SAH and negatively correlated with tHcy. Tobacco use was positively correlated with tHcy.

The dietary intakes showed all positive correlations with each other varying from r=0.313 to r=0.867 (p-value<0.01) without any significant correlation with the biomarkers (data not shown). The correlations between dietary intakes, demographics and lifestyles revealed that the level of education was positively correlated with intake of vitamin B6 (r=0.181, p-value=0.001), folate (r=0.162, p-value=0.003) and vitamin B3 (r=0.148, p-value=0.007). Also alcohol use was positively correlated with the intake of protein (r=0.308, p-value<0.001) and with all vitamins (varying from r=0.256 to r=0.348, all p-value<0.001). Use of coffee was positively associated with vitamin B3 intake (r=0.245, p-value<0.001). Use of tobacco was negatively correlated with intake of proteins (r=-0.144, p-value=0.009) and vitamin B6 (r=-0.188, p-value=0.001). In addition, ethnicity was negatively correlated with intake of proteins (r=-0.242, p-value<0.001) and with all vitamins except for folate (varying from r=-0.167 to r=-0.280, all p-value<0.001). The polymorphisms MTHFR C677T and A1298C were also correlated (r=-0.517, p-value<0.001). However, none of the polymorphisms were correlated with the biomarkers (data not shown).

From the GLM analyses, SAM was positively associated with BMI, also after Bonferroni adjustment (*Table 4*). BMI, miscarriage, stillbirth, and the NNMT GG genotype were positively associated and vitamin B6 intake was negatively associated with SAH. After Bonferroni adjustment, no significant association remained. The SAM/SAH ratio was positively associated with vitamin B6 intake and negatively with NNMT GG, but disappeared after Bonferroni adjustment. tHcy was positively associated with having more than five liveborns, use of tobacco and MTHFR 677TT and negatively associated with serum vitamin B12, serum folate, RBC folate, non European origin, the use of B vitamin supplements, protein intake, and MTRR 66AG. After Bonferroni adjustment, the associations of tHcy and the use of B vitamin supplements, protein intake, MTHFR 677TT, and serum vitamin B12 remained significant. None of the interaction terms between the genetic polymorphisms and serum vitamin B12, serum folate, and RBC folate were significant.

TABLE 2 Biomarkers in blood and dietary nutrient intakes in women

Biomarkers	***n***	**Median (range)**	**References** [a]
SAM (nmol/L)	336	80.6 (41.7 - 121.2)	70 - 128
SAH (nmol/L)	336	13.9 (7.6 - 34.2)	9 - 20
SAM/SAH	336	5.9 (2.2 - 10.3)	4.7 - 9.0
tHcy (µmol/L)	331	9.9 (5.3 - 42.0)	6 - 19
Vitamin B12 (pmol/L)	333	256 (76 - 933)	145 - 637
Folate, serum (nmol/L)	335	14.7 (5.9 - 86.7)	8 - 28
Folate, RBC (nmol/L)	331	637 (153 - 2620)	390 - 1560
Nutrients			**References** [b]
Total energy (MJ/d)	336	8.6 (1.6 - 17.7)	8.1
Proteins (g/d)	336	75.8 (12.4 - 139.6)	86.3
Folate (µg/d)	336	198 (38 - 375)	153
Vitamin B2 (mg/d)	336	1.4 (0.3 - 3.0)	1.4
Vitamin B3 (mg/d)	336	14.8 (2.4 - 29.1)	-
Vitamin B6 (mg/d)	336	1.7 (0.3 - 3.2)	1.6
Vitamin B12 (µg/d)	336	3.6 (0.6 - 16.6)	3.3

[a] Reference values for SAM, SAH, tHcy and folate were established in the Clinical Chemistry laboratory at Erasmus University Medical Centre, Rotterdam, the Netherlands. Reference values for vitamin B12 were used as stated in "Reference Ranges for Adults and Children" (20).

[b] References are from the "Dutch National Food Consumption Survey 2003" of women aged 17-50 years (9).

TABLE 3 Genetic polymorphisms in women

MTHFR C677T	***n* = 325**
CC	147 (45)
CT	135 (42)
TT	43 (13)
HWE	0.1795
MTHFR A1298C	***n* = 327**
AA	155 (47)
AC	138 (43)
CC	34 (10)
HWE	0.6899
MTRR A66G	***n* = 327**
AA	78 (24)
AG	163 (50)
GG	86 (26)
HWE	0.9645
NNMT IVS1 -151G>A	***n* = 328**
AA	199 (61)
AG	115 (35)
GG	14 (4)
HWE	0.6074

Values are number (percentage). HWE, Hardy Weinberg Equilibrium.

DISCUSSION

This study demonstrates the influence of demographic, lifestyle, dietary and genetic determinants on methylation biomarkers in blood of women of reproductive ages. SAM was positively correlated with serum folate, but not with the other biomarkers. Also SAH was not correlated with one of the biomarkers. Vitamin B12 and RBC and serum folate were all negatively correlated with tHcy. The multivariable model revealed that BMI was the strongest determinant of SAM and SAH. Vitamin B6 intake was a significant determinant of SAH, but disappeared after Bonferroni adjustment. The strongest determinants of tHcy were serum vitamin B12, MTHFR 677TT, B vitamin supplement use, and protein intake.

We observed a positive correlation between serum folate and SAM, which was not significant in the GLM analysis. Becker et al. show that in contrast to tHcy, serum SAH and the SAM/SAH ratio were not associated with serum concentrations of folate, vitamin B12, or vitamin B6 (21). In contrast to the latter study and ours is a Brazilian study of pregnant women in which a low serum vitamin B12 concentration was associated with a low SAM/SAH ratio (22). A possible explanation is the high frequency of vitamin B12 deficiency, i.e., 52%, compared with 5% in our study population, when using the same cut-off value. The tissue specificity, the pregnant condition and variations in age, gender, lifestyles and diets may explain the conflicting results.

The dietary intake of vitamin B6 was inversely associated with SAH, although this association became borderline significant after Bonferroni adjustment. This is in line with the function of vitamin B6 as cofactor for the transsulphuration enzymes cystathionine β–synthase (CBS, EC 4.2.1.22) and γ–cystathionase (EC 4.4.1.1). Interestingly, dietary vitamin B6 was not associated with tHcy. This was possibly due to the stronger effect of the B vitamin supplement on tHcy. Studies in rats also showed that dietary vitamin B6 deficiency increases SAH in the liver and thymus (23, 24). Others showed in a small group of men (n=33), but not in women (n=33), that dietary vitamin B6 was inversely correlated with tHcy but not with SAM and SAH (25). Remarkably, none of these studies observed associations between B vitamin supplement use and SAM or SAH, while it is the strongest determinant of tHcy. Of further interest is a study by Stabler *et al.* who found that vitamin B12 supplementation only in an elderly population decreased the SAH concentration significantly, but much less than it lowered the tHcy concentration (26). A possible explanation could be that the effect lies within the range of intake available from food, meaning that additional supplement use would not increase this effect in case of adequate intake. Thus, if these biomarkers of cellular methylation are much closer related to health and disease than hyperhomocysteinemia and not influenced by B vitamin supplements, the preventive effect of B vitamin supplements might be smaller than expected. It would, therefore, be of interest to further study this hypothesis in human trials that show whether B vitamin supplementation alters the methylation biomarkers.

BMI appeared a strong independent determinant of SAM and a less strong determinant of SAH. To unravel whether it was weight or height in this composite determinant, we performed an additional multivariable GLM analysis. Height revealed the strongest determinant for SAM showing an inverse association (standardized β=-29.020, *p*-value=0.016), whereas weight was positively associated with SAH (standardized β=0.044, *p*-value=0.007). These findings are new and interesting, but hard to explain with the current knowledge. Therefore, replication studies and longitudinal studies on SAM, SAH, weight, height, and DNA methylation of genes involved in weight and height are needed to further unravel these findings.

Nevertheless, some mechanisms are suggested to be involved in the association of weight, height and the methylation biomarkers. First of all, the physiological concentration of SAH in these healthy premenopausal women is high enough to reflect a cellular state of hypomethylation. Two interesting studies showed an association between hypomethylation and weight. Sinclair *et al.* (27) demonstrated that male offspring from ewes who were periconceptionally fed a methyl-deficient diet, were fatter, more frequently insulin resistant and showed a higher percentage of unmethylated or hypomethylated loci than offspring who were fed a normal diet. Also Waterland *et al.* showed that hypomethylated agouti mice were more prone to gain weight; an effect that could be prevented by hypermethylating dietary supplements (28, 29). Moreover, there are some interesting studies on the guanidinoacetate methyltransferase (GAMT) enzyme. This enzyme converts guanidoacetate to creatine, using SAM as the methyl donor. In a knock-out mouse model, especially the female $GAMT^{-/-}$ animals consistently weighed less than control littermates (30). It could be that the association between weight and SAH is actually determined by increased GAMT activity. Unfortunately, the GAMT and creatinine measurements were not available. The finding does, however, merit further investigation. It has been reported that women with a high BMI tend to develop insulin resistance. This effect was simulated in the Zucker rats, in which an accompanying increase of SAM was observed (31). Also the activity of the glycine N-methyltransferase (GNMT) enzyme, involved in the supply of methyl groups, was increased. Interestingly, growth hormone seems to impact GNMT expression, SAM and SAH concentrations and as such it may contribute to the association

Table 4 Determinants of SAM, SAH, SAM/SAH ratio and tHcy in women

Biomarkers	Variables[a]	Standardised Beta	*p*-value	Bonferroni *p*-value	Partial eta-squared
SAM	BMI	19.145	<0.001	<0.001	0.071
SAH	BMI	3.241	0.010	ns	0.025
	Vitamin B6 intake	-2.390	0.007	ns	0.027
	Miscarriage	1.029	0.035	ns	0.016
	Stillbirths	2.479	0.022	ns	0.019
	NNMT IVS1 -151G>A GG	1.980	0.040	ns	0.016
SAM/SAH	Vitamin B6 intake	0.742	0.025	ns	0.018
	NNMT IVS1 -151G>A GG	-0.767	0.034	ns	0.018
tHcy	Serum vitamin B12[b]	-0.178	<0.001	<0.001	0.077
	Serum folate[b]	-0.166	0.002	ns	0.076
	RBC folate[b]	-0.182	0.002	ns	0.011
	Ethnicity – Non-European	-0.117	0.025	ns	0.021
	Multiparae >5	0.432	0.031	ns	0.023
	Tobacco use	0.119	0.006	ns	0.027
	B vitamin supplement use	-0.156	<0.001	<0.001	0.054
	Protein intake	-0.011	<0.001	<0.001	0.045
	MTHFR 677 TT	0.195	0.001	0.028	0.044
	MTRR AG	-0.083	0.045	ns	0.025

Ns, non significant. Only the variables with *p*-value< 0.05 are shown here.

[a] All demographics and lifestyles, nutrient intakes and genetic polymorphisms were tested in two multivariable general linear models. The first model included SAM, SAH and tHcy as dependent variables. The second model included the SAM/SAH ratio and tHcy as dependent variables. Both models did not include serum vitamin B12, folate and RBC folate as independent variables.

[b] A separate model was made with serum vitamin B12, folate and RBC folate as independent variables together with the genetic polymorphisms and interaction terms.

between height and SAM (32). The homeostatic balance between methylation capacity and the endocrine pathway, in which insulin and growth hormone are involved, seems of interest and needs further investigation.

This study confirms previous studies in which strong associations have been shown between tHcy and B vitamin supplements, tobacco use, MTHFR 677 TT genotype, dietary intake of proteins and even ethnicity (1, 33, 34).

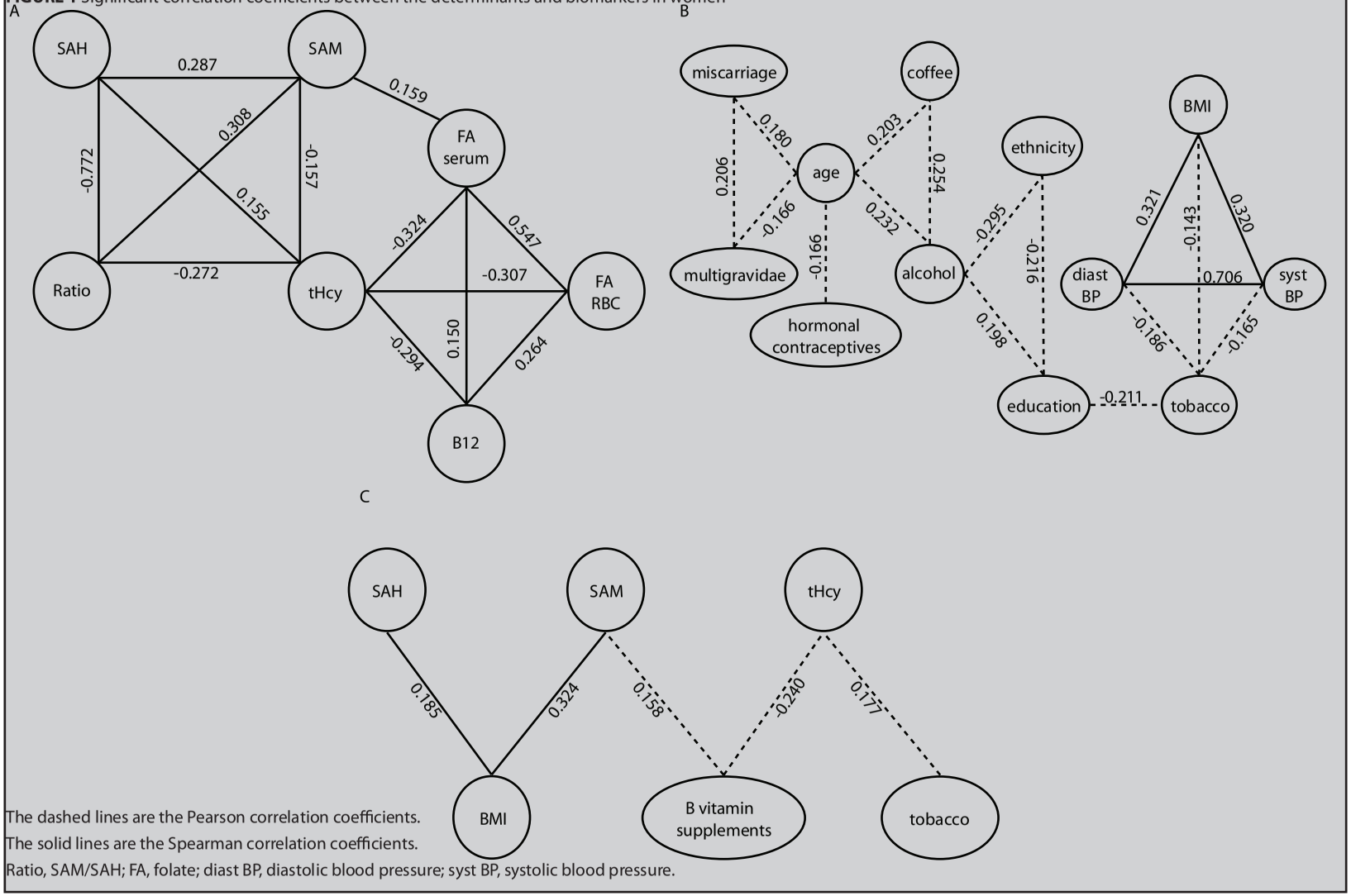
Our study reveals that the factors determining the biomarkers of cellular methylation are different from those of tHcy. Experimental studies demonstrated the importance of SAH and the SAM/SAH ratio as major predictors of methylation capacity (35, 36). Recently, it has been argued that an altered cellular methylation state instead of hyperhomocysteinemia is the primary mechanism involved in many diseases (13, 37). This is supported by recent studies showing that SAH is a more sensitive indicator of cardiovascular disease than tHcy (38, 39).

The strength of the current study is that we included a large population of 336 non pregnant women from the western part of the Netherlands and standardized collected fasting blood samples. Furthermore, demographics, lifestyles, dietary intakes and genetic polymorphisms were evaluated at the same time. The adjustment for multiple testing enabled us to show the strongest predictors of biomarkers for cellular methylation. External validity was also studied by using the best available reference groups. Our study group comprised more non Europeans than the reference populations, which might explain the lower percentage of alcohol use. The lower medication use might be due to the application of a strict exposure definition in which only medication on prescription was only recorded. The dietary intakes were only slightly different from those reported in the Dutch National Food Consumption Survey (9). This may be due to ethnic differences and changes in dietary patterns since 2003 (7). Furthermore, all genetic polymorphisms were in Hardy Weinberg Equilibrium and the frequencies of the variant alleles were all comparable with those reported in other studies, supporting the absence of population selection. Therefore, these data are a good representation of the general Dutch female population of reproductive ages.

Some limitations have to be considered. Some of the demographics and lifestyle factors were dichotomously classified as users or nonusers through which dose-response relationships with the biomarkers could have been missed. Inherent to the study population, the observed associations might be different in women or men of different ages. Furthermore, the inclusion of other measurements of obesity and fat distribution, such as waist-to-hip ratio should be considered.

In conclusion, several factors determine the cellular methylation state, which are different from those determining tHcy. Moreover, we emphasize that at least BMI should be included in future epidemiological studies on methylation in association with outcomes, such as ageing, disease and reproduction.

FIGURE 1 Significant correlation coefficients between the determinants and biomarkers in women



The dashed lines are the Pearson correlation coefficients.
The solid lines are the Spearman correlation coefficients.
Ratio, SAM/SAH; FA, folate; diast BP, diastolic blood pressure; syst BP, systolic blood pressure.

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Chapter

MATERNAL GLOBAL METHYLATION STATUS AND RISK OF CONGENITAL HEART DEFECTS

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ABSTRACT

Background: Maternal hyperhomocysteinemia is a risk factor for congenital heart defects (CHD). It is not clear whether homocysteine or the accompanying hypomethylation, reflected by low S-adenosylmethionine and high S-adenosylhomosyteine, is detrimental for cardiogenesis.

Objective: To investigate the association between the maternal methylation status and CHD offspring.

Design: As part of a case-control study in the western part of the Netherlands, we evaluated 231 case mothers of a child with a CHD, and 315 control mothers of a nonmalformed child. The total case group was analyzed and stratified into isolated (n = 180) and non-isolated CHD (n = 51). The latter subgroup was further subdivided into non-syndromic (n = 20), Down syndrome (n = 19) and other syndromes (n = 12). A multivariate general linear model was used to test for differences between case and control groups. Odds ratios and 95% confidence intervals were computed using logistic regression analysis. All analyses were adjusted for current B vitamin supplement use.

Results: Plasma total homocysteine (tHcy) was significantly different between the total case group (median (range) 10.3 (4.0-43.8) p-value = 0.026) and the non-isolated cases (11.1 (5.5-43.8) p-value = 0.006) versus the controls (10.0 (5.3-42.0)). The subgroup of Down syndrome presented significantly higher tHcy and SAH concentrations, and a lower SAM/SAH ratio than controls. This resulted in a 30 percent lower risk for each unit increase of the SAM/SAH ratio.

Conclusion: Maternal hyperhomocysteinemia is a strong risk factor for having a child with CHD. Maternal hypomethylation, however, seems to be associated with offspring having CHD and Down syndrome.

INTRODUCTION

Congenital heart defects (CHD) account each year for over one million affected newborns worldwide (1). Over 85% of CHD are thought to result from complex interactions involving genetic susceptibilities and environmental exposures (2). Since cardiogenesis takes place in the first weeks of pregnancy, maternal metabolic derangements during the periconception period and early pregnancy can detrimentally affect the embryonic processes involved (3). We and others have shown that maternal hyperhomocysteinemia is associated with an increased risk of CHD offspring, especially with outflow tract defects (4-7). In line with this observation, periconception use of multivitamins containing folic acid decreases the homocysteine (tHcy) concentration and reduces the risk of CHD offspring (8). Folic acid seems to be the key factor, since the use of folate antagonists by the mother increases risk of CHD offspring, which can be prevented by a folic acid supplement (9). So far, it is not clear whether homocysteine itself is teratogenic or just an epiphenomenon of a deranged one-carbon metabolism.

Hyperhomocysteinemia also leads to the accumulation of S-adenosylhomocysteine (SAH), a potent inhibitor of methyltransferases using S-adenosylmethionine (SAM) as the main methyl donor (10, 11). These methyltransferases are important in DNA methylation, which is the best known epigenetic mechanism regulating gene expression without changing DNA sequences (12). DNA hypomethylation is believed to initiate chromosome instability and to alter gene expression, cell differentiation and apoptosis during embryogenesis (13). It thereby affects many biological pathways at different time points in several tissues by gene silencing and also chromosome segregation. As a consequence, major and subtle genetic aberrations can be differentially expressed resulting in various CHD. A recent study identified tHcy, SAH and methionine as the most important predictive biomarkers for the included CHD (14). However, it is not clear whether this accounts for isolated CHD or perhaps also for non-isolated CHD.

In the present study, we conducted a case-control triad study in the western part of the Netherlands to test the hypothesis that maternal hypomethylation reflected in SAM/SAH ratio, SAM and SAH concentrations rather than hyperhomocysteinemia is a risk factor for CHD offspring. We investigated the CHD cases as a total group in comparison to the controls, and in subgroups.

MATERIALS AND METHODS

Subjects

The subjects are enrolled in the HAVEN study, of which the name is a Dutch acronym for the ongoing study designed to investigate determinants in the pathogenesis and prevention of CHD. It is a case-control triad study that has been conducted from June 2003 at the Department of Obstetrics and Gynecology/Division of Obstetrics and Prenatal Medicine at the Erasmus University Medical Center in Rotterdam, the Netherlands. Case children and both parents are enrolled in collaboration with the Departments of Pediatric Cardiology of four collaborating University Medical Centers in the Netherlands. Control children together with their parents are recruited in collaboration with the child health centers of 'Thuiszorg Nieuwe Waterweg Noord' in the western part of the Netherlands. These child health centers are part of the Dutch Health Care system where all newborns are standardized and regularly checked on for health, growth, and development by physicians specialized in child health care. The domain population comprised both case children and control children living in the western part of the Netherlands. All children were between 11 and 18 months of age and there was no familial relationship between cases and controls. The materials and methods of this study have been described before and are summarized hereafter (7).

Cases are included if they had a CHD diagnosed by a pediatric cardiologist and confirmed by echocardiography, and/or cardiac catheterization and/or surgery after birth. The CHD phenotypes included (n = 231) comprised tetralogy of Fallot (n = 35), transposition of the great arteries (n = 31), atrioventricular septal defect (n = 21), perimembranous ventricular septal defect (n = 66), coarctation of the aorta (n = 26), aortic valve stenosis (n = 4), pulmonary valve stenosis (n = 39) and hypoplastic left heart syndrome (n = 9). These phenotypes are selected because experimental and epidemiological studies showed that hyperhomocysteinemia and related gene-environment interactions are involved in their etiology (4, 8, 14). The total case group consisted of 180 isolated and 51 non-isolated defects. The 51 non-isolated CHD consisted of 20 non-syndromic cases, 19 with Down syndrome, and 12 with other syndromes: 22q11 deletion syndrome (n = 5), insertion 1>3 (n = 1), Noonan (n = 1), Turner (n = 1), Alagille (n = *1*), Saethre-Chotzen (n = 1), CHARGE (n = 1), and Beckwith-Wiedemann syndrome (n = 1).

Children were eligible as controls if they did not have a major congenital malformation or chromosomal abnormality according to the medical records and regular health checks by the physician at the child health centers. Cases and controls were excluded if the index-pregnancy was a plural birth, if they were not familiar with the Dutch language in writing and reading and if the mother was pregnant, breastfeeding or reported to have a different diet at the study moment than in the periconception period. Both groups had corresponding proportions of female and male children. The study protocol was approved by the Central Committee on Research involving Human Subjects ("Centrale Commissie Mensgebonden Onderzoek", CCMO) and the Institutional Review Boards (Medical Ethics Committees) of all participating hospitals. All participants gave their written informed consent.

Data collection

At a fixed study moment of approximately 16 months after the index pregnancy, mothers filled out a general questionnaire at home, which was checked for completeness and inconsistency at the hospital visit. Information was obtained about educational level, ethnicity, and both current use of alcohol, tobacco, medication, and B vitamin supplements and use of these products during the periconception period. Current use was defined as any use in the previous 4 weeks. The periconception period was defined as 4 weeks prior until 8 weeks after the conception. Educational level was classified according to the definitions of Statistics Netherlands (15). Mothers were classified as Dutch Natives, European Others and Non Europeans. Maternal height (anthropometric rod; SECA, Germany) was measured up to 0.1-cm accuracy and weight (weighing scale; SECA, Germany) up to 0.5-kg accuracy. Body mass index (BMI) was defined as weight divided by the square of height.

EDTA-blood was taken for determination of tHcy, SAM and SAH. After withdrawal, blood was kept on ice and centrifuged at 4˚C within two hours. Plasma aliquots were stored at -80˚C until analysis. THcy was determined using isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS; Waters Acquity UPLC Premier XE, Milford, MA, USA) by a method adapted from Ducros *et al* (16). For chromatographic separation we used a Waters Symmetry C_8 column (2.1 x 100 mm, reference WAT 058961, Waters, Etten-Leur, the Netherlands) with a precolumn (Waters, reference 205000343). The column was eluted at 0.25 mL/min and no splitter was used. Calibration was performed with aqueous standards because the results were similar to those of plasma-based standards. SAM and SAH were also determined using LC-MS/MS by a method adapted from Gellekink *et al* (17). In short, non-acidified EDTA-plasma was stored at -80˚C and 200 µL plasma was used for sample clean-up. Samples (10 µL) were injected on a 50 x 2.1 mm Atlantis C_{18} column (Waters) and eluted in a gradient of methanol in aqueous acetic acid (0.1%). The retention times were 0.6 min (SAM) and 1.4 min (SAH). Standards were dissolved in 1 mmol/L HCl; pool sera were SAM and SAH depleted by SPE and spiked with the calibrator. Calibration curves for SAM and SAH were linear until 500 nmol/L.

Statistical analysis

Time after pregnancy, maternal age and BMI are presented as medians and compared between the different case groups and controls using the Mann-Whitney U test. Differences in frequencies were tested using the chi-square test. The biochemical parameters are presented as medians and ranges and because the distributions were positively skewed, all biomarker data were log-transformed (natural log) before analysis. Normality of the transformed data was verified for all case groups and the control group by using the Kolmogorov-Smirnov test. Differences between the case groups and controls were tested with a general linear model (UNIANOVA). Current B vitamin supplement use of the mother was considered a confounding factor and was therefore included in the model. Probability values of p-value< 0.05 were considered statistically significant. Moreover, all comparisons were adjusted for multiple testing by the method of Bonferroni. All analyses were performed using SPSS for Windows software (version 15.0; SPSS Inc., Chicago, IL, USA).

The results on maternal homocysteine levels in association with congenital heart defects have already been published within our study, though in a much smaller sample size and only in the total case group (7).

RESULTS

The general characteristics of the total case and control group were not significantly different (*Table 1*). The general characteristics of the subgroups of cases were also determined (data not shown). Mothers of non-syndromic cases used significantly more medication in the periconception period than mothers of controls

TABLE 1 General characteristics of mothers of a CHD child (cases) and nonmalformed child (controls)

	Cases ***n*** **= 231**	**Controls** ***n*** **= 315**	***p*-values**
Study moment			
Time after pregnancy (mo)	15.8 (14.9 - 18.1)	16.0 (15.1 - 18.0)	0.661
Maternal age (y)	32.7 (29.7 - 36.2)	32.6 (28.5 - 34.9)	0.081
BMI (kg/m^2)	24.3 (22.0 - 28.2)	24.3 (22.0 - 27.3)	0.488
Educational level[a] [*n* (%)]			0.473
Low	64 (28)	77 (24)	
Intermediate	106 (46)	161 (51)	
High	61 (26)	77 (24)	
Ethnicity [b]			0.767
Dutch Native	183 (79)	249 (79)	
European Others	13 (6)	14 (4)	
Non European	35 (15)	52 (17)	
Parity > 1	142 (62)	170 (54)	0.080
Family history of CHD	24 (10)	19 (6)	0.063
Use of [*n* (%)]			
Alcohol	116 (50)	180 (57)	0.109
Tobacco	45 (20)	61 (19)	0.973
Medication	45 (20)	63 (20)	0.880
B vitamin supplements	42 (18)	64 (20)	0.533
Periconception period			
Use of [*n* (%)]			
Alcohol	79 (34)	106 (34)	0.894
Tobacco	44 (19)	70 (22)	0.367
Medication	60 (26)	66 (21)	0.169
B vitamin supplements	115 (50)	160 (51)	0.816

Values are median (interquartile range) or number (percentage). BMI, body mass index.

[a] Categorized as low (primary/lower vocational/intermediate secondary education), intermediate (higher secondary/intermediate vocational education) or high (higher vocational/university education) (15).

[b] 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 or is from European origin and living in the USA, Australia or Indonesia. Non European: all others.

(cases: number of users 8 (40%), controls: number of users 66 (21%); *p*-value < 0.05). However, after adjusting for multiple testing, it became not significant. Mothers of children with Down syndrome were significantly older (median 35.0 years, range 31.3-41.2 years) compared with control mothers (median 32.6 years, range 28.5-34.9 years; *p*-value < 0.01). All other general characteristics of the subgroups of cases compared with the control group were not significantly different. We also looked at maternal medical diseases in the periconception period associated with CHD, such as Diabetes and epilepsy. Both diseases were present in 2 case mothers and 4 control mothers. Also morbid BMI (≥40 kg/m^2) was considered a potential confounder: there were 3 case mothers and 5 control mothers with a morbid BMI. These were very small numbers and not different between cases and controls (data not shown). After adjusting for multiple testing, all variables became not significant; accept for the variable maternal age in the Down Syndrome group versus the controls.

In *Tables 2.1* and *2.2* comparisons of plasma biomarker concentrations are presented between the different case subgroups and the controls. *Table 2.1* reveals a significant difference in tHcy concentration between the total case group and the non-isolated cases versus the controls. In *Table 2.2* the non-isolated cases are divided in three different subgroups: non-syndromic, Down syndrome, and other syndromes. The other syndrome group presented significantly higher levels of tHcy than the control group. Moreover, mothers of a child with Down syndrome presented not only significantly higher levels of tHcy, but also of SAH and a lower SAM/SAH ratio than the control group. All comparisons remained significant after adjusting for multiple testing, except for the comparison of tHcy concentrations between the total case group and the Down syndrome group versus the controls.

DISCUSSION

In this study we confirmed our previous finding that maternal tHcy concentrations were significantly higher in the total group of CHD offspring than in the control group. The non-isolated CHD cases, however, and more specifically the subgroups of Down Syndrome and Other Syndromes seem to be entirely responsible for this finding. Of most interest is the new observation that a maternal status of hypomethylation, reflected by a high tHcy and SAH level and low SAM/SAH ratio, is significantly associated with an increased risk of having a child with CHD and Down syndrome.

So far, only Hobbs *et al*. associated maternal hypomethylation with CHD risk. The children in that study had a non-syndromic CHD comprising a septal, conotruncal, or right or left-sided obstructive defect. The most important difference with our results is that we found this profile of hypomethylation in the syndromic case group only, and especially in mothers of a child with Down syndrome and CHD, and not in the non-syndromic case group.

There could be several explanations for these different results. Firstly, Hobbs *et al*. included mothers at various points within a period of 0.1 to 52.2 months after the index-pregnancy, while we included mothers at a fixed study point at around 15 months within a much shorter period of 11 to 18 months after the index-pregnancy. The wide period of sampling and data collection might have introduced bias by covariates that affected the tHcy levels and were not reported, such as breastfeeding and age (18). If more case mothers than control mothers were breastfeeding at the time of sampling, the tHcy levels could have been higher and the SAM/SAH ratio lower, thereby producing an overestimation of the associated risk estimates. This might have been the case, because fewer case mothers participated more than 6 months after the index-pregnancy, whereas in our study all breastfeeding mothers were excluded. Another explanation can be that the CHD phenotypes and the omitted numbers in the case groups are different, since they were not reported.

Nevertheless, our main finding was the altered SAM/SAH ratio as a marker of hypomethylation in mothers of a child with CHD and Down syndrome. A higher SAH concentration and a lower SAM/SAH ratio are indicators of a lower methylation capacity (19). Hypomethylation has been associated with chromosome instability (20-22). Moreover, folate deficiency also reduces synthesis of SAM, leading to DNA hypomethylation (23). Only one case report described a child with Down syndrome and neural tube defect (24). The mother of this child had a three-fold higher SAH level; this led to a markedly reduced SAM/SAH ratio and to a significant hypomethylation of lymphocytes. Moreover, both mother and child were homozygous for the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism, which is associated with mildly increased tHcy and decreased folate levels, particularly in situations of deprived folate status. In other studies, this MTHFR polymorphism has also been associated with Down syndrome (25, 26). In an additional analysis we therefore evaluated the methylation biomarkers together with the maternal MTHFR C677T genotypes. In the total case group, mothers with the homozygous variant not only had lower SAM/SAH ratios (data not shown and not significant), they also showed significantly higher tHcy concentrations [median (range): 14.8 (4.0-43.8) μmol/L; *n* = 19] than the heterozygous

TABLE 2.1 Plasma biomarkers concentrations of cases versus controls

	Total group of cases			Isolated cases			Non-isolated cases			Controls	
Biomarker	***n***	**Median (range)**	***p*-value[b]**	***n***	**Median (range)**	***p*-value[c]**	***n***	**Median (range)**	***p*-value[d]**	***n***	**Median (range)**
tHcy (µmol/L)[a]	229	10.3 (4.0 - 43.8)	0.026	175	10.1 (4.0 - 31.2)	0.157	51	11.1 (5.5 - 43.8)	0.006	310	10.0 (5.3 - 42.0)
SAM (nmol/L)	231	78.5 (46.7 - 150.2)	0.338	180	77.8 (46.7 - 127.6)	0.208	51	79.6 (60.6 - 150.2)	0.827	315	80.3 (41.7 - 121.2)
SAH (nmol/L)	231	14.0 (6.4 - 95.8)	0.478	180	13.9 (6.4 - 27.5)	0.950	51	14.5 (10.2 - 95.8)	0.091	315	14.0 (7.2 - 34.2)
SAM/SAH	231	5.6 (0.6 - 10.3)	0.210	180	5.7 (1.7 - 10.3)	0.395	51	5.6 (0.6 - 9.0)	0.127	315	5.9 (2.20 - 11.64)

All comparisons are adjusted for current maternal B vitamin supplement use

[a] Fewer number of tHcy because of lost samples (5 cases and 5 controls)

[b] Comparison of total group of cases with controls

[c] Comparison of isolated cases with controls

[d] Comparison of non-isolated cases with controls

TABLE 2.2 Plasma biomarker concentrations of non-isolated cases versus controls

	Non-isolated cases									Controls	
	Non-syndromic			Down Syndrome			Other syndromes				
Biomarker	***n***	**Median (range)**	***p*-value[b]**	***n***	**Median (range)**	***p*-value[c]**	***n***	**Median (range)**	***p*-value[d]**	***n***	**Median (range)**
tHcy (µmol/L)[a]	20	10.3 (5.5 - 21.3)	0.664	19	11.1 (6.7 - 43.8)	0.034	12	12.7 (8.3 - 23.6)	0.004	310	10.0 (5.3 - 42.0)
SAM (nmol/L)	20	80.8 (66.4 - 117.3)	0.490	19	79.9 (60.6 - 150.2)	0.388	12	75.7 (64.4 - 91.0)	0.129	315	80.3 (41.7 - 121.2)
SAH (nmol/L)	20	14.3 (10.2 - 21.8)	0.989	19	16.0 (10.6 - 95.8)	0.001	12	12.3 (11.2 - 17.6)	0.554	315	14.0 (7.2 - 34.2)
SAM/SAH	20	5.9 (4.6 - 9.0)	0.669	19	5.6 (0.6 - 6.7)	0.007	12	5.5 (4.0 - 7.5)	0.710	315	5.9 (2.2 - 11.6)

All comparisons are adjusted for current maternal B vitamin supplement use

[a] Fewer number of tHcy because of lost samples (5 controls)

[b] Comparison of Non-syndromic cases with controls

[c] Comparison of Down Syndrome cases with controls

[d] Comparison of Other syndromes with controls

case group [10.4 (5.1-26.1) µmol/L; *n* = 95] and than wildtype carriers [10.1 (6.5-31.2) µmol/L *n* = 97): *p*-value = 0.007, Kruskal Wallis Test]. The group of mothers of a child with Down syndrome and a CHD was small, and only one mother carried the homozygous variant. She had a lower median SAM [homozygous: 74.8 nmol/L versus heterozygous: 83.1 nmol/L versus wildtype 77.4 nmol/L], a lower SAM/SAH ratio [homozygous: 4.5 versus heterozygous: 6.1 versus wildtype 5.6], a higher SAH [homozygous: 16.5 nmol/L versus heterozygous: 16.1 nmol/L versus wildtype 14.5 nmol/L], and also a higher tHcy level [homozygous: 43.8 µmol/L versus heterozygous: 11.3 µmol/L versus wildtype 11.1 µmol/L].

These results are substantiated by the literature and suggest an association between the hypomethylation status of the mother and the risk of having a child with Down syndrome and CHD. Because of our definition of the case group, we were unable to distinguish between the risk of Down syndrome without a CHD and risk of a CHD in children with Down syndrome. Therefore, further studies are needed to unravel the role of hypomethylation in these separate groups.

Low maternal folate status, MTHFR 677TT carriership and hyperhomocysteinemia might result in a decreased SAM/SAH ratio, thereby inducing general hypomethylation and increasing the risk of offspring with Down syndrome and CHD. The absence of this association in the other CHD phenotypes suggests that maternal hypomethylation may be less important. As shown by Becker *et al*., the folate status is a strong determinant of tHcy levels, but not of the SAM/SAH ratio (27). This may suggest that the treatment of hyperhomocysteinemia by folic acid does not reduce the risk of having offspring with Down and CHD, because it does not affect the hypomethylation status. On the other hand, Ingrosso *et al.* showed that hypomethylation of lymphocytes could be restored by folic acid treatment (28). Although several studies suggest that periconception intake of folic acid may reduce the risk of a broad range of CHD the results are not conclusive (8, 29). Therefore, we suggest that, due to differences in cardiac genes sensitive to folate shortage, hyperhomocysteinemia or hypomethylation, folic acid treatment may not reduce the risk of all CHD phenotypes.

Some methodological limitations have to be considered. Despite our efforts to minimize the potential biases inherent to a case-control study focusing on congenital malformations, our results do not allow concluding that the alterations in maternal biomarkers were also present during the embryogenesis of the heart. However, our findings are substantiated by the fact that biomarkers of the tHcy pathway are rather stable in the periconception period and over a period of 1-2 years (30). Moreover, the subgroup biomarker analyses not showing a difference with controls are likely underpowered, which means that there could be a difference.

A strength of our study is the standardized study moment of around 15 months after the index-pregnancy: it does not significantly interfere with the maternal physiology, metabolism and endocrinology and it diminishes the chance of misclassifying cases and controls, because most malformations are completely diagnosed in the first year of life. Moreover, compared to other studies, our study moment is relatively soon after pregnancy, which is important to minimize recall bias. Another strength is that we selected CHD phenotypes on the basis of evidence from experimental and epidemiological studies showing associations with maternal hyperhomocysteinemia and related gene-environment interactions (4, 8, 14). This is reinforced by the hypothesis underlying the HAVEN study, which is that most human teratogenic exposures are derived from maternal endogenous metabolic or endocrine derangements or exogenous harmful exposures, which adversely affect the embryogenesis of her child (3). This adverse effect is dependent on the time window and sensitivity of the exposures. By analysing the CHD phenotypes separately and after pooling, we produced comparable results that substantiated the homogeneity of the CHD group.

In conclusion, our findings suggest an association between maternal hypomethylation status reflected in high tHcy and SAH levels and a low SAM/SAH ratio, and offspring with Down syndrome and CHD. Since this subgroup was very small, this needs to be confirmed in a much larger population, focussing on mothers whose children have Down syndrome with or without a CHD. It is important to extend this knowledge with new risk factors for CHD, because this is the only manner to improve the preconception counselling and care of future women who want to become pregnant.

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Chapter

5

CONGENITAL HEART DEFECTS IN VERY YOUNG CHILDREN AND ALTERATIONS IN BIOMARKERS OF METHYLATION

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Submitted

ABSTRACT

Objectives: To investigate associations between congenital heart defects (CHD) and the global methylation status in blood.

Methods: In a case-control study, we included 143 children with a CHD (cases) from 4 acedemic medical centers in the western part of the Netherlands and 186 children without a congenital malformation in collaboration with the Child Health Care centers in Rotterdam. Biomarkers of the global methylation state, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), total homocysteine (tHcy), folate, vitamin B12, were compared and adjusted for age, medication, vitamin use, and family history of CHD. Odds ratios estimating the risk of CHD associated with levels of biomarkers were calculated. 5,10-Methylenetetrahydrofolate reductase (MTHFR; 677C>T and 1298A>C genotype) polymorphism determination was done by DNA sequencing.

Results: We observed in nonisolated syndromic CHD significantly higher median concentrations of SAM (*p*-value = 0.002), SAH (*p*-value = 0.006) and serum folate (*p*-value = 0.01) compared with other CHD subgroups and controls. Significant correlations were established between SAM and SAH (r = 0.283, *p*-value = 0.001), SAM and red blood cell (RBC) folate (r = 0.227, *p*-value = 0.007), SAH and serum folate (r = 0.307, *p*-value < 0.001), and serum and RBC folate (r = 0.432, *p*-value < 0.001). The higher methylation status in CHD was not related to the MTHFR polymorphisms. High SAM and serum folate revealed increased risks for CHD, odds ratio (95% confidence interval) 1.71 (0.96-3.07) and 1.71 (0.96-3.09), respectively.

Conclusion: A status of global hypermethylation in very young children is associated with nonisolated CHD, in particular syndromic CHD, in which the high folate status seems the most important determinant.

INTRODUCTION

Every year more than 1 million children are born with a congenital heart defect (CHD) of which the causes are largely unknown (1). So far, several studies have shown the beneficial effects of the maternal use of synthetic folic acid in the periconception period and the harmful effects of a diet low in vitamin B12 on the risk of CHD offspring (2, 3). The underlying mechanisms, however, are largely unknown.

It reveals from a meta-analysis that maternal hyperhomocysteinemia 3-4 fold increases the risk of CHD, in particular of cardiac outflow tract defects (4). Both a low folate and low vitamin B12 status cause a mild to moderate hyperhomocysteinemia, which can be treated successfully by synthetic folic acid and/or vitamin B12. These associations are supported by studies in the chicken embryo showing that homocysteine exposure induces CHD by decreasing apoptosis and myocardialization of the cardiac tissues in a time and concentration-dependent manner (5, 6). In addition, a study in mice demonstrated that a reduced activity of the folate gene 5,10-methylenetetrahydrofolate reductase (MTHFR) also leads to CHD offspring (7). These observations are in line with the results from knock out studies in mice showing that ablation of the folate receptor 1 (Folr1) and reduced folate carrier 1 (Slc19a1) gene results in CHD (8).

Evidence is accumulating that the association between periconception hyperhomocysteinemia and CHD may be explained by alterations in the intermediate methylation biomarkers of the homocysteine pathway in the mother, i.e., S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and the SAM/SAH ratio. Hobbs *et al*. observed a higher SAH and lower SAM concentration in mothers of CHD offspring (9). In a previous study, we found a lower SAM/SAH ratio in mothers of offspring with CHD and Down syndrome (10). Hyperhomocysteinemia is also a risk factor for cardiovascular disease (11). Considering the vascular hypothesis in the pathogenesis of CHD, a high SAH, decreased SAM/SAH ratio and global DNA hypomethylation have been associated with vascular diseases (11-13).

We hypothesize that the global methylation status in children with CHD is altered due to a derangement in intrauterine metabolic programming as a consequence of periconception exposure to hyperhomocysteinemia and global hypomethylation. DNA-methylation is the best-characterized epigenetic mechanism to explain interactions between nutrients, metabolites and genes. The observations that derangements in DNA methylation due to alterations in the global methylation status in blood and other tissues during pregnancy and post-weaning can modify embryonic, fetal and metabolic development are herewith in line (14-16).

Therefore, this study investigates the global methylation status in blood of very young children with CHD, stratified in CHD subgroups, and a control group of healthy nonmalformed children.

MATERIALS AND METHODS

Subjects

This study included the children enrolled in the HAVEN study, designed to unravel the etiology of CHD. The detailed information about the study methods used is described previously (17). The case group consisted of 143 children with CHD derived from the western part of the Netherlands. The CHD phenotypes comprised tetralogy of Fallot ($n = 15$), transposition of the great arteries ($n = 28$), perimembraneous ventricular septal defect ($n = 43$), coarctation of the aorta ($n = 15$), atrioventricular septal defect ($n = 8$), hypoplastic left heart syndrome ($n = 2$), pulmonary valve stenosis ($n = 27$) and aortic valve stenosis ($n = 5$). We divided the CHD cases in isolated CHD ($n = 119$) and nonisolated CHD ($n = 24$). The subgroup of nonisolated CHD was further subdivided into syndromic CHD ($n = 16$), including Down syndrome ($n = 8$), 22q11 deletion syndrome ($n = 3$), 22q13 duplicate ($n = 1$), insertion 1>3 ($n = 1$), Turner ($n = 1$), CHARGE syndrome ($n = 1$), and Alagille syndrome ($n = 1$), and nonsyndromic CHD ($n = 8$). CHD diagnoses were confirmed after birth by echocardiography and/or cardiac catheterization and/or surgery. 186 Healthy children without a birth defect were randomly recruited from the child health care centers of 'Thuiszorg Nieuwe Waterweg Noord' at the same time and in the domain of the case group. Child health care centers are part of the Dutch Health Care system where physicians specialized in child health care regularly check all newborns at standardized moments on health, growth and development.

We excluded children who were related and of which the parents were not familiar with the Dutch language in writing and reading. The study protocol was approved by the Central Committee on Research involving Human Subjects, and the Institutional Review Boards of all participating hospitals. All parents gave their written informed consent before participation.

Data collection

At the standardized study moment of around 17 months after delivery of the CHD and control child, we collected data of the mothers and children comprising demographics, gender, family history of CHD, exposures and for cases the CHD phenotype. The use of medication and vitamins of the child at the study moment was recorded, because of the potential effects on the biomarkers of methylation and B vitamins in blood. A positive family history of CHD was defined as the child having a CHD relative to the 3rd degree.

At the hospital visit, venous blood samples were drawn from the children to measure the concentrations of SAM, SAH, homocysteine (tHcy), serum and red blood cell (RBC) folate, serum vitamin B12 and the MTHFR 677C>T and 1298A>C polymorphisms. EDTA-blood was kept on ice and centrifuged within 2 hours after withdrawal at 4 °C. Plasma aliquots were stored at -80 °C until analysis. To determine SAM and SAH we used liquid chromatography tandem mass spectrometry (LC-MS/MS; Waters acquity UPLC premier XE, Milford, MA, USA) as previously described (14). In short, EDTA-plasma was stored at -80 °C and 200µL plasma was used for sample clean up. Samples (10µL) were injected on a 50 x 2.1-mm Atlantis C18 column (Waters) and eluted in a gradient of methanol in aqueous acetic acid (0.1%). The retention times were 0.6 minutes (SAM) and 1.4 minutes (SAH). Samples were dissolved in 1 mM/L HCl; pool sera were SAM and SAH depleted by SPE and spiked with the calibrator. Calibration curves for SAM and SAH were linear until 500 nmol/L. tHcy was also determined using LC-MS/MS. For chromatographic separation, we used a Waters Symmetry C8 column (2.1 x 100 mm, reference WAT 058961, Waters, Etten-Leur the Netherlands) with a precolumn (Waters, reference 205000343). The column was eluted at 0.25mL/min and no splitter was used. Calibration was performed with aqueous standards because the results were similar to those of plasma-based standards. Serum folate and vitamin B12 were routinely determined by immuno electrochemiluminescence immunoassay (ECLIA) on the Roche Modular E170 (Roche Diagnostics GmbH, Mannheim Germany). RBC folate was measured after direct hemolysis of whole-blood in ascorbic acid. The RBC folate concentration was corrected for the hematocrite and the serum folate concentration. The between-run coefficient of variation for SAM was 4.4% at 70.8 nmol/L, 4.4% at 100.8 nmol/L en 4.8% at 143.2 nmol/L. These coefficients of variation were 4.2% at 24.2 nmol/L for SAH, 5.9% at 15.3 µmol/L en 3.4% at 39.3 µmol/L for tHcy, 9.5% at 8.3 nmol/L en 3.2% at 20.2 nmol/L for folate and 5.1% at 125 pmol/L and 2.9% at 753 pmol/L for vitamin B12.

To determine the MTHFR 677C>T and MTHFR 1298A>C polymorphism genomic DNA was isolated from 0.2mL EDTA-whole blood with the Total Nucleic Acid Extraction kit on a MagNA PureLC (Roche Molecular Biochemicals, Mannheim, Germany) these data are previously described in a subgroup of CHD and controls (18).

Statistical analysis

Age of the child, the biomarkers of methylation and vitamins in blood are presented as medians and ranges because of skewness even after transformation. They were compared with the Mann-Whitney U test. Characteristics and exposures presented in frequencies were tested using the Chi-square test. Child's age, family history of CHD, medication and vitamin use at the study moment were considered as possible confounders. We tested deviation of the genotype frequencies from those expected under Hardy Weinberg Equilibrium for cases and controls separately using the chi-square test. Allele and genotype frequencies were compared between cases and controls. Pearson and Spearman correlation analysis were performed between the general characteristics and the biomarkers. The differences in biomarker concentrations were tested by multivariable logistic regression analysis. The biomarkers of methylation and B vitamins were dichotomized according to cut-off values based on the 85th percentile of the controls. Crude and adjusted odds ratios (OR) with 95% confidence interval (95% CI) were calculated by multivariable logistic regression analysis. All analyses were performed with SPSS for Windows software (version 15.01; SPSS Inc., Chigago, IL, USA).

RESULTS

Children with CHD showed more often a positive family history of CHD (*p*-value=0.020). They used significantly less vitamin preparations than controls (*p*-value=0.003), comprising combinations of vitamin A, D and E. Only 2 cases and 1 control used a multivitamin containing folic acid. The distribution of ethnicity was comparable between the groups (*Table 1*). The general characteristics stratified for the CHD subgroups showed that nonisolated CHD compared with controls were almost one month older, 18.1 (15.2-27.0) and 17.3 (13.0-24.9), respectively, (*p*-value=0.034), and used more medication 58.3% and 14.5%, respectively (*p*-value<

TABLE 1 General characteristics of the study population of children at the study moment

	Cases (*n* = 143)[a]	Controls (*n* = 186)[a]
Age (m)	17 (11.4 - 27.0)	17.3 (13.0 - 24.9)
Male gender, [n (%)]	87 (61.7)	104 (55.9)
CHD family history, [n (%)]	18 (12.8)[b]	10 (5.4)
Use of medication, [n (%)]	29 (20.3)	27 (14.5)
Use of vitamins, [n (%)]	83 (58.0)[c]	139 (74.7)
Ethnicity[d]:		
Dutch native, [n (%)]	110 (76.9)	141 (75.8)
European others, [n (%)]	10 (7.0)	11 (5.9)
Non European, [n (%)]	23 (16.1)	34 (18.3)

CHD, Congenital heart disease.

[a] Values are expressed as medians (range) or number (percentage).

[b] *p*-value < 0.05.

[c] *p*-value < 0.005.

[d] Dutch Natives were defined as those of whom both parents and grandparents were born in the Netherlands, or one of the parents was born in another country, but both grandparents were born in the Netherlands. European others were defined as those of whom one of the parents or grandparents was born in a European country, Indonesia, or was from European origin and living in the USA or Australia. Non-Europeans were defined as all others (27).

TABLE 3 Biomarkers of methylation and vitamins in blood of children with nonisolated CHD and controls[a,b]

Biomarkers	Nonisolated CHD		
	Nonsyndromic (*n* = 8)	Syndromic (*n* = 26)	Controls (*n* = 186)
SAM (nmol/L)	111.9 (100.3 - 156.5)[c]	132.1 (99.8 - 224.8)[d]	104.4 (50.8 - 164.0)
SAH (nmol/L)	17.0 (10.6 - 24.1)	22.1 (12.8 - 39.6)d	15.9 (8.9 - 76.4)
SAM/SAH	6.8 (6.0 - 9.9)	6.2 (3.2 - 9.0)	6.5 (0.7 - 13.0)
tHcy (µmol/L)	5.5 (3.9 - 8.7)	6.3 (4.8 - 8.3)	6.2 (3.7 - 12.1)
Folate, serum (nmol/L)	32.4 (15.1 - 79.8)	43.7 (23.4 - 100.3)[c]	28.5 (8.4 - 99.6)
Folate, RBC (nmol/L)	1084 (561 - 2213)	1097 (752 - 2351)	916 (342 - 2460)
Vitamin B12 (pmol/L)	581 (201 - 1104)	420 (278 - 911)	481 (135 - 1232)

SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine, tHcy, total homocysteine; RBC, red blood cell.

[a] All comparisons are adjusted for age, family history of CHD, medication and vitamin use at the study moment.

[b] Values are expressed as medians (range).

[c] *p*-value < 0.05.

[d] *p*-value < 0.01.

0.001). In isolated CHD a positive family history of CHD was more often observed than in controls, 12.7% versus 5.4% (*p*-value=0.024). After adjusting for multiple testing with the Bonferroni method, only medication use remained significant in nonisolated CHD (data not shown).

All comparisons are adjusted for child's age, positive family history of CHD, and medication and vitamin use at the study moment. The total CHD group showed significantly higher concentrations of SAM (*p*-value=0.01), serum folate (*p*-value=0.02) and RBC folate (*p*-value=0.03) than the control group. In the CHD subgroups only nonisolated CHD showed significantly higher SAM (*p*-value<0.001), SAH (*p*-value=0.01), serum folate (*p*-value=0.01) and an almost significantly higher RBC folate (*p*-value=0.05) concentration compared with controls. These biomarker and B vitamin concentrations were also higher than in isolated CHD (*Table 2*).

Spearman correlation analysis revealed significant correlations in the total CHD group between SAM and SAH (r=0.283, *p*-value=0.001), SAM and RBC folate (r=0.227, *p*-value=0.007), SAH and serum folate (r=0.307, *p*-value<0.001), and serum and RBC folate (r=0.432, *p*-value<0.001). In controls the correlations were significant between tHcy and SAM/SAH ratio (r=-0.153, *p*-value=0.04), tHcy and serum folate (r=-0.252, *p*-value=0.001), tHcy and RBC folate (r=-0.171, *p*-value=0.02), and between serum and RBC folate (r=0.605, *p*-value<0.001).

In nonisolated nonsyndromic CHD, SAM was significantly higher (*p*-value=0.03) and tHcy tended to be lower (*p*-value=0.06) than in controls. In nonisolated syndromic CHD, SAM (*p*-value=0.002), SAH (*p*-value=0.006) and serum folate (*p*-value=0.01) were significantly higher (*Table 3*). The concentrations of these biomarkers in the group of nonisolated syndromic CHD were highest compared with all other CHD subgroups. A separate analysis of 8 children with Down syndrome in the nonisolated syndromic subgroup revealed the highest concentrations of SAM (median [range], 132.1 nmol/L (110.8-193.1), *p*-value= 0.004) and SAH (25.3 nmol/L (17.1-35.0), *p*-value=0.002), RBC folate (1544 nmol/L (752-2065), *p*-value=0.04) and lowest tHcy (5.8 µmol/L (4.8-8.1)) and vitamin B12 (412 pmol/L (282-593)) compared to controls. After adjusting for multiple testing SAM and SAH of nonisolated CHD and nonisolated syndromic CHD remained significant. SAM or serum folate concentrations above the 85th percentile were both associated with a 1.7-fold increased risk for CHD (*Table 4*).

MTHFR polymorphisms were assessed in 139 CHD and 183 controls. Genotype distributions for the total CHD and control group were in Hardy-Weinberg (*p*-value>0.05) equilibrium and the genotype frequencies were not significantly different between CHD and controls. In CHD the frequencies for the MTHFR 677 CC/CT/TT genotypes were 46%/47.5%/6.5% and in controls 50.3%/41.5%/8.2%, respectively. In CHD the frequencies for the MTHFR 1298 AA/AC/CC genotypes were 49.6%/41%/9.4% and 41%/49.2%/9.8% in controls. In the CHD group, 9 children carried the MTHFR 677TT genotype of which 8 had an isolated CHD. The nine 677TT carriers showed a significantly lower vitamin B12 concentration compared with the MTHFR 677CC carriers, 419 pmol/L (192-490), and 516 pmol/L (149-1147), *p*-value=0.022, respectively. In the CHD group, 13 children carried the MTHFR 1298CC genotype with significantly lower tHcy than MTHFR 1298 AA carriers, 5.7 µmol/L (4.1-7.3) and 6.3 µmol/L (4.0-11.7), *p*-value=0.04, respectively.

DISCUSSION

Recently, we reported the association between a status of global hypomethylation in mothers, determined by the same biomarkers in blood, of a child with nonisolated CHD and Down syndrome (10). In the present study, we observe in particular in the children of nonisolated CHD a status of global hypermethylation reflected by increased SAM, SAH and folate concentrations in blood. This finding was most pronounced in children with nonisolated syndromic CHD. It is intriguing to establish that in the same phenotype but now in the children themselves the nonisolated syndromic CHD and Down syndrome phenotypes show the highest hypermethylation status. This is exactly opposite of the hypomethylation status in their mothers. Furthermore, the correlations between the biomarkers in CHD and controls were different. Assuming that the maternal global hypomethylation status was also present during the periconception period, this may suggest that the global hypermethylation status in the children might be explained by alterations in metabolic imprinting (19, 20). This is in line with the Developmental Origins hypothesis of Health and Disease, in which exposure to malnutrition and metabolic derangements during pregnancy lead to phenotypic and metabolic derangements in later life.

Methylation is also an important process in the segregation of chromosomes during conception (21). Of most interest therefore is the relatively large group of children with nonisolated syndromic CHD and Down syndrome showing the highest global hypermethylation status.

Remarkable is the significantly higher folate concentration in the total CHD group, nonisolated CHD, nonisolated syndromic CHD, and in the nonisolated CHD with Down syndrome. After adjustment for a positive family history of CHD, age, medication and vitamin use, the folate concentrations remained significantly higher.

TABLE 2 Biomarkers of methylation and vitamins in blood of children with CHD, after stratification in CHD subgroups, and controls [a,b]

Biomarkers	CHD (*n* = 143)	Isolated CHD (*n* = 119)	Nonisolated CHD (*n* = 24)	Controls (*n* = 186)
SAM (nmol/L)	107.5 (53.5 - 224.8)[c]	106.1 (53.5 - 221.8)	130.0 (99.8 - 224.8)[d]	104.4 (50.8 - 164.0)
SAH (nmol/L)	16.8 (7.6 - 51.8)	16.3 (7.6 - 51.8)	20.9 (10.6 - 39.6)[c]	15.9 (8.9 - 76.4)
SAM/SAH	6.5 (1.0 - 13.5)	6.61 (1.0 - 13.5)	6.4 (3.2 - 9.9)	6.5 (0.7 - 13.0)
tHcy (µmol/L)	6.2 (3.9 - 12.3)	6.2 (4.0 - 12.3)	6.3 (3.9 - 8.7)	6.2 (3.7 - 12.1)
Folate, serum (nmol/L)	32.0 (11.3 - 113.7)[c]	30.6 (11.3 - 113.7)	37.5 (15.1 - 100.3)[c]	28.5 (8.4 - 99.6)
Folate, RBC (nmol/L)	1032 (397 - 2353)[c]	1031 (397 - 2353)	1097 (561 - 2351)[e]	916 (342 - 2460)
Vitamin B12 (pmol/L)	511 (149 - 1147)	508 (149 - 1147)	521.(201 - 1104)	481 (135 - 1232)

CHD, Congenital heart disease; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine, tHcy, total homocysteine; RBC, red blood cell.

[a] All comparisons are adjusted for age, family history of CHD, medication and vitamin use at the study moment.

[b] Values are expressed as medians (range).

[c] *p*-value < 0.05.

[d] *p*-value < 0.001.

[e] *p*-value < 0.01.

TABLE 4 Biomarker concentrations and the risk of CHD

Biomarkers	Cut-off value[a]	CHD / controls	Crude OR (95% CI)	Adjusted OR (95%CI)[b]
SAM (nmol/L)	>123.5	35/28	1.86 (1.01 - 3.26)[c]	1.71 (0.96 - 3.07)
SAH (nmol/L)	>23.1	27/28	1.27 (0.71 - 2.28)	1.12 (0.61 - 2.06)
SAM/SAH	>8.52	27/28	1.33 (0.74 - 2.37)	1.32 (0.72 - 2.40)
tHcy (µmol/L)	>7.8	18/28	0.82 (0.43 - 1.55)	0.71 (0.36 - 1.37)
Folate, serum (nmol/L)	>44.4	36/27	1.86 (1.06 - 3.26)[c]	1.72 (0.96 - 3.09)
Folate, RBC (nmol/L)	>1340.7	30/26	1.58 (0.89 - 2.83)	1.37 (0.75 - 2.52)
Vitamin B12 (pmol/L)	>766	13/27	0.59 (0.30 - 1.20)	0.56 (0.27 - 1.16)

SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine, tHcy, total homocysteine; RBC, red blood cell. OR, odds ratio; CI, confidence interval.

[a] The cutoff values of the biomarker concentrations are calculated from the 85th percentile of the control values.

[b] Adjusted for age, CHD family history, medication and vitamin use at the study moment.

[c] *p*-value < 0.05.

With a similar size of the CHD group and larger control group this finding confirms our previous study in which the same CHD phenotypes were studied (17). Because of the significant correlations between SAM and SAH and the folate status it is likely that the high folate concentrations are responsible for the global hypermethylation status. This is substantiated by the significant correlations observed between SAM, SAH and folate in healthy human by Hirsch *et al.* (22).

Comparisons of the biomarker concentrations of methylation and vitamins in these very young children with that of adults reveals that except for tHcy overall the concentrations are much higher in children, and in particular in those with CHD. The maturation of the metabolic organs, such as the liver and kidney, nutrition and lifestyle change with age and may explain the different levels.

The MTHFR 677C>T and 1298A>C polymorphisms significantly affect the function of the MTHFR protein resulting in a slightly lower folate status and higher tHcy and SAH concentrations. Theoretically a cause of the higher folate status in children with CHD could be a higher frequency of the MTHFR 677CC and or 1298AA genotypes compared with controls. The frequency of these genotypes, however, was lower. Although associations have been shown between the maternal MTHFR genotypes and the risk of Down syndrome, the aberrant genotypes seem not to be more prevalent in the children with Down syndrome (23). In 8 out of 9 carriers of the MTHFR 677TT, the genotype with the strongest effect on folate and homocysteine in blood, the child had an isolated CHD. This contributes to the lower folate status in isolated versus nonisolated CHD. However, differential carriership of the aberrant MTHFR polymorphisms does not explain the global hypermethylation status in nonisolated CHD. If due to nutritional intake and vitamin use the nutritional status would be different between children with CHD and controls, we would have expected lower folate and higher tHcy concentrations in CHD, due to e.g., lower nutritional intake and more medication use. However, we found the opposite even after adjustment for current vitamin and medication use. We could also not explain these higher folate levels by a higher frequency of formula feeding containing folic acid in children with CHD (data not shown).

Vitamin B12 as methyl group acceptor is also involved in the remethylation of tHcy into methionine and subsequently SAM and SAH. In nonisolated nonsyndromic CHD, tHcy was lower compared with controls and the other CHD subgroups. It is likely that this is due to the high vitamin B12 and high folate concentrations which both reduce tHcy. Elaborating on the role of vitamin B12 in methylation, in our previous study we observed a low maternal vitamin B12 concentration and intake that increased CHD risk nearly 2-fold (3, 17). In the current study in children with CHD the vitamin B12 concentration was slightly higher, albeit not significantly, than in controls. These findings are in the same direction as the biomarkers of methylation and the folate concentrations in mothers and children. This may support our speculation of the intrauterine influence of maternal global hypomethylation on the metabolic imprinting of also the folate and vitamin B12 levels of the child.

We cannot state that the biomarkers of methylation and vitamins are an accurate reflection of tissue-specific epigenetic mechanisms. However, several studies indicate that the concentrations of SAM and SAH determined in blood very well reflect tissues concentrations, as well as global DNA methylation (11, 24, 25). We studied a population-based large group of CHD children and controls, and we collected the data in a standardized manner thereby maximizing the validity of the data. All biomarkers in cases and controls were precisely and randomly measured in the same manner. Despite the limitation of the relatively small CHD subgroups, the difference in biomarkers remained significant. It would be interesting, however, to investigate in future studies the separate CHD phenotypes as well. Other strengths are the homogeneity of the CHD phenotypes, the ethnicity in CHD and controls, and the use of a standardized study moment at a relatively early age of 17 months. We selected CHD phenotypes on the basis of evidence from experimental and epidemiological studies showing associations with maternal hyperhomocysteinemia and related gene-environment interactions thereby improving homogeneity in origin and phenotype (6, 9, 26). The standardized study moment is important, because it reduces the misclassification of CHD and controls, since CHD is mostly diagnosed in the first year of life. Moreover, metabolism and nutrition change with age. A prospective preconception study would be most ideal to perform. However, this is not yet feasible because of the large population needed to include enough CHD and the accompanied high costs.

In conclusion, we show a status of global hypermethylation in very young children with nonisolated CHD, in particular nonisolated syndromic CHD, compared with controls. Further research is warranted to elucidate if the increased biomarker concentrations of methylation and folate are due to an (epi)genetic disorder.

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Genetic polymorphisms,

lifestyle, and nutrients

Chapter

TWO MTHFR POLYMOPHISMS MATERNAL B VITAMIN IN-TAKE AND CONGENITAL HEART DEFECTS

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ABSTRACT

Background: The 5,10-methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms are associated with complex congenital malformations. Whether these polymorphisms are associated with congenital heart defects (CHD) is not clear.

Objective: We studied both MTHFR polymorphisms, folate and vitamin B2 by maternal food intake and supplements, and CHD risk.

Design: 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 standardized questionnaires on periconception 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 analyzed by logistic regression analysis and odds ratios (OR) and 95% confidence intervals (CI) were calculated. For the interaction analysis the dominant model was used.

Results: The risk estimates for the MTHFR 677 CT genotypes were 1.4 (0.9-2.0) in mothers, 1.1 (0.8-1.6) in fathers and 1.2 (0.8-1.7) in children and for the MTHFR 677 TT genotypes 0.9 (0.6-1.2), 1.4 (1.0-1.9) and 1.0 (0.7-1.3), respectively. The MTHFR 1298 CC genotype in fathers and the MTHFR 1298 AC genotype in children significantly reduced CHD risk, 0.6 (0.5-0.9) and 0.6 (0.4-0.9), respectively. Of interest is the significant interaction (*p*-value = 0.008) towards a nearly 2-fold increased risk in mothers carrying the MTHFR 1298 C allele and using a periconception folic acid supplement.

Conclusions: The MTHFR C677T and A1298C polymorphisms are not strong risk factors for CHD.

INTRODUCTION

Congenital heart defects (CHD) are the most frequent birth defects and account each year for over one million affected newborns worldwide (1). Genetic risk factors for CHD can be identified in the mother, father and the child by a candidate gene approach based on the molecular biological 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 organ development of the unborn child as well.

Periconception use of multivitamins seems to contribute to the prevention of CHD whereby folic acid may be the key compound (2, 3). The protective effect seems most distinct for outflow tract defects and ventricular septal defects (4). Evidence from experimental studies reveals that during early embryogenesis, hyperhomocysteinemia deranges the migration of neural crest cells from the neural tube to the tissues forming the cardiac outflow tract (5, 6). Low folate and low vitamin B2 intakes result in a mild hyperhomocysteinemia (7). In addition, genetic factors are also implicated in the folate-homocysteine pathway. The 5,10-methylenetetrahydrofolate reductase (MTHFR) 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 (8). The association between the MTHFR C677T polymorphism and CHD risk is not clear. Several studies investigated other neural crest related malformations, such as cleft lip with or without cleft palate, and found small but strong interactions between the MTHFR C677T polymorphism and periconception folic acid use, but also in combination with a low food folate intake (9, 10). Only two studies reported on the association between the MTHFR A1298C polymorphism and CHD in Norway and Italy (11, 12).

Therefore, we hypothesize that the functional MTHFR C677T and A1298C polymorphisms in the mother and/or the child result in a higher risk of CHD either independently or in combination with low periconception intake of folate and vitamin B2. Since it is not yet feasible to study this hypothesis in a preconception 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 periconception period, because food intake is stable during life except for periods of dieting, breastfeeding and extreme growth (13, 14). The study was performed in an ethnically homogeneous case-control family study in the Netherlands.

MATERIALS AND METHODS

Subjects

The HAVEN study, a Dutch acronym for the investigation of genetic and environmental factors in the etiology and prevention of CHD, was conducted from June 2003 onwards and coordinated by the project team of the Department of Obstetrics and Gynecology/Division of Obstetrics and Prenatal Medicine of Erasmus MC, University Medical Center in Rotterdam, the Netherlands. The methods for this study are extensively described previously and are summarized here (15). The study populations consisted of case and control family triads (child, mother, and father). All are living in the western part of the Netherlands. Cases were derived from four University Medical Centers and controls were enrolled in collaboration with child health centers in the Rotterdam area. These child health centers are typically Dutch where all newborns are regularly checked on growth development and health by physicians specialised in child health care. We used a standardized study moment of approximately 17 months after pregnancy and all children were between 11 and 18 months old.

For analysis, we only included families of which DNA was available. These families comprised 229 children affected with a CHD and their parents, indicated as cases, case mothers and case fathers, respectively. The CHD phenotypes were tetralogy of Fallot (n = 24), transposition of the great arteries (n = 42), atrioventricular septal defect (n = 21), perimembranous ventricular septal defect (n = 62), coarctation of the aorta (n = 22), aortic valve stenosis (n = 6), pulmonary valve stenosis (n = 44) and hypoplastic left heart syndrome (n = 8). The included phenotypes were originally selected because experimental and epidemiological studies have shown that hyperhomocysteinemia and related geneenvironment interactions are involved in their etiology (5,16 17). The pediatric cardiologist in each center confirmed the diagnoses by echocardiography and/or cardiac

catheterization and/or surgery.

Nonmalformed children and both parents (n = 251) served as controls. Controls were excluded if they had a congenital malformation or chromosomal abnormality ascertained by their physician at the child health center. 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.

Data collection

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) covering the food intake the month before the study moment. All questionnaires were reviewed and checked for completeness and consistency by the researcher at the hospital visit. The general questionnaires were subdivided in parts concerning two different periods in time. The first period referred to the periconception period, which is defined as 4 weeks before until 8 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 periconception period. Inconsistent users or mothers who used supplements only during a part of the periconception period were considered as nonusers. 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, ethnicity, educational level, family history of CHD 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) (18). With regard to ethnicity, we classified our participants into three different groups: Dutch natives (n = 426), European others (n = 55) and nonEuropeans (n = 95) (19). 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 nonEuropean group from further analysis.

The standardized and validated FFQ comprised of questions concerning maternal dietary intake of the month before the study moment (20). 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.

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 8 cases, 2 case fathers and 1 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 and the A1298C polymorphism in the MTHFR gene from isolated DNA using real-time polymerase chain reaction (PCR) (Taqman®, Applied Biosystems, Foster City, CA, USA). PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) with Hinf1 digestion was used in 8 individuals of whom only buccal swaps were available. These results were checked by 2 blinded observers. For every 90 genotyped individuals we used 3 individuals as positive controls since their genotype was already known and proven by sequencing. Moreover, we checked the data for Mendelian inconsistencies, which resulted in the exclusion of 10 individuals. The genotyping success rate was 100%.

Statistical 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. Using the Chi-square test, we tested deviation of the genotype frequencies from those expected under Hardy Weinberg Equilibrium for cases and controls separately.

Allele and genotype frequencies were compared between cases and controls. Odds ratios (OR) with

TABLE 1 General characteristics.

	Mothers		Fathers	
	Cases (*n* = 230)	**Controls (*n* = 251)**	**Cases (*n* = 230)**	**Controls (*n* = 251)**
Periconception[a]				
Supplement use [*n* (%)][b]	125 (54)	149 (59)	32 (14)	33 (13)
Study moment				
Age (yrs)	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)
Parity >1	129 (56)	130 (52)	-	-
Daily dietary intake of	**Cases (*n* = 175)**	**Controls (*n* = 210)**		
Total energy (MJ)	8.4 (7.3 - 10.2)	8.6 (7.3 - 10.3)	-	-
Folate (µg)	157 (118 - 200)	165 (129 - 198)	-	-
Adjusted Folate (µg)[e]	152 (128 - 186)	160 (134 - 191)	-	-
Vitamin B2 (mg)	1.4 (1.0 - 1.6)	1.4 (1.1 - 1.6)	-	-
Adjusted Vitamin B2 (mg)[e]	1.3 (1.1 - 1.6)	1.4 (1.1 - 1.5)	-	-
	Children			
Study moment	**Cases (*n* = 230)**	**Controls (*n* = 251)**		
Age (m)	16.8 (15.4 - 20.0)	16.7 (15.3 - 18.4)		
Male gender [*n* (%)]	133 (58)	142 (57)		
Family history of CHD [*n* (%)][f]	20 (9)	11 (4)		
Ethnicity[g]				
Dutch Natives	201 (87)	225 (90)		
European Others	29 (13)	26 (10)		

CHD: congenital heart defect. Values are medians (interquartile range) or number (percentage).

[a] Periconception 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*-value ≤ 0.05 (Mann-Whitney U test).

[d] Categorized as low (primary/lower vocational/intermediate secondary), intermediate (higher secondary/intermediate vocational) or high education (higher vocational/university) (18).

[e] Energy-adjustment by the residual method of Willett (21).

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

[g] 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 (19).

TABLE 2 Distribution of the MTHFR C677T and A1298C genotypes

Genotypes	**Cases**	**Controls**	**OR (95% CI)**
	Mothers		
MTHFR C677T	(*n* = 230)	(*n* = 251)	
TT	22 (9)	36 (14)	0.9 (0.6-1.2)
CT	117 (51)	104 (42)	1.4 (0.9-2.0)
CC	91 (40)	111 (44)	1.0 (Reference)

95% confidence intervals (CI) were computed for the associations of case-control status and genotype using the additive model in a univariate logistic regression analysis. We used the dominant model for both MTHFR polymorphisms in the interaction analyses to increase power. Moreover, we performed a family based association test (FBAT) that compares transmission frequencies of a given allele with the assumption of random transmission, and a haplotype analysis.

We excluded all pregnant (n = 50) and lactating women (n = 12) as well as those who reported a different diet at the study moment compared with the periconception period (n = 24) in order to analyze 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 case mother pairs and 210 control mother pairs. 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 (21). Gene-environment interactions between the MTHFR genotypes and periconception supplement use and between the MTHFR genotypes and (energy adjusted) dietary intake were tested under the multiplicative assumption using a multivariable logistic regression model. For these analyses, dietary intakes of folate and vitamin B2 were dichotomized into low and high intakes. The cut-off values were based on the median intakes of the control mothers. We used the MTHFR 677 CC and MTHFR 1298 AA genotypes as the reference categories. All analyses were performed with SPSS for Windows software (version 11.5; SPSS Inc., Chicago, IL, USA) except for the transmission disequilibrium and haplotype tests, which were performed with FBAT (version 2.0.2C for Windows; free available at http://www.biostat.harvard.edu/~fbat/default.html) (22). An additional analysis of the hybrid design that incorporates both transmission disequilibrium and casecontrol analyses was performed using the LEM software program (version 1.0 for Windows; free available at http://www.uvt.nl/faculteiten/fsw/organisatie/departementen/mto/software2.html (23, 24). This method was also used to test for parental imprinting effect.

RESULTS

In *Figure 1* the flowchart of the study population is shown. General characteristics of case and control-triads both at the study moment and in the periconception 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 containing supplements or dietary intake of folate and vitamin B2 and CHD risk. Other potential confounders, such as education, 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 CHD was twice as high in cases as in controls.

Table 2 presents the genotype frequencies of the MTHFR polymorphisms. The MTHFR 677 T-allele frequencies were respectively 35%, 34% and 34% in case mothers, case fathers and case children and 25%, 28% and 31% in control mothers, control fathers and control children. The MTHFR 1298 C-allele frequencies in the case triads were respectively 33%, 30% and 31% and in the control triads 33%, 39% and 36%. Genotype distributions for case and control groups separately were in Hardy Weinberg Equilibrium. Univariate logistic regression analysis was performed to test for the independent effects of the MTHFR genotypes. Case fathers with the MTHFR 677 TT genotype had an OR (95% CI) of 1.4 (1.0-1.9). Overall, more case mothers, case fathers and cases than controls carried the MTHFR 677 CT or TT genotypes, although these OR were not significantly different. The MTHFR A1298C polymorphism in case mothers was not significantly associated with CHD risk. However, the MTHFR 1298 AC and CC genotypes in case fathers showed a significantly reduced CHD risk (p-value=0.006). The MTHFR 1298 AC and CC genotype in cases showed a comparable significant effect (p-value=0.024). The transmission disequilibrium and haplotype tests revealed no distortions in allele transmissions (data not shown). The log-linear likelihood approach confirmed the results of the logistic regression. Imprinting effects were also analyzed and did not yield any significant results (data not shown).

The periconception use of folic acid containing 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 the genotypes of the mother or the child and maternal periconception supplement use and between the genotypes and maternal (energy-adjusted) dietary intake of folate and vitamin B2. In *Figure 2*, the risk estimates are shown for the MTHFR A1298C polymorphism in the mother and child, which are stratified for periconception supplement use of the mother. The OR (with 95% CI) of the mothers carrying the MTHFR AC and CC genotypes in the supplemented versus the nonsupplemented

TABLE 2 Distribution of the MTHFR C677T and A1298C genotypes

Genotypes	Cases	Controls	OR (95% CI)
MTHFR A1298C	(*n* = 230)	(*n* = 251)	
CC	24 (10)	31 (12)	0.9 (0.7-1.3)
AC	102 (45)	104 (42)	1.1 (0.7-1.6)
AA	104 (45)	116 (46)	1.0 (Reference)
Fathers			
MTHFR C677T	(*n* = 229)	(*n* = 251)	
TT	30 (13)	19 (8)	1.4 (1.0-1.9)
CT	94 (41)	103 (41)	1.1 (0.8-1.6)
CC	105 (46)	129 (51)	1.0 (Reference)
MTHFR A1298C	(*n* = 228)	(*n* = 251)	
CC	19 (8)	37 (15)	0.6 (0.5-0.9)
AC	98 (43)	123 (49)	0.7 (0.4-0.96)
AA	111 (49)	91 (36)	1.0 (Reference)
Children			
MTHFR C677T	(*n* = 229)	(*n* = 251)	
TT	27 (12)	25 (10)	1.1 (0.8-1.5)
CT	103 (45)	107 (43)	1.2 (0.8-1.7)
CC	99 (43)	119 (47)	1.0 (Reference)
MTHFR A1298C	(*n* = 229)	(*n* = 251)	
CC	27 (12)	25 (10)	1.0 (0.7-1.3)
AC	90 (39)	129 (51)	0.6 (0.4-0.9)
AA	112 (49)	97 (39)	1.0 (Reference)

MTHFR, methylenetetrahydrofolate reductase; CHD, congenital heart defect; OR, odds ratio; CI, confidence interval. Values are number (percentage).

group was 1.8 (1.01-3.1) versus 0.6 (0.3-1.1), respectively. This interaction was found significant in a multivariable logistic regression model (*p*-value=0.008). No significant interaction was found for the MTHFR A1298C polymorphism in the child and periconception folic acid containing supplementation, with OR of 0.9 (0.5-1.6) and 0.5 (0.3-0.99) in respectively the supplemented and nonsupplemented group. 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).

DISCUSSION

In this case-control family study of an ethnically homogeneous European population, we examined the impact of two common polymorphisms in the MTHFR gene on CHD risk. The genetic polymorphisms were analyzed, both independently and in combination with maternal periconception vitamin supplement use and dietary intake of B vitamins. We demonstrated that the MTHFR 1298 C-allele of the father and the child decreased CHD risk by 40% and 30% respectively. Of interest is the nearly 2-fold increased CHD risk of the MTHFR 1298 C-allele in mothers and periconception folic acid supplementation.

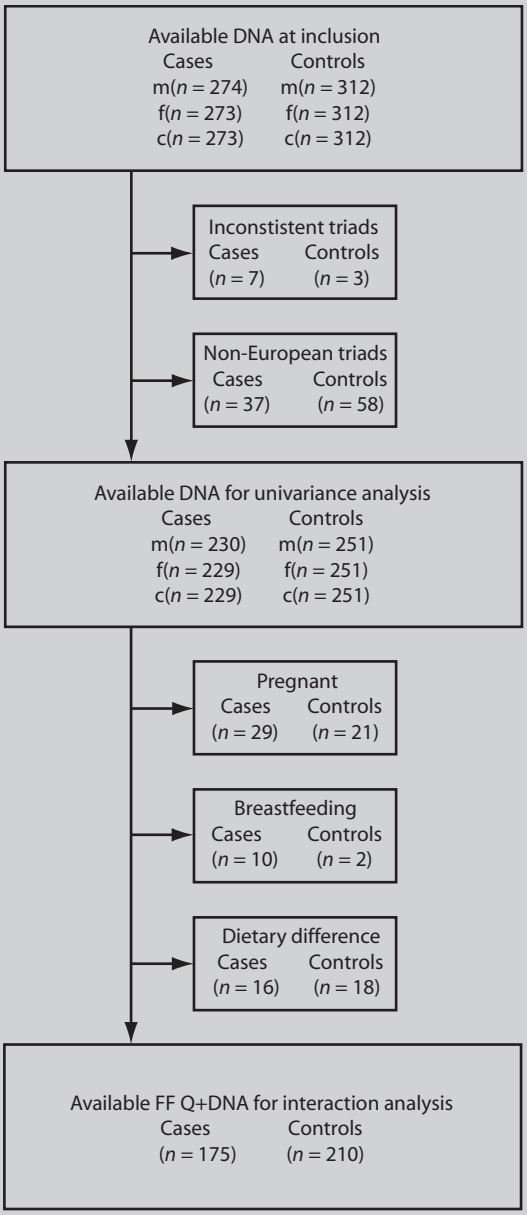
So far, four studies were performed on the association between the MTHFR A1298C polymorphism and CHD risk. In two studies a similar case-control family design was used but no significant association was found between the MTHFR A1298C polymorphism in the mother and the risk of CHD offspring. This might have been due to the small sample sizes of 103 (12) and 25 cases (11), ethnic heterogeneity and differences in the included phenotypes. In two case-only family studies the linkage approach was used, in which only the heterozygous case parents are informative (25, 26). McBride *et al*. (26) did not show a significant association between the MTHFR A1298C polymorphism in the mother and CHD risk. Hobbs *et al*. (25), however, demonstrated a protective effect of the fetal MTHFR 1298 C-allele on CHD risk. Moreover, they confirmed the linkage 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. These results are in line with our finding of the significant association between the MTHFR 1298 C-allele of the child and reduced CHD risk. The significant reduced CHD risk of the paternal MTHFR 1298 C-allele is herewith in line and may suggest that genetic imprinting is involved. However, the additional log-linear approach did not demonstrate imprinting effects (27, 28). Therefore, the paternal role requires further investigation.

The MTHFR 1298 C-allele showed also a strong and significant inverse association between the MTHFR 1298 C-allele and colon cancer (29, 30). Previous research suggests that the MTHFR 1298 CC genotype causes a decreased activity of the MTHFR enzyme (31). The MTHFR enzyme determines the balance between the different forms of folate for DNA synthesis and DNA methylation (32). Therefore, a decreased MTHFR enzyme activity inhibits the conversion of 5,10-methylenetetraydrofolate to 5-methyltetrahydrofolate, resulting in a surplus of 5,10-methylenetetrahydrofolate that leads to more incorporation of thymidine than uracil in 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 CHD. In addition, a low MTHFR enzyme activity might lead to DNA hypomethylation, which silences gene expression (33). Therefore, we speculate that pathologically expressed genes implicated in cardiogenesis are silenced due to the reduced MTHFR activity. From this view we would expect an association with MTHFR C677T and not MTHFR A1298C. Therefore, these results should be carefully interpreted and further studies with larger sample sizes are warranted. We realize that selective survival could have diluted our findings. Particularly of interest is that Reyes-Engel *et al*. observed an increased frequency of the MTHFR 677 T and 1298 C-allele in children, which coincided with a generally increased folate intake by pregnant women. This genetic selection may lead to a higher frequency of mutated individuals in the population (34).

The interaction analysis showed an interesting opposing effect of the maternal MTHFR A1298C genotype in supplemented and nonsupplemented mothers. A protective effect of the MTHFR 1298 C-allele is shown in nonsupplemented mothers, which is in line with the demonstrated independent protective effects of the MTHFR 1298 C-allele in fathers and children. This effect is also present in children with the MTHFR 1298 C-allele in the nonsupplemented group (OR of 0.5 (0.3-0.99). An additional analysis revealed that periconception folic acid use increased CHD risk in MTHFR 1298 C-allele carriers only. This is in contrast to the studies showing a beneficial effect of folic acid supplementation on CHD risk. Nevertheless, folic acid at a high dose may not always be beneficial when interacting with certain pathologically expressed genes (35), but this finding requires further confirmation.

No significant associations were observed between CHD risk and the MTHFR C677T polymorphism, neither independently nor in combination with maternal periconception supplement usage or dietary intake of folate

FIGURE 1 Flowchart of the study population



M, mothers; f, fathers; c, children; FFQ, food frequency questionnaire.

Inconsistent non-European triads were excluded from the families 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 reported to have used a different diet at the study moment than in the periconception 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.

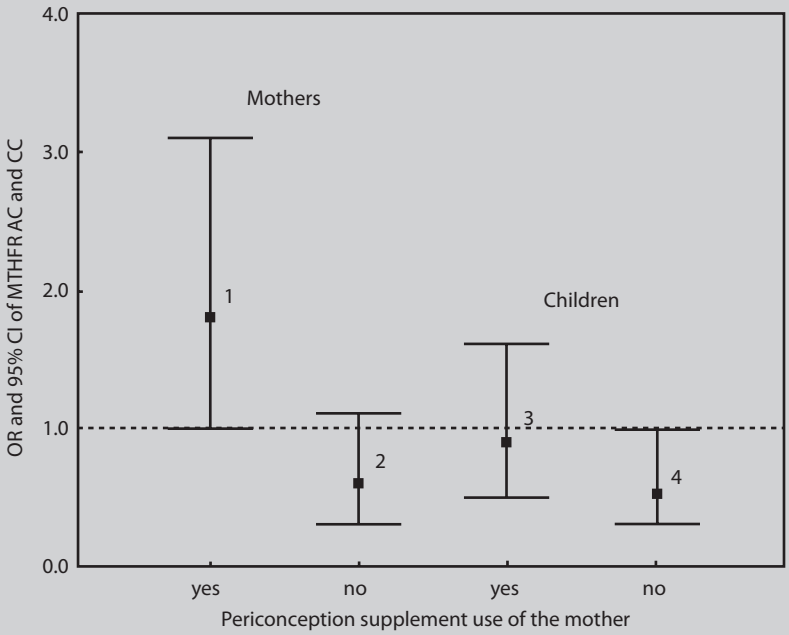
or vitamin B2. The meta-analysis by Verkleij-Hagoort *et al*. revealed an overall OR of 1.0 (0.8-1.3) in mothers and 1.1 (0.9-1.5) in children for the MTHFR C677T polymorphism and CHD risk, thereby supporting our finding (36).

We have to consider some 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 enrolled in the study, because they did not know their genotype on beforehand. Moreover, the allele frequencies in controls were comparable with the allele frequencies in the European population (11). Secondly, nondifferential misclassification might have occurred, because periconception 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 and Jacques who showed that differential misclassification is rare in case-control studies (37). Moreover, participants were unaware of the detailed study objectives. Because of small sample sizes the individual phenotype analysis was not possible. Nevertheless, we performed a subgroup analysis of outflow tract defects and non-outflow tract defects showing that the independent effects of the MTHFR polymorphisms were similar to the total group (data not shown). Other limitations that we have to consider are multiple testing and possibility of false positive results. It would therefore be very interesting to confirm our findings in a very large database.

Our study has also several strengths. The large sample size of 230 cases and 251 controls enabled us to detect a 40% significant risk reduction of the gene only, 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 also based on a controlled and standardized study design, which has successfully been used for many times in the investigation of gene-environment interactions in other complex malformations (15, 38, 39). 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 at the beginning of pregnancy and two to four years later. In general, no differences occur in the dietary patterns between the periconception period and one year postpartum (13, 14). Moreover, at this study moment most of the CHD diagnoses are completed. Thereby, the risk of undiagnosed and less severe CHD in the control group is minimized and misclassification is reduced. In contrast to other countries, such as the United States of America and Australia (voluntary), the food in the Netherlands is not yet fortified by folic acid, which assures the reliability of the estimation of the true dietary folate intake. Furthermore, to increase the homogeneity of the study population, we included only Dutch natives and European families.

In conclusion, our findings suggest that the MTHFR 1298 C-allele of fathers and children might be protective for the investigated CHD. Of interest is the observed interaction between the maternal MTHFR A1298C polymorphism and periconception folic acid supplementation. Future studies with larger sample sizes should be performed to confirm our findings and to investigate the maternal–fetal interactions with regard to the most favorable balance between DNA synthesis and DNA methylation, thereby affecting fetal cardiovascular embryogenesis.

FIGURE 2 Stratified interaction between the MTHFR A1298C genotype in mothers or children and periconception supplement use of the mother.



OR, odds ratio; CI, confidence interval.

MTHFR 1298 AA genotype was used as reference category. Supplement use in the periconception 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. A significant interaction was found between maternal periconception supplement use and genotype of the mother (*p*-value=0.008).

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Chapter

7

GENETIC AND LIFESTYLE FACTORS RELATED TO THE PERICONCEPTION VITAMIN B12 STATUS AND CONGENITAL HEART DEFECTS: A DUTCH CASE-CONTROL STUDY

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ABSTRACT

Background: Maternal hyperhomocysteinemia is associated with congenital heart defects (CHD) in the offspring. A low periconception vitamin B12 status is determined by genetic and lifestyle factors and causes hyperhomocysteinemia.

Objective: We investigated methionine synthase reductase (MTRR) and transcobalamin II (TC) genes and maternal intake and serum concentrations of vitamin B12 in association with CHD risk.

Design: Seventeen months after the index-pregnancy, we studied 230 children with a CHD and 251 nonmalformed children and their parents. Data were collected on current and periconception 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 maternal serum vitamin B12 concentrations. Transmission disequilibrium tests and univariate and multivariate analyses were applied. Allele transmissions were not significantly distorted.

Results: The MTRR and TC genotypes did not significantly affect CHD risk. Neither polymorphisms in mothers and/or children revealed significant interactions nor in combination with low vitamin B12 intake. Low maternal serum vitamin B12 combined with the maternal or child's MTRR 66 GG genotype resulted in odds ratios 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 and children revealed risk estimates of 2.2 (0.7–7.1) and 1.9 (0.5–7.4), respectively.

Conclusions: The MTRR 66 GG and TC 776 GG genotypes in mothers and children may contribute to the risk of CHD, particularly when the maternal vitamin B12 status is low. The future enlargement of our sample size might demonstrate significant associations.

INTRODUCTION

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

We and others demonstrated a strong association between maternal hyperhomocysteinemia 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 among others 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 hyperhomocysteinemia (7,8). A part of the preventive effect of periconception folic acid supplementation on the occurrence of CHD and other malformations can be explained by the normalization of low folate and moderate homocysteine 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 hyperhomocysteinemia 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 transcobalamin I, also known as R-binder or haptocorrin. Approximately 10–25% of vitamin B12 in blood is bound to transcobalamin II (TC), which is the biologically active fraction required for the cellular uptake of vitamin B12 (13).

The biochemical phenotypes of the MTRR A66G polymorphism (EC 1.16.1.8) result from altered affinities for the redox partner, methionine synthase (EC 2.1.1.13). Olteanu *et al*. revealed that there are subtle changes in the redox properties of the flavin cofactors in the polymorphic variant. However, compared with the large thermodynamic barrier for electron transfer to methionine synthase, it is unlikely that the relatively small redox changes have a significant effect on the biochemical phenotypes (14). The MTRR polymorphism presumably exerts only a mild effect on the homocysteine metabolism, considering the common occurrence of the A66G substitution in healthy human subjects.

Regarding the TC C776G polymorphism (dbSNP rs1801198, MIM #275350), the data of Namour *et al*. show that TC C776G phenotypic variability is a multifactor-dependent phenomenon that includes a specific cell folding of TC (15). Disturbed folding of TC might lead to impaired binding of vitamin B12 to TC (16). Alternative signal peptide splicing is another proposed mechanism for the genetic basis of the TC phenotype, but it is less likely. In summary, the TC polymorphism is associated with a reduced cellular expression level of TC in relation with a decreased level of transcripts and a reduced concentration of plasma TC (15).

So far, few studies showed a significant effect of these MTRR and TC polymorphisms on 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-value < 0.05) (17). Others observed hyperhomocysteinemia in TC 776 CG heterozygotes (15) and reported 15% (p-value = 0.021) (18) and 39% (p-value = 0.002) (16) lower transcobalamin concentrations for the GG genotype compared with the CC genotype.

A low maternal vitamin B12 status is associated with a higher risk of neural tube defects (NTD) (19,20) and orofacial clefts (21). 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 (22). 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. During 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.

From this background, the aim of the current study was to investigate: (1) associations between MTRR A66G and TC C776G genetic polymorphisms and CHD risk, and (2) the effects of periconception vitamin B12 intake on biochemical determinants of the homocysteine pathway.

MATERIALS AND METHODS

Subjects

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 = 33), transposition of the great arteries (n = 46), atrioventricular septal defect (n = 27), perimembranous ventricular septal defect (n = 75), coarctation of the aorta (n = 23), 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 catheterization and/or surgery. The eligible 312 control children 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 center.

Families visited the hospital at the standardized study moment of around 17 months after the index-pregnancy. This study moment is based on the fact that in general, metabolism and lifestyle factors including nutritional habits are rather constant and do not change except for periods of illnesses, dieting and increased needs during pregnancy and breastfeeding (24). Moreover, most congenital malformations are diagnosed in the first year of life. Therefore, a study moment between 11 and 18 months after the index-pregnancy best mimics the preconception maternal biochemical status and lifestyle, thereby reducing information and recall bias as well as the misclassification of undiagnosed less severe CHD in the control group (20,21,25).

The Central Committee on Research in Humans, The Hague, the Netherlands and the Medical Ethics Committees of the participating hospitals approved the study protocol and written informed consent was obtained from every parent.

Data collection

At the study moment, the general characteristics, maternal food frequency data and lifestyle exposures were collected by questionnaires. The data comprised 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 nonEuropeans (26). Educational level was categorized according to the definitions of Statistics Netherlands (27).

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 of the mother. This includes the intake during the periconception period of the index-pregnancy as well. We used a validated semiquantitative food frequency questionnaire (FFQ) (28). In a standardized manner, the questionnaire data were checked for completeness and consistency by the researcher during the hospital visit. Standardized 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 vitamin supplements, tobacco and alcohol, which were evaluated both at the study moment and during the periconception period. The periconception period was defined as four weeks before until eight weeks after conception. Vitamin supplements were used daily and included supplements containing folic acid only or multivitamins.

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 from 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, WI, USA). The MTRR A66 G (rs1801394) and TC C776G (rs1801198) polymorphisms were analyzed by the Taqman_ system, according to protocols provided by the manufacturer (Taqman, Applied Biosystems, Foster City, CA, USA). Approximately 3% of the samples were re-genotyped to check for genotype calling consistency. The genotyping success rate was more than 96%.

TABLE 1 Maternal characteristics and lifestyle factors at the study moment and during the periconception period

	Cases (*n* = 230)	Controls (*n* = 251)
Study moment		
Maternal age (y)	33.5 (30.6 - 36.7)	32.8 (29.2 - 35.4)[a]
Time after index-pregnancy (m)	16.8 (14.4 - 20.0)	16.7 (15.3 - 18.4)
Body mass index (kg/m^2)	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* (%)]		
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)
Periconception period[g]		
Use of [*n* (%)]		
Vitamin supplements	125 (54)	149 (59)
Tobacco	46 (20)	57 (23)
Alcohol	94 (41)	97 (39)

CHD, congenital heart defect. Values are median (interquartile range) or number (percentage).

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

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

[c] CHD in family members of the first, second or third degree.

[d] Vitamin supplements contained folic acid only or multivitamins.

[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 periconception period are excluded.

[f] Energy-adjustment by the residual method (29).

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

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 (29). 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 periconception period to minimize 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 analyze transmission of the parental MTRR 60 G-allele and the TC 776 G-allele in case families (30). 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 periconception vitamin B12 intake by food and periconception vitamin supplement use in association with CHD risk. Because the use of folic acid supplements only and multivitamins was not significantly different between case mothers and control mothers, we did not separate out these groups in the further analyses. We used a recessive model for the genetic analyses (16,18,31,32). Odds ratios (OR) 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 the reference value (wildtype or heterozygous genotype in combination with normal vitamin B12 intake) to the highest 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 periconception vitamin supplement use.

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 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, IL, USA) with an exception for the transmission disequilibrium test (30).

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%, nonEuropeans 14%) and controls (73%, 8% and 19%), respectively. However, the distributions of the genetic polymorphisms were significantly different in nonEuropeans compared with Dutch natives and Europeans, particularly for MTRR genotypes in control mothers, case and control fathers and case children. Therefore, nonEuropean families (n = 95) were excluded from further analysis, which resulted in an ethnic more homogeneous dataset of 230 case and 251 control families.

In *Table 1*, the general characteristics and lifestyle factors are presented of 230 case mothers and 251 control mothers. The median age of the case mothers was 0.7 years higher than of the controls (p-value = 0.042). The time after the index-pregnancy, body mass index and educational level were comparable in both groups. There were no significant differences in the use of vitamin supplements and tobacco at the study moment. However, compared with cases, 10% more control mothers used alcohol (p-value = 0.026). After exclusion of the pregnant and lactating mothers and those with a changed diet compared with the periconception period, the analyses of energy and vitamin B12 intake and biomarkers were based on 175 case mothers 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 vitamin supplements, tobacco and alcohol was comparable between cases and controls. In the periconception period, there were no significant differences either concerning the use of 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

TABLE 2 Distribution of the MTRR A66G and TC C776G genotypes of the families and the association with CHD risk

Genotype		Mothers Cases/controls		Fathers Cases/controls		Children Cases/controls	
		(*n* = 230/251)	OR (95% CI)	(*n* = 229/251)	OR (95% CI)	(*n* = 229/251)	OR (95% CI)
MTRR 66	AG/GG	181/204	1.0 (0.7 - 1.5)	196/206	1.3 (0.9 - 1.9)	191/199	1.3 (0.9 - 1.8)
	AA	49/50	1.0 (Reference)	33/45	1.0 (Reference)	38/52	1.0 (Reference)
TC 776	CG/GG	147/167	1.0 (0.6 - 1.5)	148/156	1.0 (0.6 - 1.6)	139/163	0.9 (0.6 - 1.5)
	CC	74/71	1.0 (Reference)	77/86	1.0 (Reference)	79/83	1.0 (Reference)

CHD, congenital heart defect; OR, odds ratio; CI, confidence interval.

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

	Case mothers				Control mothers			
MTRR A66G	**All (*n* = 137)**	**AA (*n* = 26)**	**AG (*n* = 68)**	**GG (*n* = 43)**	**All (*n* = 203)**	**AA (*n* = 40)**	**AG (*n* = 102)**	**GG (*n* = 61)**
Folate, serum (nmol/L)	15.3 (12.0 - 19.4)	14.2 (11.3 - 18.4)	15.0 (11.3 - 18.4)	15.5 (14.1 - 21.5)	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)	676 (533 - 802)	645 (494 - 880)	676 (543 - 813)	697 (536 - 777)[a]	670 (533 - 819)	728 (565 - 1044)	645 (521 - 793)	671 (537 - 770)
B12 (pmol/L)	276 (222 - 366)	284 (212 - 343)	274 (225 - 372)	272 (185 - 397)	249 (202 - 359)	241 (205 - 367)	266 (206 - 370)	245 (187 - 319)[c]
tHcy (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)	10.3 (8.4 - 12.1)	10.1 (8.4 - 11.7)[c]	10.0 (8.4 - 12.2)	10.7 (8.4 - 12.3)[c]
TC C776G	**All (*n* = 130)**	**CC (*n* = 45)**	**CG (*n* = 58)**	**GG (*n* = 27)**	**All (*n* = 193)**	**CC (*n* = 54)**	**CG (*n* = 94)**	**GG (*n* = 45)**
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)	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)	678 (536 - 798)	679 (550 - 821)	682 (521 - 836)	640 (539 - 770)	670 (533 - 808)	673 (530 - 773)	639 (531 - 792)	702 (543 - 948)
B12 (pmol/L)[d]	278 (222 - 371)	285 (238 - 420)	299 (235 - 378)[d]	234 (171 - 272)[d]	253 (202 - 364)	239 (195 - 334)	249 (201 - 341)[e]	281 (203 - 420)
tHcy (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)	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]

RBC, red blood cell; B12 serum vitamin B1; tHcy, plasma total homocysteine.

[a] RBC folate, *n* = 42

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

[c] GG vitamin B12, *n* = 60; AA tHcy, *n* = 41; GG tHcy, *n* = 60.

[d] *p*-value = 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.

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

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% 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*-value = 0.23). For the TC 776 C and G-allele, these data were 107 and 90 times (Chi-square 0.294, *p*-value = 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.

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*-value = 0.042). The MTRR genotypes in case mothers did not significantly affect the folate, vitamin B12 and tHcy concentrations.

Compared with the CC and CG genotypes, the maternal TC 776 GG genotype in cases demonstrated significantly lower vitamin B12 concentrations (*p*-value = 0.006) without a significant change in tHcy concentrations. 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 vitamin supplements, tobacco and alcohol at the study moment, which did not significantly change the biochemical data. The multivariate logistic regression analyses showed no significant interactions between the MTRR and TC genotypes (gene–gene interaction), nor in combination with low maternal periconception vitamin B12 intake by food and vitamin supplements (gene-environment interaction). 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 OR 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

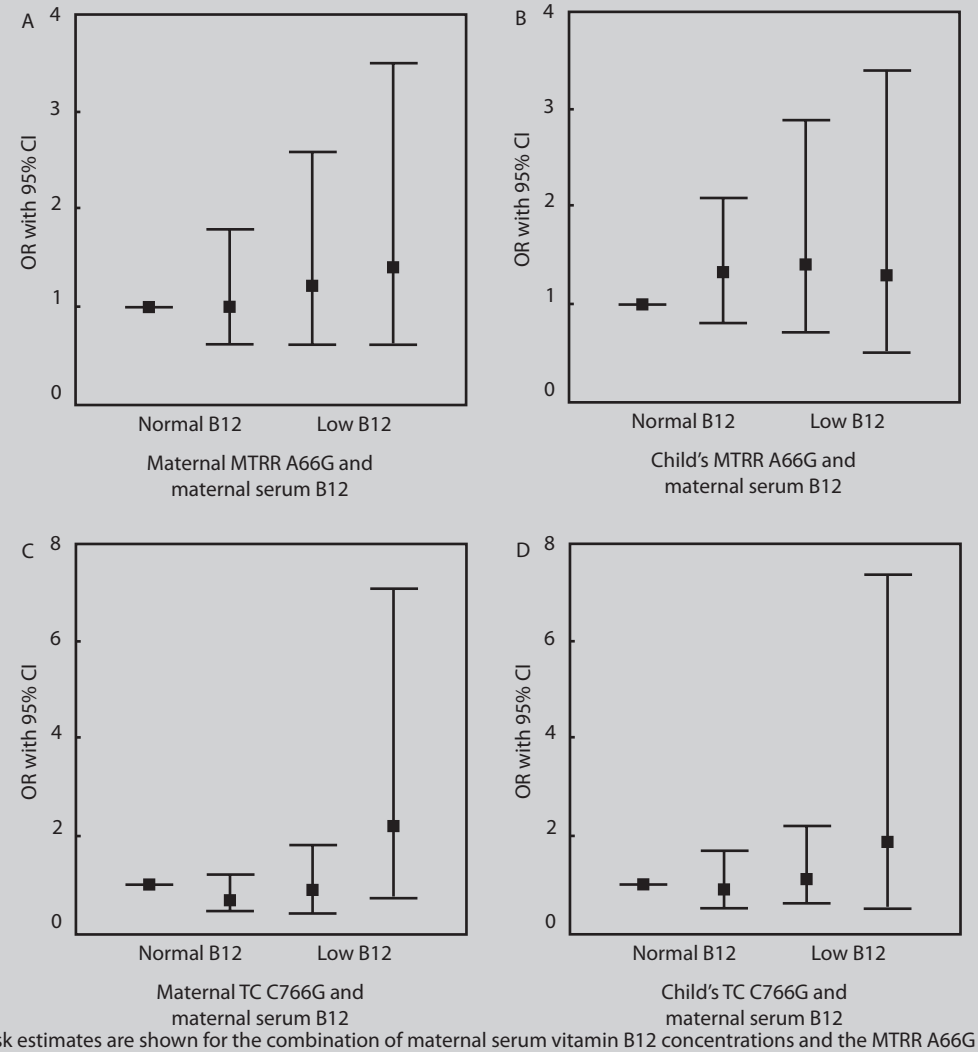
In this case-control study conducted in the western part of the Netherlands, we showed that the MTRR and TC genetic polymorphisms in mothers and children are no independent risk factors for CHD. The maternal TC genotype significantly influenced serum vitamin B12 in case mothers only, which was not due to confounding by the use of vitamin supplements at the study moment. 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 carefully interpreted, because the risks are small and not significant. Nevertheless, they are interesting and should be further investigated in other ethnic groups and countries.

We could not demonstrate a significant association between the MTRR and TC polymorphisms in families and CHD risk. Only one study with a smaller sample size than ours investigated the MTRR genotypes and CHD risk before without significant results as well (33). This is in line with the inconclusive results of studies investigating MTRR and TC polymorphisms in related malformations, such as NTD and orofacial clefting (16,31,32,34–39).

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 finding is supported by others (15,16,18). 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 (16). 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 (18).

The weak trend for hyperhomocysteinemia across the TC genotypes confirms the data of other studies (15,16). 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 (15,16). Thus, this TC polymorphism may affect cellular

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. The cut-off value for a low serum vitamin B12 concentration is the 15th percentile (175 pmol/L) of serum vitamin B12 concentration in control mothers.

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 hyperhomocysteinemia 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 nonsignificant 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. Therefore, we conclude that besides the low maternal vitamin B12 status, the additive effect of the MTRR and TC genotypes on the risk of CHD offspring is small.

A high plasma methylmalonic acid (MMA) 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 MMA concentration was associated with a 3-fold increased CHD risk, albeit not significantly (33). 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 (31,32). In addition, the ethnic background was not clear for 25% of the study population (31) or not described at all (32). This is important information, because differences in allele frequencies of genetic polymorphisms are striking between and within continents (11,31,40). With concern to confounders, Wilson *et al*. excluded vitamin supplement users (31). Van der Linden *et al*. adjusted for significant differences in maternal age, but did not consider use of vitamin supplements at the study moment (32).

One of the strengths of our study is that we standardized 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 10–11 years (31) or even at different time intervals in controls and cases (8 and 16 years after pregnancy) (32). We previously showed the importance of considering the time interval between delivery and the post-partum study moment (41). A standardized 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 periconception period and to reduce recall bias (3,20,21,23,25). An earlier study moment after birth would significantly interfere with the maternal physiology, metabolism and endocrinology. It may 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 hyperhomocysteinemia as well as the inclusion of only Dutch native and European families in the analyses in order to increase the homogeneity of the ethnicity in 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 some limitations of the 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 (42). 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 (43). Finally, we only showed trends towards an association, which may be due to the sample size. Calculations revealed that the sample size of both the case group 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%. Enlargement of the size of the control group up to 3-fold is another option.

In conclusion, our findings suggest that interactions between TC 776 GG and MTRR 66 GG genotypes, and low periconception vitamin B12 status may be involved in the pathogenesis of CHD 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 favorable 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 periconception 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 CHD.

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Chapter

EIGHT-FOLD INCREASED RISK FOR CONGENITAL HEART DEFECTS IN CHILDREN CARRYING THE NICOTINAMIDE N-METHYLTRANSFERASE POLYMORPHISM AND EXPOSED TO MEDICINES AND LOW NICOTINAMIDE

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ABSTRACT

Background: Congenital heart defects (CHD) have a multifactorial origin, in which subtle genetic factors and periconception exposures interact.

Objective: We hypothesize that derangements in the homocysteine and detoxification pathways, due to a polymorphism in the nicotinamide N-methyltransferase (NNMT) gene, low maternal dietary nicotinamide intake, and medicine use in the periconception period, affect CHD risk.

Design: In 292 case and 316 control families, maternal periconception medicine use and low dietary intake of nicotinamide (≤13.8 mg/day) were independently associated with CHD risk [odds ratio (95% confidence interval) 1.6 (1.1–2.3) and 1.5 (1.03–2.3), respectively].

Results: No significant association was found for the NNMT AG/AA genotype in mothers [0.9 (0.7–1.3)], fathers [1.1 (0.8–1.6)], or children [1.1 (0.8–1.6)]. However, the combination of periconception medicine use, low dietary nicotinamide intake, and the NNMT AG/AA genotype in mothers or children showed risk of 2.7 (1.02–8.1) and 8.8 (2.4–32.5), respectively.

Conclusion: Children carrying the NNMT A-allele face additional CHD risk in combination with periconception exposure to medicines and/or a low dietary nicotinamide intake. These findings provide a first set of data against which future studies with larger sample sizes can be compared with.

INTRODUCTION

Congenital heart defects (CHD) are among the most common congenital malformations and are a leading cause of perinatal mortality. Over 85% of CHD have a multifactorial origin, involving genetic factors, maternal nutrition and lifestyle factors during embryogenesis (1).

One important risk factor for CHD is maternal hyperhomocysteinemia, which can be caused by subtle variations in genes, such as methylene tetrahydrofolate reductase (MTHFR) (2) and a low dietary folate intake (3, 4). It is very likely that other nutrients involved in this pathway are implicated as well (5, 6). Furthermore, it is increasingly apparent that medicines in general exert side effects sometimes due to interference with the nutrient status. Interestingly, medicines and nutrients can be metabolized by the same detoxification pathways (7).

Against this background, it is interesting to note that a new candidate gene for hyperhomocysteinemia was identified in a genome-wide study (8). Linkage was shown in chromosomal region 11q23, where the nicotinamide N-methyltransferase (NNMT, E.C. 2.1.1.1) gene is located; it was explained by one single nucleotide polymorphism (SNP) in this gene (dbSNP rs694539, minor allele frequency (MAF) 16.7% in European population). The NNMT enzyme catalyzes the N-methylation of nicotinamide and other pyridines. The methyl group used in this reaction is generated during the conversion of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH); SAM and SAH are both important intermediates in the homocysteine pathway. This NNMT enzyme is important for energy supply, but also for detoxification processes of medicines that undergo methylation via methyltransferases, such as tricyclic antidepressants (9). Nicotinamide (vitamin B3) is a water-soluble B vitamin, essential for energy supply and the substrate for NNMT.

As a mother is the environment of the developing foetus, we hypothesized that the NNMT polymorphism in a mother or child and a low maternal dietary intake of nicotinamide and medicine use in the periconception period may be risk factors for CHD. This hypothesis was tested in a case-control family study in an ethnically homogeneous population in the western part of the Netherlands.

MATERIALS AND METHODS

Subjects

This study is part of the HAVEN study, whose name is a Dutch acronym for the ongoing investigation of genetic and environmental factors in the aetiology and prevention of CHD. From June 2003 this study has included cases from four University Medical Centers and controls in collaboration with the child health centers of 'Thuiszorg Nieuwe Waterweg Noord' in the Rotterdam area. The domain population comprised case children and control children living in the western part of the Netherlands. The materials and methods for this study are described previously and summarized hereafter (10).

In the analyses we included case children and control children with their parents from whom DNA was available. All children were aged between 11 and 18 months, of European origin and no familial relationship existed between cases and controls (11). Successful DNA analysis was performed in 283 case children, 291 case mothers and 292 case fathers. The CHD phenotypes included were tetralogy of Fallot ($n = 30$), transposition of the great arteries ($n = 51$), atrioventricular septal defect ($n = 30$), perimembranous ventricular septal defect ($n = 81$), coarctation of the aorta ($n = 28$), aortic valve stenosis ($n = 6$), pulmonary valve stenosis ($n = 50$) and hypoplastic left heart syndrome ($n = 16$). The selection of the included CHD phenotypes was based on experimental and epidemiological studies that showed that hyperhomocysteinemia and related gene-environment interactions are involved in their etiology (12-14). Two pediatric cardiologists diagnosed the CHD using echocardiography and/or cardiac catheterization and/or surgery data.

DNA was available from 316 control children, 313 control mothers and 314 control fathers. These children had no major congenital malformations or chromosomal abnormalities according to the medical record and regular health checks by physicians of the child health centers. A smaller group was used for the combined analyses, because we excluded mothers who were pregnant or lactating, and those who reported that their diet at the time of the study moment was different from that during the periconception period.

The study protocol was approved by the Central Committee on Research involving Human Subjects and the Institutional Review Boards (Medical Ethics Committees) of all participating hospitals. In addition, written informed consent was obtained from every participant.

Data collection

All parents filled out a general questionnaire about 16 months after the birth of the index child. At this fixed study moment, mothers also filled out a standardized and validated food frequency questionnaire (FFQ) on their food intake over the previous four weeks (15). At the same time, blood or buccal swabs were obtained to extract DNA from all children and their parents. The questionnaires were filled out at home and checked for completeness and consistency by the researcher during the hospital visit.

The general questionnaire referred to two different time periods. The first period was the periconception period, which was defined as four weeks prior to conception until eight weeks after conception. The second period was defined as four weeks before the study moment, i.e. around 16 months after the index pregnancy. We collected sociodemographic characteristics such as age, ethnicity and educational level, and also obtained information on lifestyle factors, such as the use of medicine, alcohol, tobacco and B vitamin supplements in both time periods. Medicine use was defined as any prescribed use of medicine. Overall, the different medicines reported were mainly antibiotics, anticonvulsants, anti-inflammatory medicines, hormones and antimycotics. Women were defined as 'tobacco users' if they reported having used at least 1-10 cigarettes and/or cigars per day. Alcohol use was defined as any use of alcohol. The use of B vitamin supplements in the periconception period was defined as the daily use during the complete period. Inconsistent users or mothers who used B vitamin supplements only during a part of the periconception period were classified as nonusers.

The FFQ filled out by the mothers covered their daily dietary intake over four weeks prior to the study moment, i.e. approximately 16 months after the index pregnancy. The dietary intake collected at this moment is comparable with that in the periconception period. This is supported by others (16, 17). From the FFQ we extracted total energy, dietary nicotinamide and folate intake for analysis. At the hospital visit, maternal weight (weighing scale, SECA, Hamburg, Germany) and height (anthropometric rod, SECA, Hamburg, Germany) were measured.

DNA from mothers, fathers and children was obtained 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). Of 4 case children, 1 case father and 1 control father DNA was isolated from buccal swabs instead of blood samples because of logistical 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). NNMT genotyping was performed using an Assays-on-Demand (SNP ID rs694539, Applied Biosystems, Foster City, CA, USA) allelic discrimination assay on a Taqman 7000 analyzer (Applied Biosystems, Foster City, CA, USA) according to manufacturers' instructions (http://www.appliedbiosystems.com). Polymerase chain reaction (PCR) was performed using 384-well plates. Each genotype plate contained no DNA template (water) controls and a total of 75 randomly chosen duplicate samples. The reproducibility was 100 %.

Statistical analysis

Sociodemographic and lifestyle characteristics both at the study moment and in the periconception period were compared between cases and controls using the Chi-square test for categorical variables and the Mann-Whitney U test for continuous variables. All continuous variables are presented as medians with interquartile range, because some of them were positively skewed even after transformation.

Genotype data were checked for Mendelian segregation errors. Inconsistent triads (9 case triads and 6 control triads) were excluded from analysis. Deviation from Hardy Weinberg equilibrium was tested with the Chi-square test. Univariate logistic regression was used to compute odds ratios (OR) and 95% confidence intervals (CI) for the association between case-control status and the dichotomous variables NNMT polymorphism, medicine use and nicotinamide intake. We used the dominant model by which the NNMT AG/AA genotype group was considered the risk group. Moreover, a subgroup analysis was performed on risk of NNMT polymorphism for each CHD phenotype separately.

Linkage and/or association between the NNMT polymorphism and CHD risk was tested using the family-based association test (FBAT), which looks for distortions in the transmission frequencies of a given allele, compared to the assumed transmission frequencies of random transmission (18). FBAT is attractive because it is robust against population admixture or stratification.

Logistic regression analyses were performed to assess the additive effects of the NNMT polymorphism, medicine use and nicotinamide intake on the risk of CHD. Firstly, we coded separate categories for the risk of the genotype of mother or child in combination with periconception medicine use. NNMT GG carriers without

TABLE 1 Sociodemographic and lifestyle characteristics

	Mothers			Fathers		
Study moment	**Cases *n* = 291**	**Controls *n* = 313**	***p*-value**	**Cases *n* = 292**	**Controls *n* = 314**	***p*-value**
Age (y)	33.2 (30.6-36.4)	32.8 (29.3-35.1)	0.019	35.0 (32.1-38.6)	35.6 (32.1-38.6)	0.906
Body mass index, (kg/m^2)	24.4 (22.0-27.7)	24.1 (22.0-27.1)	0.453			
Educational level[a]			0.235			0.111
Low	65 (22)	54 (17)		69 (24)	70 (22)	
Intermediate	133 (46)	160 (51)		103 (35)	136 (43)	
High	93 (32)	99 (32)		120 (41)	108 (34)	
Use of						
Medicine	57 (20)	60 (19)	0.897			
Alcohol	152 (52)	189 (59)	0.075			
Tobacco	48 (17)	52 (17)	0.969			
B vitamin supplements	210 (72)	226 (72)	0.991			
Daily dietary intake of	***n* = 213**	***n* = 247**				
Total energy (MJ)	8.7 (7.7-10.3)	8.9 (7.5-10.4)	0.698			
Folate (µg)	197 (156-242)	202 (167-239)	0.340			
Nicotinamide (mg)	14.6 (12.6-17.0)	15.3 (13.2-17.8)	0.036			
Periconception						
Use of						
Medicine	86 (30)	69 (22)	0.027	43 (15)	47 (15)	0.933
Alcohol	119 (41)	115 (37)	0.295	247 (85)	260 (83)	0.552
Tobacco	57 (20)	62 (20)	0.946	93 (32)	108 (34)	0.506
B vitamin supplements	129 (44)	129 (42)	0.439	42 (14)	45 (14)	0.985
	Children					
Study moment	***n* = 283**	***n* = 316**				
Age (m)	16.2 (15.0-19.0)	16.1 (15.1-18.0)	0.119			
Male gender	162 (57)	168 (53)	0.316			
Family history of CHD[b]	25 (9)	17 (5)	0.098			
Ethnicity[c]			0.991			
Dutch Natives	258 (91)	288 (91)				
European Others	25 (9)	28 (9)				

Values are medians (interquartile range) or number (percentage).

[a] Categorized as low (primary/lower vocational/intermediate secondary), intermediate (higher secondary/intermediate vocational) or high education (higher vocational/university) (31).

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

[c] 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, or is from European origin and living in the USA, Australia or Indonesia (11).

periconception medicine use were expected to have the lowest risk and therefore considered as the reference category. The highest risk group comprised NNMT AG/AA carriers and periconception exposure to medicine. Secondly, different categories were created of the combined risk of the genotype with maternal dietary nicotinamide intake. Therefore, nicotinamide intake was divided into low and high intake by using the lowest tertile of the control-mothers as a cut-off point (≤ 13.8 mg/day). The different subgroups thus ranged from the combination of the NNMT-GG polymorphism with high maternal dietary nicotinamide intake (reference group) to the NNMT AG/AA polymorphism with low maternal dietary nicotinamide intake. We computed adjusted OR with 95% CI in a multivariable logistic regression model. These OR were adjusted for family history of CHD, maternal age, dietary folate intake, and periconception use of alcohol, tobacco and B vitamin supplements, because they were considered potential confounders.

We also tested multiplicative interaction between NNMT genotype (coded as GG=0 and AG/AA=1 of mothers or children), dietary intake of nicotinamide (both as a continuous variable and as a dichotomized variable) and periconception medicine use. This was done using multivariable logistic regression models in which we included the interaction terms 'Medicine use x NNMT genotype', 'Nicotinamide intake x NNMT genotype', and 'Medicine use x Nicotinamide intake x NNMT genotype' and calculated *p*-values for interaction. The additive effects and the interaction analyses were adjusted for multiple testing by the method of Bonferroni. Probability values of *p*-value < 0.05 were considered statistically significant and all tests were two-sided.

Analyses were performed with SPSS for Windows software (version 15.0; SPSS Inc., Chicago, IL, USA) or FBAT 3.2.

RESULTS

Figure 1 depicts the study population flowchart.

The sociodemographic and lifestyle characteristics of mothers, fathers and children are presented in *Table 1*. Maternal age was slightly, albeit significantly, different between cases and controls, and was included as a putative confounder in the further analysis. Case mothers used more medicines in the periconception period; this led to an adjusted OR (95% CI) of 1.5 (1.0-2.3) with a crude *p*-value of 0.027 and an adjusted *p*-value of 0.032. After dietary nicotinamide intake has been categorised into low or high intake, mothers on a diet low in nicotinamide showed a 1.6-fold higher CHD risk (95%CI 1.0-2.5) crude *p*-value = 0.042, adjusted *p*-value = 0.039. The adjusted OR (95% CI) for nicotinamide intake as a continuous variable was 0.94 (0.87-1.00) crude *p*-value = 0.210, adjusted *p*-value = 0.056. The adjusted OR and *p*-values were adjusted for maternal age, periconception use of tobacco, alcohol and B vitamins, family history of CHD, total dietary energy and folate intake. There were no significant differences in sociodemographic and lifestyle characteristics between case and control children and fathers.

Table 2 presents the distribution of the genotype frequencies and the risk estimates of the NNMT polymorphisms of mothers, fathers and children. All genotype distributions were in Hardy Weinberg Equilibrium. NNMT frequencies were not different between cases and controls. FBAT did not reveal any statistically significant association between the NNMT genotype and CHD risk (data not shown).

Table 3 presents the distribution of the genotype frequencies and the risk estimates of the NNMT polymorphisms of mothers, fathers and children for each CHD phenotype separately. There are no significant effects; though of interest are the borderline significant increased risk estimates for transposition of the great arteries, which are consistent for mothers, fathers and children.

Figure 2 presents the risk estimates in mothers and children carrying the NNMT genotypes, periconception exposure to medicines (left panels), and low or high nicotinamide intake (right panels). Children who carry the NNMT GG genotype and have been periconceptionally exposed to medicines have an almost two-fold significantly increased risk of having a CHD than nonexposed children with the same genotype (adjusted *p*-value = 0.018, Bonferroni adjusted *p*-value = 0.036). Moreover, mothers who carry the NNMT GG genotype and have a low dietary nicotinamide intake show a two-fold significantly higher risk than mothers with the same genotype who have a high nicotinamide intake (adjusted *p*-value = 0.012, Bonferroni adjusted *p*-value = 0.036).

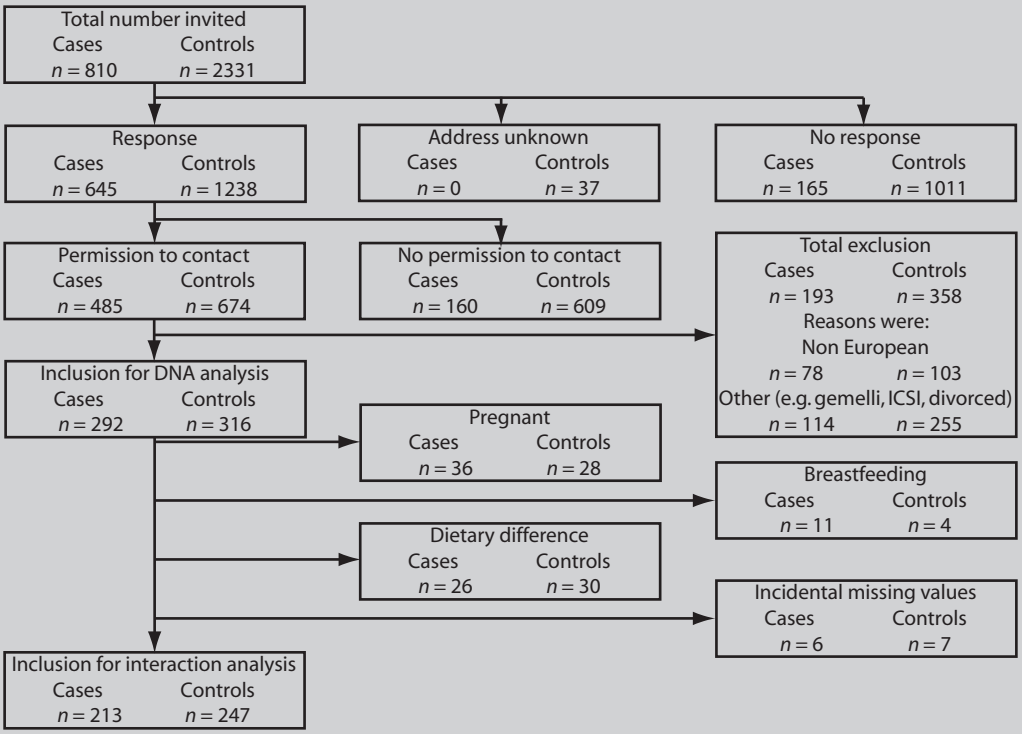
Figure 3 shows the results of medicine and nicotinamide intake and the NNMT genotypes in mothers and children. The combination of the mother's NNMT AG/AA genotype with low maternal dietary intake of nicotinamide and use of medicines showed the highest relative risk, (OR (95%CI); 3.2 (1.02-10.2), adjusted *p*-value = 0.048, Bonferroni adjusted *p*-value = 0.144). Moreover, the CHD risk was almost nine times higher in children carrying the NNMT AG/AA genotype who were periconceptionally exposed to medicines and low maternal intake of nicotinamide (95% CI=2.3-33.0, adjusted *p*-value = 0.002, Bonferroni adjusted *p*-value =

TABLE 2 Distribution of the NNMT genotypes in families

	Cases	Controls	OR (95% CI)
Mothers	***n* = 291**	***n* = 313**	
NNMT, AG/AA	106 (36)	118 (38)	0.9 (0.7 - 1.3)
GG	185 (64)	195 (62)	1.0 (Reference)
HWE *p*-value	0.5666	0.6067	
G-allele frequency	466 (80)	496 (79)	
A-allele frequency	116 (20)	130 (21)	
Fathers	***n* = 292**	***n* = 314**	
NNMT, AG/AA	98 (34)	97 (31)	1.1 (0.8 - 1.6)
GG	194 (66)	217 (69)	1.0 (Reference)
HWE *p*-value	0.7496	0.7526	
G-allele frequency	475 (81)	523 (83)	
A-allele frequency	109 (19)	105 (17)	
Children	***n* = 283**	***n* = 316**	
NNMT, AG/AA	99 (35)	102 (32)	1.1 (0.8 - 1.6)
GG	184 (65)	214 (68)	1.0 (Reference)
HWE *p*-value	0.4724	0.2000	
G-allele frequency	454 (80)	524 (83)	
A-allele frequency	112 (20)	108 (17)	

Values are numbers (percentage), tested by the Chi square test.

FIGURE 1 Study population flowchart.



The numbers are the absolute numbers of case mothers / control mothers.

0.006).

We did not detect any significant multiplicative interactions between the NNMT genotype and either of the environmental factors (data not shown).

DISCUSSION

This is the first study to investigate associations between the NNMT polymorphism and the combined periconception exposure to medicine and/or a diet low in nicotinamide on CHD risk. The NNMT AG/AA genotypes did not affect CHD risk. The association with the subgroup of transposition of the great arteries, albeit not significant, is interesting. However, the results of the separate CHD phenotypes should be further investigated in much larger data sets. Periconception medicine and low dietary nicotinamide intake independently almost two-fold increased the risk of CHD. This is supported by the additive effect between the NNMT AG/AA genotypes of both mothers and children and periconception medicine or low nicotinamide intake, of which the environmental factors seem to have the largest contribution. An almost nine-fold increased CHD risk was found for children carrying the NNMT AG/AA genotype who were exposed to both periconception medicines and low nicotinamide intake.

So far no epidemiological studies reported on associations between the NNMT polymorphism and CHD or other congenital malformations. However, up to now three papers have been published on the effects of the NNMT polymorphism on plasma homocysteine levels. Souto *et al*. found strong evidence that the NNMT gene is a major determinant of plasma homocysteine in a Spanish population (8). Evidence on the functionality of the NNMT polymorphism is still conflicting. Zhang *et al*. found that Japanese men (≥40 years) with low plasma folate between 1.5 and 4.8 nmol/L, who carried the NNMT GG genotype, had mildly elevated plasma homocysteine concentrations (19). In a Danish population, no significant effect of the NNMT polymorphism on homocysteine levels was shown (20). In that study it is suggested that the MTHFR gene is responsible for almost all variation in the homocysteine level attributable to genetic factors. The exact function of NNMT in the homocysteine pathway is not completely understood. NNMT is a SAM-dependent methyltransferase and predominantly metabolises nicotinamide to N-methyl nicotinamide. NNMT binds the methyl group generated from the conversion of SAM into SAH, which are both precursors of homocysteine. Therefore, alterations in the activity of NNMT may affect the SAH and homocysteine level. The maternal homocysteine levels were also available in this study, which enabled us to perform an additional analysis tot test whether homocysteine is an effect modifier of CHD risk. In the interaction analysis the interaction term NNMT x homocysteine level was included. The *p*-value of 0.681 indicates that homocysteine is not an effect modifier of CHD risk. Although we cannot show effect modification by homocysteine, it is an interesting issue that should be further investigated.

We demonstrated that periconception medicine use significantly increased CHD risk. Others reported associations between anticonvulsants and unspecified CHD phenotypes, and nonsteroidal anti-inflammatory medicines and transposition of the great arteries and ventricular septal defects (21, 22). We hypothesize that children are in particular at increased risk for CHD when their detoxification pathway is also compromised due to polymorphisms in methylated genes, such as NNMT (23). Since, NNMT plays a role in the detoxification of medicines that undergo methylation (24). Therefore, if the NNMT polymorphism leads to a decreased enzyme activity resulting in an altered detoxification of methylated medicines, it may enhance CHD risk. This is supported by the additive effect shown especially in the children (*Figure 2*). Unfortunately, we were not able to make a distinction between the different types of medicines because of the small numbers. Further research with larger sample sizes is needed to explore these specific associations.

We also demonstrated that a maternal dietary intake of nicotinamide below 13.8 mg/day almost two-fold increased the risk of CHD. In epidemiological and experimental studies levels of nicotinamide intake comparable to our study have been shown to be a risk factor for oral facial clefts and spina bifida (25-27). Nicotinamide is important for cellular maintenance, antioxidant activity, DNA repair mechanisms and methylation processes, which are important biological processes in embryonic cardiac development. The Dutch Recommended Daily Allowance (RDA) of nicotinamide is 13 mg nicotinic amide equivalents per day for women above 18 years (28). The cut-off value that we used (≤13.8mg/day) was based on the lowest tertile of the control group and is just slightly above the RDA. In our population 23% of the control mothers and 27% of the case mothers had a nicotinamide intake below the Dutch RDA. This is a high proportion of which underreporting is not a likely explanation, because the FFQ has been validated twice and after energy adjustment the results remained the same. It is possible that the association is due to potential confounders, such as folate, because nicotinamide and folate are present in liver, fruits and vegetables. Folate intake was not significantly different between case mothers and control mothers (*Table 1*). Therefore, it is very unlikely that an accompanying low folate intake

TABLE 3 Subgroup analysis of the association between the NNMT AG/AA polymorphism and each CHD phenotype

CHD phenotypes	Mothers[a]			Fathers[b]			Children[c]		
	Number (*n* = 291)	Cases [d] AG/AA	OR (95%CI)	Number (*n* = 292)	Cases [d] AG/AA	OR (95%CI)	Number (*n* = 283)	Cases [d] AG/AA	OR (95%CI)
Tetralogy of Fallot	30	10 (33)	0.8 (0.4 - 1.8)	30	9 (30)	1.0 (0.4 - 2.2)	29	8 (28)	0.8 (0.3 - 1.9)
Transposition of the great arteries	51	22 (43)	1.3 (0.9 - 2.3)	51	19 (37)	1.3 (0.7 - 2.5)	51	22 (43)	1.6 (0.9 - 2.9)
Atrioventricular septal defect	30	12(40)	1.1 (0.5 - 2.4)	30	6 (20)	0.6 (0.2 - 1.4)	27	5 (19)	0.5 (0.2 - 1.3)
Perimembranous ventricular septal defect	80	21 (26)	0.6 (0.3 - 1.02)	81	28 (35)	1.2 (0.7 - 2.0)	80	25 (31)	1.0 (0.6 - 1.6)
Coarctation of the aorta	28	10 (36)	0.9 (0.4 - 2.1)	28	9 (32)	1.1 (0.5 - 2.4)	27	9 (33)	1.0 (0.5 - 2.4)
Aortic valve stenosis	6	3 (50)	1.7 (0.3 - 8.3)	6	0	-	6	2 (33)	1.0 (0.2 - 5.8)
Pulmonary valve stenosis	50	23 (46)	1.4 (0.8 - 2.6)	50	19 (38)	1.4 (0.7 - 2.5)	47	20 (43)	1.6 (0.8 - 2.9)
Hypoplastic left heart syndrome	16	5 (31)	0.8 (0.3 - 2.2)	16	8 (50)	2.2 (0.8 - 6.1)	16	8 (50)	2.1 (0.8 - 5.7)

OR, odds ratio; CI, confidence interval.

[a] Control mothers NNMT AG/AA genotype: 118 (38); Total number = 313

[b] Control fathers NNMT AG/AA genotype: 97 (31); Total number = 314

[c] Control children NNMT AG/AA genotype: 102 (32); Total number = 316

[d] Cases are numbers (percentages) of the NNMT AG/AA genotype. The NNMT GG genotype was the reference group.

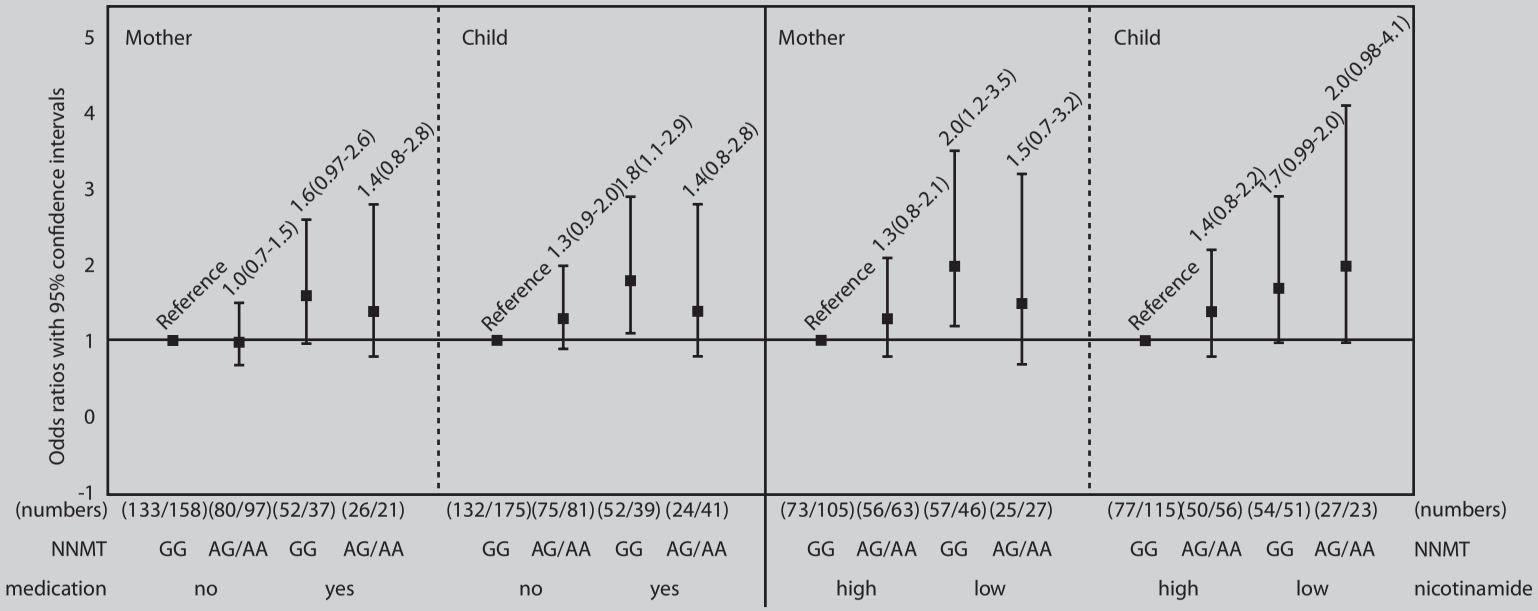
explains the risks associated with nicotinamide intake.

Epigenetics is a mechanism in which nutritional factors regulate gene expression, whereby methylation is the best understood. NNMT and nicotinamide play a role in the transfer of methyl groups to genes and as such are involved in the epigenetics of mother and child. Therefore, we suggest that the demonstrated additive risks of the NNMT AG/AA genotype and nicotinamide intake may affect the control of specific embryonic cardiac genes. This needs, however, detailed experimental studies.

Strengths and weaknesses of our study have to be considered as well. Recall bias is one of the pitfalls of case-control designs. However, this is not frequently present in case-control studies on congenital malformations (29, 30). Our sample size of 283 case families might have been too small to detect a 1.5-fold increased CHD risk in children carrying the NNMT AG/AA genotype (risk allele frequency of 20%, type 1 error of 0.05, CHD population risk of 0.008 resulted in a power of 65%). Moreover, the fixed study moment, as strength of our study, minimises recall bias. Finally, we show an effect of medicine only in carriers of the A-allele. As mothers and children are not aware of their genotypes, this association cannot be explained by recall bias. Other strengths of our study are the inclusion of CHD phenotypes associated with hyperhomocysteinemia and the ethnic homogeneity of the families. The latter is particularly important when studying genetic factors and cultural determined lifestyle factors, such as diet.

In conclusion, we identified new risk factors for complex CHD and gained new insights in its multifactorial etiology. Our results provide a first set of data against which future studies with larger sample sizes can be compared with.

FIGURE 2 Periconception medicine use or dietary nicotinamide intake combined with the NNMT genotypes of the mother or the child and CHD risk



The first group in each panel represents the reference group. In the left two panels the OR with 95% CI are shown of the different subgroups with combinations of maternal medicine use in the periconception period and NNMT genotype of the mother (first panel) or the child (second panel).
In the right two panels the OR with 95% CI are shown of the combinations of maternal dietary nicotinamide intake with NNMT genotype of the mother (first panel) or the child (second panel).

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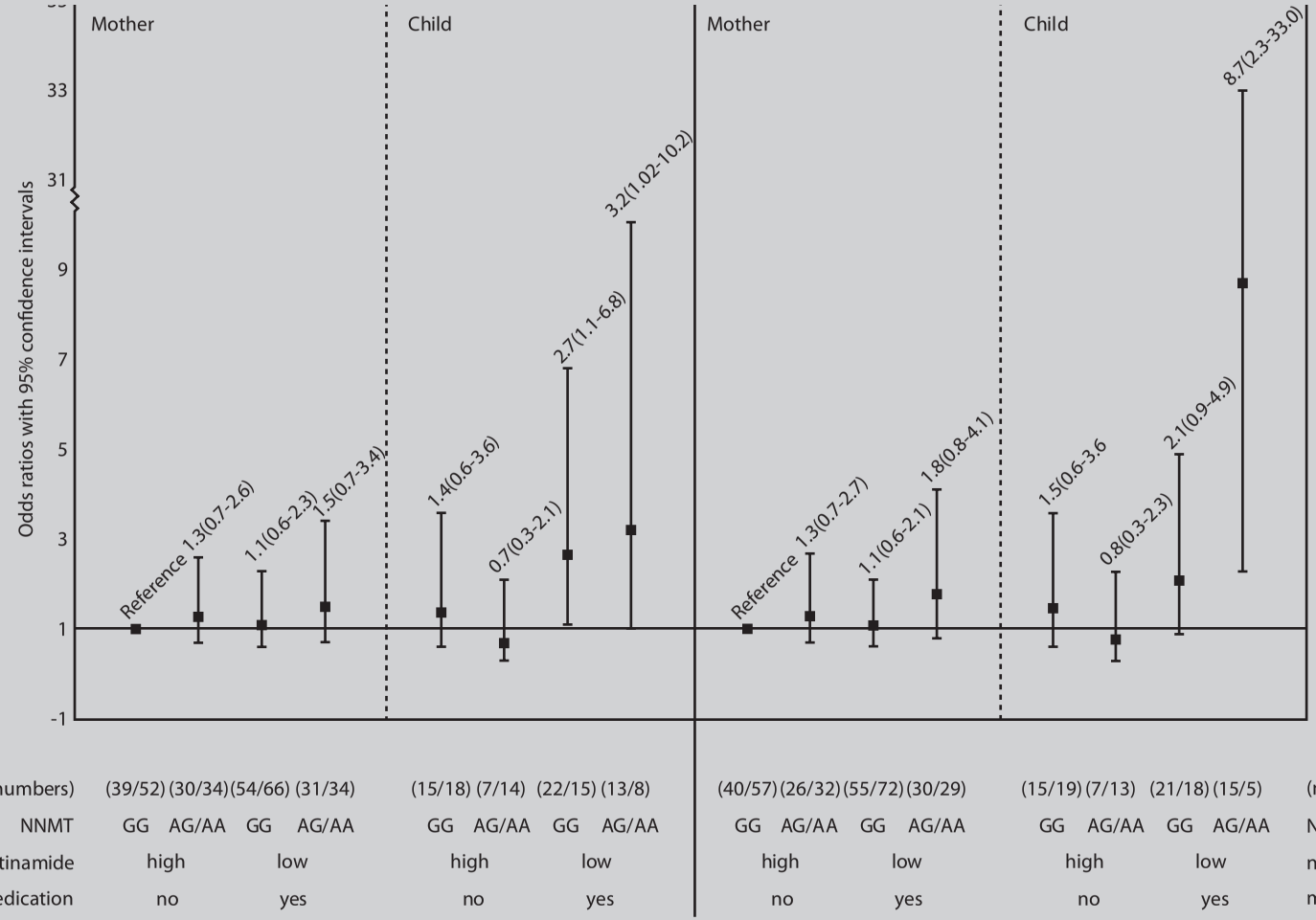
FIGURE 3 Periconception medicine use, dietary nicotinamide intake and the NNMT genotype



The first category in each panel is the reference category. These consist of mothers who did not use any medicines in the periconception period, who had a high dietary intake of nicotinamide based on the 30th percentile of the control group and who carried the NNMT GG-variant in the left panel and the NNMT GG-variant in children in the right panel.

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Chapter

GENERAL DISCUSSION

General discussion

Prevention of congenital heart diseases (CHDs) is our main goal. Some of the most important reasons are the high birth prevalence rate (1), the high morbidity and mortality (2, 3), the great impact on the affected children and their families (4), and the high related health care costs (2). However, before being able to launch any prevention strategy, knowledge about the pathogenesis of CHDs is of crucial importance. Up until now, only approximately 15% of the CHDs can be attributed to a known cause. The remaining 85% is thought to result from interactions between genetic predispositions and periconception environmental exposures (3). Genetic predispositions of the mother and/or the child in utero might interact with environmental exposures from the mother, since the mother is the environment of the developing embryo. Recently, interesting studies have indicated that derangements in the one-carbon metabolism might be involved in the aetiology of congenital malformations, such as neural tube defects, orofacial clefts and CHDs (6-8). Deranged expressions of paternal, maternal and/or embryonic functional polymorphisms of genes involved in this pathway may influence the early heart development. Also lifestyle factors, such as the maternal nutritional status or use of medicines, may play a role.

Therefore, this thesis aimed to demonstrate associations between paternal, maternal and embryonic genetic and lifestyle factors related to the one-carbon metabolism and CHDs.

Of each study, the merits and limitations have been described in the previous chapters. The current chapter will discuss the methodological issues, inference of the main findings, and speculate on implications for public health and clinical practice, considered in the light of current knowledge and ongoing research. Finally, we will make suggestions for future research.

METHODOLOGICAL ISSUES

Study design

When studying associations between risk factors and disease, a large prospective cohort study would be the first choice. However, the major drawback of such a design, when studying rare outcome, is the very large number of study participants needed. In the case of CHD, which occurs in about 6 of 1,000 newborns, thousands of participants need to be included to achieve enough power. Consequently, this design would be very expensive. Even a bigger challenge would be to include the couples at the moment they are planning pregnancy. To include them before they are actually pregnant, is very important when investigating the association between lifestyle factors and CHD, since the heart develops in the first 8 weeks of pregnancy. Therefore, the case-control design is the next-best option. This design is widely used in observational epidemiological research, since it is a relatively cheap, rapid, and a reliable design of establishing evidence of an association between an 'exposure' and a rare outcome. However, we have to consider some important biases inherent to the case-control design. Further on in this thesis, we will address the role of these biases and the way we dealt with them, with respect to the study moment, inclusion of participants and measurements of nutrient status and genetic factors.

Study moment

There are several reasons why we have chosen for one study moment of approximately 15 months after the index-pregnancy. First of all, one fixed study moment relatively soon after pregnancy, compared with other studies, minimises recall bias: that is, the longer the recall period, the more likely people will not accurately remember their exposures. This could subsequently result in non-differential misclassification of exposure; meaning that misclassification errors apply equally to cases and controls. Although also in our study non-differential misclassification might have occurred, this type of bias is generally considered not so much pernicious, because it tends to attenuate an existing association (4). Most CHDs are detected and completely diagnosed during the first year of life. Therefore, choosing an even earlier study moment might lead to misclassification of cases and controls and would thus underestimate the results. Another reason for choosing this study moment is the metabolic state of the mothers. It is known that maternal metabolism changes during pregnancy and breastfeeding, and that it will have returned to preconceptional values at about 1 year after delivery (7, 8). This is especially important with regard to the measurements of the biomarkers and dietary energy and nutrient intakes, which we measured after pregnancy assuming to represent the preconceptional values. This assumption was based on several studies showing that, in general, metabolism and nutritional

habits are rather constant over time and do not change except for episodes of illnesses, dieting and increased needs during pregnancy or breastfeeding (9-11). Moreover, the results of this thesis (Chapter 2) show that in a prospective study, the preconceptionally measurements of several biomarkers as well as dietary energy and nutrient intakes are indeed comparable with those approximately one year after delivery.

Study participants

We recruited the case families, consisting of a mother, father and child, 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. Although the cases are derived from four different medical centres, there are only two paediatric cardiologists who diagnose the case children and both are trained in the same medical centre. This enhances the uniformity of the diagnosis of the CHD phenotypes. Diagnoses were confirmed by echocardiography and/or cardiac catheterisation and/or surgery after birth. All diagnoses were made and verified before the families were invited to participate in the HAVEN study. The following CHDs were included in this study: tetralogy of Fallot, transposition of the great arteries, atrioventricular septal defect, perimembranous ventricular septal defect, coarction of the aorta, aortic valve stenosis, pulmonary valve stenosis, and hypoplastic left heart syndrome. These phenotypes were selected, because experimental and epidemiologic studies showed that hyperhomocysteinaemia and related gene-environment interactions are involved in their causation (5-7). We have already explained that the fixed study moment of about 15 months after delivery reduces misclassification of cases. Other data further substantiating the validity of our case group, and thereby minimising the possibility of diagnostic bias, is that the rate of third degree family members with a CHD is higher in the case group (10% versus 6%, P=0.063: Chapter 4).

A very important aspect of a case-control design is the selection of controls. Originally, at the start of the study, we aimed to recruit the control families via the Departments of Otorhinolaryngology at the participating hospitals. However, the Medical Ethics Committees of these hospitals did not give permission because of the burden for the control families. Therefore, we recruited the control group, consisting of healthy children and both parents, in close collaboration with a physician of the child health centres of "Thuiszorg Nieuwe Waterweg Noord" in the surroundings of Rotterdam. This was at that moment the best way to derive both cases and controls from the same study base: the western part of the Netherlands. The child health centres are part of the Dutch Health Care system, where all newborns are regularly checked on for health, growth, and development by physicians specialized in child health care. Children were eligible as controls if they did not have a major congenital malformation or chromosomal defect according to these regularly checks. Another exclusion criteria was familial relationship between cases and controls.

Another possible selection bias will be introduced when the association of exposure with disease is different for participants and non-participants. In our study, the response rates are 79% for cases and 55% for controls (Chapter 8). These different response rates can partially be explained by different motivations to participate. Parents of case children often want to know more about the aetiology of CHDs and the possibilities to prevent a second affected pregnancy. Parents of control children, on the other hand, are mostly interested in participating because they know someone with an affected child, they want to check their health status, or because of altruism. The reasons not to participate are rather similar between cases and controls: 1) practical problems, such as the distance that they have to travel or difficulties taking a day off; 2) emotional problems, such as upcoming divorce; and 3) medical problems, such as chronic disease of a family member.

Another consideration when comparing cases with controls is differential misclassification. This is the case when parents of case children have a better recall, because they actively search for an explanation of the disease of their child. This might be the case for some exposures, but only when the exposure is well known to be associated with the disease or socially undesirable (8). However, even for alcohol use and smoking, which are considered socially undesirable with respect to pregnancy, differential misclassification has only minor effect (9).

Comparing the general characteristics between cases and controls is a way of checking the comparability and to see whether selection bias might play a role; this indicates that the series are representative of the same study base. Selection bias seems not an important issue in our study, since all general characteristics are comparable between cases (n = 292) and controls (n = 314; Chapter 8), except for maternal age and periconceptional use of medication. Mothers of cases are significantly, though slightly, older than mothers of control children (33.2 and 32.8 years, respectively). Therefore, in all analyses investigating environmental exposure effects, we adjusted for maternal age.

Another issue that is not specifically related to case-control design is whether our study population is a

good representative of the general population: a matter of external validity. In other words: will the results be applicable to the general population. This has been pointed out nicely in Chapter 3, in which we compared the general characteristics of 336 control mothers with national reference data of women between 15 and 45 years of age from Statistics Netherlands (10). Since the general characteristics of the case group did not significantly differ from the control group, the following comparisons do also apply to the case group. Our study group comprised more non-European women than the Dutch reference population, which was according to the expectations since a higher percentage of non-Europeans live in the western part of the Netherlands. Moreover, all genetic polymorphisms were in Hardy Weinberg Equilibrium and the frequencies of the variant alleles were all comparable with those reported in other studies (11, 12).

Measurements

We measured three related groups of potential risk factors other than those included in the general characteristics, namely biomarkers, dietary nutrient intake and genetic polymorphisms. Each and every one of them requires special attention with respect to measurement errors.

Biomarkers: The blood sampling and determination of SAM, SAH, tHcy, vitamin B12 and folate in serum and folate in RBC are done in a standardized way. All samples are analyzed in the same clinical chemistry laboratory at Erasmus University Medical Centre, Rotterdam, the Netherlands. After withdrawal, one EDTA-tube is kept on ice and centrifuged at 4°C within 2 hours. This is extremely important for accurate measurement of SAM, SAH and tHcy and to reduce the risk of haemolytic samples (13). Moreover, all samples are analyzed in batches and, thereby, possible measurement errors are random. The methods that we use to determine the biomarkers are all reliable, valid and established methods (14, 15). An important form of measurement error is the between-run coefficient of variation (CV). All CVs were below 10%, indicating acceptable reliability: SAM 4.7%; SAH 4.4%; tHcy 5.9% at 15.3 µmol/L and 3.4% at 39.3 µmol/L; vitamin B12 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; folate 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L. In addition, all concentrations are comparable with reference values established at the clinical chemistry laboratory (16) (Chapter 3).

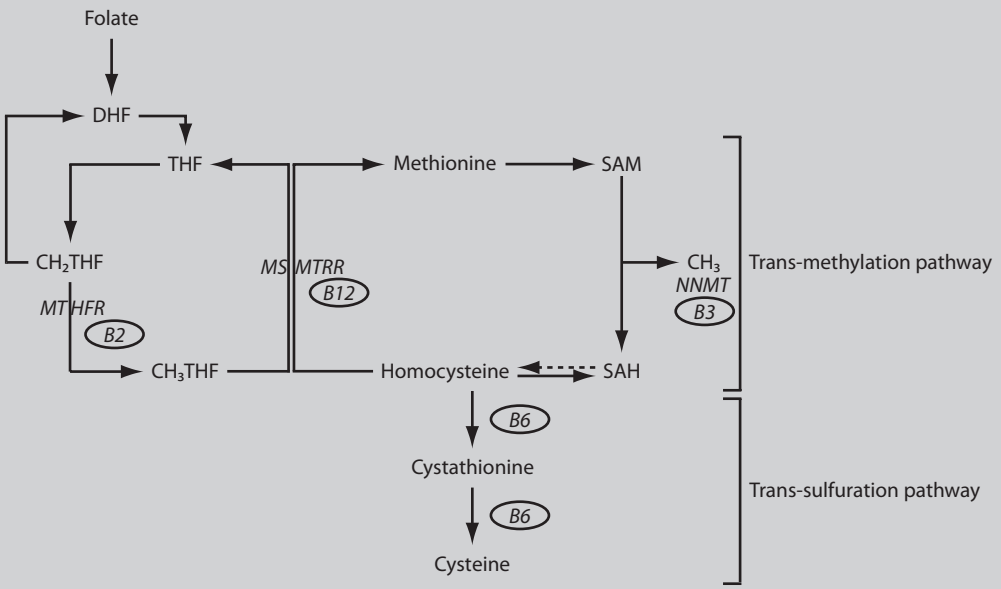
Dietary nutrient intakes: During the hospital visit, the food frequency questionnaire (FFQ) data have been checked for completeness and consistency by the researcher. This semiquantitative FFQ, validated by Feunekes et al. (17) and modified for the estimation of B vitamin intakes (18), is used to estimate daily habitual intake of energy, macronutrients, and micronutrients. Based on data of the Dutch national food consumption surveys in 1992 and 1998, the FFQ has been updated twice (19, 20). After modification, the FFQ covered the daily intake of each nutrient or food of interest for at last 90% of the population mean intake. Dietary assessment methods are known to have a bias towards underestimating habitual energy intake. Therefore, we investigated the overall underreporting bias by determination of the physical activity level, which was above the cut-off value of 1.35 indicating that underreporting was not likely to play a role in our study (Chapter 2; (21)).

Genetic polymorphisms: Measurement errors are not likely, because we used Taqman® techniques according to protocols provided by the manufacturer (Taqman, Applied Biosystems, Foster City, CA, USA) and we re-genotyped >3% of the samples to check for genotype calling consistency resulting in a genotyping success rate of more than 96%. In addition, all inconsistent triads were excluded from analysis. Moreover, all genotype frequencies in controls are in Hardy Weinberg Equilibrium and in line with other European studies that have investigated MTHFR (22, 23), MTRR (24, 25), TC (26, 27), and NNMT (11, 28).

Power

We have used a population CHD risk of 0.008 and a type I error of 0.05 in all power calculations (29). Calculations reveal a 40% significant risk reduction of the MTHFR 1298 A allele with a power of 78% (cases n = 229, MTHFR 1298 A allele frequency = 0.34). The sample size of both the case and the control group should be 416 for the MTRR polymorphism (risk allele frequency = 0.46) and 495 for the TC polymorphism (risk allele frequency = 0.56) to find a significant OR of 2.5 for the gene-serum vitamin B12 interaction with a power of 70%. Since we only showed trends towards an association in this study, larger numbers are needed to investigate significant gene-environmental interactions. Also the sample size of 283 case families in Chapter 8 might have been too small to detect a 1.5-fold increased CHD risk in children carrying the NNMT AG/AA genotype (risk allele frequency = 0.20, power = 65%)

Figure 1 Summary of the folate and homocysteine pathway



INFERENCES OF THE MAIN FINDINGS

Biomarkers of one-carbon metabolism

An important discovery is that periconceptional intake of multivitamin supplements containing folic acid may reduce the risk of CHD in offspring, similar to the known risk reduction for neural tube defects seen with folic acid (6). It is not quite clear which components of the multivitamin supplements account for this effect, nor the pathogenetic mechanism behind it. One of those speculated mechanisms is that B vitamin depletion leads to moderate hyperhomocysteinaemia and we and others have shown that increased concentrations of maternal homocysteine are associated with CHD in offspring (30). However, homocysteine is part of the one-carbon metabolism that provides one-carbon groups for the methylation of numerous substrates, such as DNA, lipids and proteins. The question arises whether hyperhomocysteinaemia or the methylation state is the more important cause for CHD (31).

The methylation process is catalyzed by methyltransferases that transfer methyl groups from SAM to its substrates, using folate as one of its main methyl group donors (*Figure 1*).

SAM is transmethylated into SAH. So far, SAM and SAH concentrations in blood and its ratio are the closest biomarkers of cellular methylation; low concentrations of SAM and the SAM/SAH ratio and high concentrations of SAH indicate a state of hypomethylation (32, 33). So far, there is only one study that investigated the methylation biomarkers in association with CHD. Hobbs et al. observed a higher SAH and lower SAM concentration in mothers of CHD offspring than control mothers (7). We did not find an association between any of the maternal biomarkers and isolated CHD (Chapter 4). Remarkably, a small subset of mothers who had a child with Down Syndrome and a CHD had significantly higher SAH concentrations and lower SAM/SAH ratios than mothers of control children. Apparently, hypomethylation seems not associated with CHD, but it seems indeed associated with Down syndrome and CHD. Unfortunately, inherent to the focus of our study, we cannot evaluate the association of hypomethylation and Down syndrome without the presence of CHD. From the literature, it is known that DNA methylation patterns are established during embryogenesis and that incorrect development of these patterns can lead to developmental malformations (34), chromosome instability (35), altered chromosome recombination (36) and aberrant chromosome segregation (37). Moreover, folic acid deficiency has been related to chromosome 21 aneuploidy (38). In the light of this literature and our study, DNA methylation problems may be related with the production of aneuploid gametes for chromosome 21. Future studies should verify this interesting hypothesis.

Obviously, we were very interested whether the children affected with Down syndrome and CHD would present with similar concentrations of methylation biomarkers to their mothers (Chapter 5). Interestingly, these children presented with the highest concentrations of SAM and SAH compared with control children, indicative of a state of hypermethylation and not hypomethylation. Possibly, this was due to the very high folate concentrations in the group with Down syndrome. With respect to these findings that initially seem contradictory, several pathogenetic mechanisms may be proposed. Firstly, periconceptional hypomethylation in the mother may lead to epigenetic aberrations in utero resulting in chromosomal malsegregation and altered methylation patterns within the child with Down syndrome (39). This theory is substantiated by several studies on the 'agouti' mouse model: "an epigenetic biosensor for nutritional and environmental alterations on the fetal epigenome" (40). For example, pregnant pseudoagouti Avy/a mouse dams were fed a methyl-supplemented diet, which altered agouti gene expression in their offspring, as indicated by increased agouti/black mottling of their coats (41, 42). Another hypothesis is that only children with high methylation capacity and high folate status survive the maternal hypomethylation status in utero (43, 44). A third hypothesis relates to the gene-dosage theory (45): the excessive synthesis of multiple gene products derived from overexpression of the genes present on chromosome 21 is thought to underlie both the dysmorphic features and the pathogenesis of the biochemical abnormalities that are characteristic of DS. One interesting example is the gene encoding for the enzyme cystathionine β–synthase (CBS) that is located on chromosome 21 (46). This enzyme catalyses the condensation of homocysteine and serine to cystathionine; an intermediate step in the transsulfuration pathway. A 157% increase in CBS enzyme activity has been previously documented in individuals with DS and has been associated with reduced levels of homocysteine (45). In the light of this hypothesis, Pogribna et al. found relatively hypermethylated DNA in children with Down syndrome, despite depressed SAM and SAH concentrations (47). Thus, up till now, there are only a few, contradictory studies on the biomarkers of methylation in very young children with or without Down syndrome or CHD substantiating these theories. Our findings are a valuable contribution and need to be further explored.

Getting back to the role of methylation in CHD, neither the methylation biomarkers in mothers nor in children revealed any significant association with isolated CHDs. There is, however, quite some evidence suggesting an association between imbalanced maternal methylation and congenital defects, such as neural tube defects and certain specific heart defects (48). A recent, very interesting study in this respect indicated that homocysteine-induced delay of neural tube closure in chick embryos is caused by the inhibition of transmethylation via elevation of SAH concentrations and a reduction of the SAM/SAH ratio (34). It is plausible that the one-carbon metabolism only plays a role in the development of certain specific cardiac defects. Unfortunately, we could not reliably establish these associations in our studies because of the small numbers in each CHD phenotype group. We have to take into account the very complex and multifactorial aspects of the one-carbon metabolism in relation to the origin of CHDs. By now, it is clear that the aetiology of CHDs involves complex interactions between lifestyle exposures and genetic susceptibilities. The methylenetetrahydrofolate reductase (MTHFR) gene is one of such genetic factor that plays a role in the one-carbon metabolism. We showed that the effect of the methylation biomarkers differed respectively of the common polymorphism MTHFR C677T (Chapter 4 and 5). We will discuss the inferences of this polymorphism and other genetic and environmental factors related to the one-carbon metabolism in the next paragraph.

Genetic polymorphisms, lifestyle and nutrients

Up till now, two meta-analyses have shown that the single genetic polymorphism MTHFR C677T does not independently affect the risk of CHDs (49, 50), which we also found in our study (Chapter 6). This is not very surprising considering the multifactorial aetiology of CHDs in which genetic polymorphisms are thought to be small risk modifiers. Moreover, the studies included in these meta-analyses comprised a diversity of CHD phenotypes and population background, which makes them difficult to compare. If we would have found an effect, it would have been most likely with the MTHFR 677 TT-genotype, since it strongly correlates with reduced enzyme activity, increased thermolability and significantly increased homocysteine concentrations (51). In our study, we also found the MTHFR C677T polymorphism a strong and independent determinant of homocysteine concentrations (Chapter 3). Surprisingly, not the MTHFR 677 TT-genotype, but the MTHFR 1298 C-allele in fathers and children showed an independent association with CHD risk: the C-allele in fathers decreased the CHD risk with 40% and the C-allele in children with 30% (Chapter 6). Hobbs et al. also found a protective effect of the MTHFR 1298 C-allele (52). The reverse association was not quit what we had expected, since the MTHFR 1298 CC-genotype has been found to decrease the MTHFR enzyme activity (53), which would more likely increase the CHD risk. However, the effect of the MTHFR A1298C polymorphism on homocysteine

concentrations or on cellular DNA methylation is still controversial (54-56). In these studies, the combination of the two common MTHFR polymorphisms had the strongest effects on homocysteine concentrations and DNA methylation. It would therefore be very interesting to enlarge our study groups, which would enable us to study these gene-gene interactions in more detail. The other investigated polymorphisms, namely MTRR A66G, TC C776G, and NNMT G113A, were not independently associated with CHD risk (Chapter 7 and 8). However, the association of the NNMT polymorphism and the subgroup of transposition of the great arteries, although not significant, is interesting (Chapter 8), especially since our study was the first to investigate this polymorphism in relation to a congenital defect.

Genetic polymorphisms are only thought to play a minor, modifying role in the complex aetiology of CHDs. Lifestyle factors of the pregnant mothers, on the other hand, are thought to play a major, modifying role and are therefore very interesting to investigate with respect to CHD. Moreover, unlike genetic factors, lifestyle factors are good targets for prevention, since they can be adjusted and thereby the risk of CHD can be decreased. Therefore, we focused on related lifestyle factors implicated in the one-carbon metabolism. Interestingly, low intake of vitamin B3 and use of medication in the preconception period were associated with an almost twofold increased CHD risk (Chapter 8), independent of the other investigated possible risk factors. Despite the fact that B vitamins are often simultaneously present in several food products, we showed that an accompanying low folate intake was not likely to explain the risks associated with low vitamin B3 intake. Over thirty years ago, the first animal studies were published on the teratogenic effect of vitamin B3 antagonists (57, 58). This strong teratogenic effect is not surprising, since vitamin B3 is essential for the biosynthesis of pentose, steroids, red blood cells, fatty acids and is involved in glycolysis, protein, carbohydrate and fat metabolism and in DNA repair mechanisms and methylation processes (59). Recently, epidemiological and experimental studies showed that levels of vitamin B3 intake comparable with those in our study were associated with oral facial clefts and spina bifida (60, 61); all defects involve neural crest cells and therefore share a common etiology for CHD. As a cofactor for the NNMT enzyme, vitamin B3 also plays a role in the detoxification of certain medication and toxicants (62). Maternal, prescribed use of medication was also associated with an increased CHD risk. Others reported associations between specific groups of medication and CHD risk (63). Unfortunately, we were not able to make a distinction between the different types of medication. We, however, hypothesize that children are in particular at increased risk for CHD when their detoxification pathway is also compromised due to polymorphisms in genes that play a role in the detoxification of medication undergoing methylation, such as NNMT, and diminished availability of its cofactor vitamin B3. This hypothesis is supported by the almost nine fold increased CHD risk for children carrying the NNMT AG/AA genotype who were exposed to both periconception medication and low vitamin B3 (Chapter 8). With this study, we gained new insights in the multifactorial aetiology of CHD. We suggest that the demonstrated additive risks may affect the control of specific embryonic cardiac genes. This needs, however, detailed experimental studies.

Another interesting gene-lifestyle interaction we found, was the significant interaction between the MTHFR A1298C polymorphism and maternal use of a folic acid containing supplement in the preconception period (Chapter 6). We did not find this significant association with dietary folate intake, which is not surprising because the bioavailability of natural folate is much lower compared with that of the synthetic form, i.e. folic acid in supplements (64). Surprisingly, periconception folic acid use increased CHD risk in MTHFR 1298 C-allele carriers only. This is in contrast to the studies showing a beneficial effect of folic acid supplementation on CHD risk. Nevertheless, it substantiates the theory that folic acid at high dose may not always be beneficial when interacting with certain pathologically expressed genes (65).

These studies suggest that subtle genetic and lifestyle factors implicated in the one-carbon metabolism interact and are involved in the complex pathogenesis of CHDs. The lifestyle factors, such as dietary nutrient intake and the use of supplements and medication, seem to have the largest contribution and are interesting risk factors to focus on with respect to prevention strategies.

IMPLICATIONS FOR PUBLIC HEALTH

This thesis demonstrates the association between CHD and maternal hyperhomocysteinaemia, and several genetic and lifestyle factors related to the one-carbon metabolism, such as the NNMT polymorphism, use of medication and low B vitamin intakes. This data further supports that the one-carbon metabolism is a candidate pathway impacting cardiac development. Elucidating such a candidate pathway may suggest clinical approaches that could significantly reduce the number of CHDs, which is our main goal in the end. We are not there yet, but this thesis aimed at putting some more pieces of the puzzle together.

Primary prevention programs can focus on increasing awareness through, for example public or professional

education or they can focus on identifying high risk groups through for example screening programs. The first interesting question that comes to mind when thinking about screening is whether the next step would be to screen parents-to-be for their genetic susceptibility: in other words, is it possible to determine high risk groups for having a child with a CHD based on a number of single nucleotide polymorphisms? This concept is already well known and offers one of the most promising applications in the field of individualized drug therapy: pharmacogenetics. A nice example in this respect and indirectly related to the subject is the MTHFR gene that influences patients' methotrexate levels, which can lead to various adverse drug events. Patients with the MTHFR polymorphism can therefore be identified and can subsequently be prescribed lower dosages of methotrexate, to lower the risk of adverse drug events (66). Another, greatly debated development in this respect are the personalized lifestyle health recommendations based on clients' genomic profiles: for example, companies focus on lowering the risk of cancer, type 2 diabetes, and cardiovascular disease (67). There are, however, many disadvantages of this approach that need to be figured out and solved before even thinking of implementing. The main limitation is the level of evidence for an association between the polymorphism and phenotype and the clinical relevance of the effect estimate. At this point, from this thesis and from other literature, we may conclude that genetic factors can only be seen as small modifiers of CHD risk.

The more interesting risk factors to focus on with respect to primary prevention are the lifestyle factors. We demonstrate that CHD risk increases with higher concentrations of homocysteine and the risk of having a child with Down syndrome and a CHD seems to be not only associated with hyperhomocysteinaemia, but also with maternal hypomethylation. Why not screen for these biomarkers? Nowadays, women who are at risk for having a child with a CHD, for example because they already have a child with a congenital defect, should be screened for hyperhomocysteinaemia. This is just one example of how the results of this thesis can be implemented in preconception care and counselling: a folic acid supplement is an easy remedy for hyperhomocysteinaemia or hypomethylation, which could simply eliminate these risk factors before conception. Nowadays, women trying to get pregnant are already recommended to take a folic acid supplement: the Dutch government and the ERFO-centre have tried to stimulate the use of folic acid for many years now (68). Nevertheless, only about 50% of the women in our study population have used a folic acid containing supplement in the advised period of four weeks before until eight weeks after conception. This percentage is even higher than the recently reported percentage of 37% in a large cohort study in the Netherlands (69). Thus, adequate preconception folic acid supplementation is still very poor. Besides the fact that these women still do not adequately use folic acid supplements in the preconception period, the question arises whether folic acid alone is sufficient or that we should recommend using additional B vitamin supplements or maybe even a multivitamin supplement. We show that low vitamin B12 and B3 status are associated with CHD risk. Moreover, a lot of women in our study have substantially lower intakes of those three B vitamins than the Dutch recommended dietary allowances. Therefore, we would consider recommending low dose B vitamin supplements to achieve an optimal status. A recent study by Czeizel substantiates this recommendation: he found that multivitamins containing 0.4-0.8 mg of folic acid were actually more effective reducing neural tube defects than high dose of folic acid, while both multivitamins and folic acid supplements can prevent some types of CHD (70). One important remark has to be made on the possible detrimental effect of high dose of folic acid. Our study showed a possible detrimental effect when interacting with the MTHFR A1298C polymorphism. Other studies have clearly shown potentially harmful effects of folic acid use with respect to, for example colon cancer, or by masking a vitamin B12 deficiency (71). A number of countries, including the United States of America and Canada, introduced food fortification with folate in the late 1990s. Studies have shown both beneficial and harmful effects on birth defects (72). Therefore, before implementing this advice in daily practice, we should determine the optimal dosage of folic acid and the other B vitamins in this specific group of women who want to get pregnant. It is also for the abovementioned reasons that food fortification with folic acid is still not present in the Netherlands (73). Very recently, the Health Council of the Netherlands has given a new advice on adequate intake of micronutrients (74). They state that in principal, a well balanced diet provides the general population enough vitamins and will at the same time diminish the risk of exceeding the potentially harmful threshold. However, some high risk groups, such as pregnant women, should be recommended to use additional vitamins.

Currently, the above mentioned deliberations and recommendations are put into practice through the initiative of the department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Centre in Rotterdam by means of a special outpatient clinic for preconception counselling on nutrition and lifestyle called "Gezond zwanger worden" (In English: Towards a healthy pregnancy). This is the first initiative in the Netherlands to give this special preconception care to parents-to-be who have a normal population risk for pregnancy complications (75). Hopefully, this may contribute to reducing the number of babies born with a congenital defect.

FUTURE RESEARCH

Some of the results of the studies described in this thesis can be implemented in the preconception care as described in the previous paragraph. However, there are still a lot of additional challenging issues to investigate. Our results clearly demonstrate that derangements in the one-carbon metabolism are involved in the pathogenesis of CHDs. Maternal hyperhomocysteinaemia seems associated with CHD risk and maternal biomarkers for methylation seem more associated with Down syndrome and CHD. It would be very interesting to further explore these interesting findings by investigating the actual cellular DNA methylation in both maternal, paternal and foetal tissues. Recently, some interesting new methods have been developed to study this in detail (76). Within the HAVEN-study, we already have lymphocytes available to study these associations. Yet, you would need a prospective study design to be able to study foetal tissues. As mentioned in the previous paragraph, a preconception care program recently started at the Erasmus MC, University Medical Centre. An additional advantage of this program is the ability to prospectively study the abovementioned associations in the periconception period and during embryogenesis. Another good design with respect to level of evidence would be the randomised controlled clinical trials. However, it is unethical to refrain women who want to become pregnant from using a folic acid supplement based on the current knowledge.

Another interesting future research question would be whether the cellular methylation state is causatively associated with certain specific CHD phenotypes. Since the HAVEN-study is still ongoing, we plan to increase our study population and thereby the power, enabling us to investigate these associations within several subgroups. Animal and experimental studies are needed to unravel whether the associations we found are causative or not. The optimal dosage for B vitamin supplementation to promote beneficial effects and to avoid teratogenic effects remains an issue of continuous discussion and should therefore be explored as well before implementing on a large scale.

Besides exploring these associations more in depth, it would also be interesting to broaden our knowledge. We found some new factors within the one-carbon metabolism associated with CHD. Nevertheless, there might be many more unknown factors that are involved in the one-carbon metabolism and might be related with CHDs. Tanaka et al., for example, found some new polymorphisms related to B vitamins and homocysteine (77). This study used a genome-wide analysis, which is a nice example of using new and promising techniques in unveiling genetic aetiologies of human diseases. Another way of broadening our scope is to look beyond the one-carbon metabolism into other related metabolisms that might be associated with CHDs, such as the oxidative pathway. This pathway is related to the one-carbon metabolism through the transsulphuration of homocysteine into cysteine and glutathione (*Figure 1*). Folate depletion and elevated plasma homocysteine promote oxidative stress (78), which has been associated with increased risk of CHD as well (79, 80).

In conclusion, the results of this thesis provide a lot of arguments for further research in several one-carbon-related pathways. Moreover, the proposed gene-lifestyle interactions associated with cardiovascular development, suggest a wide range of lifestyle advices and interventions to prevent CHDs in future.

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Sum

INTRODUCTION

As early as circa 30,000 years ago, Aurignacian man painted a heart-shaped spot in the middle of a mammoths body: generally assumed to be one of the first pictures of the heart. The earliest written records of often obvious and monstrous human congenital malformations are derived from Babylonian clay tablets that are suggested to date back to 2000 BC. However, less obvious malformations, such as congenital heart defects (CHD), were less likely to be noted. The first reference to a CHD was a drawing of a "perforating channel" in the inter-atrial septum by Leonardo da Vinci: now known as the atrial septal defect. Knowledge on CHD grew bigger in the 17th and 18th centuries, because of the renewed interest in the anatomy and dissection of human bodies.

In the late 1950s, one of the first population-based studies in the field of CHD was carried out. The researchers proposed a new definition of CHD that is still used today: "a gross structural abnormality of the heart or intrathoracic great vessels that is actually or potentially of functional significance". Within this definition, various types of CHD can be distinguished, ranging from small, isolated and often innocent defects to severe, complex defects that are often part of a syndrome. Because of this wide range of CHD phenotypes, it is difficult to estimate the precise birth rate prevalence. That is why the worldwide estimated prevalence varies from 4 to 50 per 1,000 live births. Nevertheless, CHD are the most common type of birth defect, with worldwide approximately 1 million children born with a CHD each year and in the Netherlands about 1,300 a year. CHD are not only the most common type of birth defect, they are also the leading cause of infant death. The large majority of children with CHD are submitted to a hospital for surgery or other complex medical treatments and often suffer from serious physical and psychological problems during their lives. Because of improved medical treatment, more children survive childhood and grow into adulthood implying all kinds of problems: a growing need for adequate medical and health care towards the adult with a CHD. This growing need takes along expanding costs: calculated yearly costs are 1,258 million dollars in the US alone. Thus, because CHD have a high birth prevalence rate, a high morbidity and mortality, a great impact on both the affected children and their families, and high related health care costs, prevention is the key term. However, before this can be achieved, knowledge about the causes of CHD is crucial.

Up until now, only approximately 15% of the CHD can be attributed to a known cause. The remaining 85% is thought to result from interactions between genetic predispositions and periconception environmental exposures. Recently, interesting studies indicated that derangements in the one-carbon metabolism might be involved in the aetiology of CHD. One of the important methyl group donors in this metabolism is folate; a B vitamin associated with neural tube defects. Several epidemiological studies reported that maternal use of folic acid containing supplements also reduces the risk of CHD in the offspring.

The folate pathway is interrelated with the homocysteine pathway. Homocysteine is remethylated into methionine, which is then converted to S-adenosylmethionine (SAM). By donating its methyl group, SAM becomes S-adenosylhomocysteine (SAH). The methyl group is transferred from SAM to acceptor substrates, such as DNA or proteins, by numerous methyltransferase enzymes, such as nicotinamide N-methyltransferase (NNMT). Low dietary intake of B vitamins or functional variations in the key enzymes increase homocysteine concentrations and disrupt the one-carbon metabolism. It is not known, however, whether hyperhomocysteinemia or the altered one-carbon metabolism influences normal cardiac development resulting in CHD. Genetic predispositions of the mother and/or child might interact with environmental exposures in utero from the mother, since the mother is the environment of the developing embryo and foetus. Therefore, we hypothesize that derangements in one-carbon metabolism due to gene-environment interactions are associated with maldevelopment of the primitive cardiovascular system resulting in CHD.

This thesis presents mainly the results of the ongoing HAVEN study. The HAVEN study was designed as a case-control triad study of children and both of their parents with a focus on nutrition, lifestyle and genes

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in the pathogenesis and prevention of CHD. This study is conducted from June 2003 at the department of Obstetrics and Gynecology/Division of Prenatal Medicine of Erasmus MC, University Medical Centrer in Rotterdam, the Netherlands. Case children with both parents were invited to participate in collaboration with the Departments of Pediatric Cardiology of Erasmus MC, Leiden University Medical Center in Leiden, VU University Medical Center and Academic Medical Center in Amsterdam. The control children with both parents were invited in collaboration with the child health centers of 'Thuiszorg Nieuwe Waterweg Noord' in the surroundings of Rotterdam.

Part I

The first part of this thesis presents two methodological studies. The study that is presented in *Chapter 2* was performed within the FOLFO study, which is a prospective periconception study focusing on the role of nutrition and lifestyles on fertilization, implantation and embryo quality. In this chapter, we compared the maternal nutritional status before pregnancy with 1 year after delivery. This study was designed to validate the standardized study moment that is used in the HAVEN study. In 30 women, we measured the biochemical concentrations of folate, vitamin B12 and plasma total homocysteine (tHcy), and conducted a general questionnaire and a food frequency questionnaire. The general questionnaire comprised questions on ethnicity, education and lifestyle, such as smoking and the use of folic acid supplements. The food frequency questionnaire was adapted by the Division of Human Nutrition of Wageningen University in Wageningen to estimate the intake of energy, nutrients and B vitamins. In this study, we showed that the concentrations of tHcy, vitamin B6 and vitamin B12 did not significantly differ between the two study moments. The folate concentration, however, was significantly higher in the preconception period than at about 1 year after delivery. This could be explained by the significantly higher use of folic acid supplements in the preconception period. The estimated nutrient intakes were comparable between the two moments, except for alcohol, carbohydrates, vitamin B2 and retinol. This leads to the conclusion that the maternal nutrient status in the preconception period is comparable with the study moment after delivery.

In *Chapter 3*, we investigated lifestyle, nutritional and genetic determinants of the concentrations of tHcy, SAM, and SAH in blood of 336 women. In addition, we measured the concentrations of folate and vitamin B12. Both vitamins did not correlate with SAM and SAH but did correlate with tHcy. BMI was found to be the most important determinant of SAM and to a lesser extent of SAH. The most important determinants of tHcy were the 5,10-methylenetetrahydrofolate reductase (MTHFR) 677TT variant, the use of B vitamin supplements and protein intake. In conclusion, the factors determining SAM and SAH differ from those determining tHcy. This is an important finding with respect to future studies on the association between methylation and disease outcome.

Part II

This part describes associations between the biomarkers of the cellular methylation state and CHD. In *Chapter 4*, we evaluated the concentrations of tHcy, SAM and SAH in the blood of 231 case mothers and 315 control mothers in relation to CHD in their child. All analyses are adjusted for the use of B vitamin supplements. The tHcy concentration was significantly higher in the case group than in the control group, which could be mainly explained by the high concentrations in the large subgroup of isolated CHD. The subgroup of mothers with a child with Down syndrome and a CHD had higher tHcy concentrations, higher SAH concentrations and a lower SAM/SAH ratio than the control group. This supports our previous finding that maternal hyperhomocysteinemia is a risk factor for CHD. A new finding is that maternal hypomethylation increases the risk of having a child with Down syndrome accompanied with CHD.

In *Chapter 5*, we evaluated the same biomarkers in 143 case children and 186 control children at the age of about 17 months. We determined the concentrations of folate, vitamin B6 and vitamin B12 and the functional MTHFR C677T and A1298C polymorphisms. All analyses are adjusted for age, use of medication and vitamin supplements, and CHD in the family. The subgroup of nonisolated CHD showed the highest concentrations of SAM, SAH and folate, compared with the control group. Moreover, the SAM, SAH and folate concentrations were all significantly correlated with each other. The state of hypermethylation could not be explained by carriership of one of the MTHFR variants. Thus, it seems that a state of cellular hypermethylation in very young children is associated with nonisolated CHD, particularly syndromal CHD. Of course, we have to further explore whether the hypermethylation is associated with CHD or with an underlying (epi)genetic disorder.

Part III

The final part describes the genetic-environmental interactions associated with CHD. In *Chapter 6*, we present data of the interactions between two MTHFR polymorphisms and maternal B vitamin intake in 230 case children and 251 control children and both parents. The MTHFR C677T polymorphism was not associated with CHD. On the other hand, the MTHFR 1298 AA genotypes in both fathers and children were associated with increased risk. In addition, children carrying the MTHFR 1298 AA genotype who were not exposed to folic acid supplements in the periconception period had a two fold increased risk of CHD compared with children carrying the MTHFR 1298 AG/GG genotype who were exposed to folic acid supplements. No significant interactions were shown between the MTHFR genotypes and dietary intakes of folate or vitamin B2.

Chapter 7 describes the analyses on the genetic polymorphisms methionine synthase reductase (MTRR) and transcobalamine (TC), and vitamin B12 related environmental factors in association with CHD. The MTRR A66G and TC C776G did not influence CHD risk, independently. Moreover, no significant interactions were shown between these genetic polymorphisms in both mothers and children, and low intakes of vitamin B12 in association with CHD. However, the maternal TC genotype seemed to affect serum vitamin B12 only in case mothers. This effect was not influenced by current use of B vitamin containing supplements. The interactions between low serum vitamin B12 concentrations and the TC 776 GG genotype and maybe also the MTRR 66 GG genotype suggested to increase CHD risk. These interactions, however, were not statistically significant.

In *Chapter 8,* three specific factors associated with CHD are described, namely the nicotinamide N-methyltransferase (NNNMT) polymorphism in 292 case children and 316 control children with both parents, periconceptional use of medicines, and low intake of vitamin B3 by the mother. The NNMT polymorphism did not reveal any significant differences. A 1.5-fold higher risk of having a child with a CHD was shown for both periconceptional use of medicines and low intake of vitamin B3 (<13.8 mg/day). Interestingly, combining the genetic and the two environmental factors revealed an increased CHD risk. An almost threefold increased risk was found if the mother was carrying the NNMT AG/AA genotype and even up to a ninefold increased risk was found if the child was carrying the NNMT AG/AA genotype. These results have not been published before and provide therefore a first set of data against which future studies with larger sample sizes can be compared with.

In the general discussion, the results of this case-control study are discussed and the objectives are evaluated (*Chapter 9*). Furthermore, we discuss the implications and recommend future research. Maternal hyperhomocysteinemia is a risk factor for CHD. Maternal hypomethylation seems a risk factor for Down Syndrome and CHD, while hypermethylation in children seems to contribute to nonisolated CHD. Periconceptional hypomethylation in the mother may lead to epigenetic aberrations in utero resulting in chromosomal malsegregation and altered methylation patterns within the developing fetus resulting in Down syndrome. The results also showed that the interaction between the MTHFR A1298C polymorphism and folate status, and possibly also the interaction between the TC C776G and MTRR C776G polymorphisms and vitamin B12 might contribute to the risk of CHD. Another example of such gene-environment interaction is that children carrying the NNMT AG/AA genotype, who were exposed to medication and low intakes of vitamin B3, had an almost nine fold increased risk of CHD. Reflecting on these results, it seems that genetic variants that play a role in the one-carbon metabolism, together with lifestyle factors, such as the use of medicines and a suboptimal vitamin B status, are involved in the development of CHD. It would therefore be advisable to recommend high risk groups to use a diet rich in vitamin B3 and B12 and probably to use a supplement that not only contains folic acid, but also vitamin B12 and B3. On the other hand, we have to be very careful with respect to overdosing. Our results also suggest that folic acid supplements together with the MTHFR A1298C polymorphism could increase CHD risk. The results of this thesis can be implemented in preconception care.

Currently, the above mentioned deliberations and recommendations are put into practice through the initiative of the department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Centre in

Rotterdam by means of a special outpatient clinic for preconception counselling on nutrition and lifestyle called “Gezond zwanger worden” (In English: Towards a healthy pregnancy). This is the first initiative in the Netherlands to give this special preconception care to parents-to-be who have a normal population risk for pregnancy complications (75). Hopefully, this may contribute to reducing the number of babies born with a congenital defect.

Samen

Introductie

Meer dan 30.000 jaar geleden werd de eerste tekening van het hart gemaakt: een grottekening van een mammoet met een hartvormige stip ter plaatse van het hart. De eerste geschreven aanwijzingen van monsterlijke aangeboren afwijkingen bij mensen dateren van 2000 voor Christus, in de vorm van Babylonische kleitabletten. Minder opvallende aangeboren afwijkingen, zoals aangeboren hartafwijkingen (CHD), zijn pas veel later beschreven. De eerste verwijzing naar een CHD was een tekening van de beroemde Leonardo da Vinci. Hij tekende een gaatje in het inter-atriale septum, nu beter bekend als het atrium septum defect. In de 17e en 18e eeuw kwam de kennis over CHD pas echt op gang door de bloeiende interesse in anatomie en dissectie van menselijke lichamen.

In 1950 werd een van de eerste grote, op populatie gebaseerde onderzoeken op het gebied van CHD uitgevoerd. De onderzoekers stelden een nieuwe, maar nu nog steeds gebruikte definitie van CHD voor, namelijk "een structurele abnormaliteit van het hart of de intrathoracale grote vaten, welke actueel of potentieel van functioneel belang is". Binnen deze definitie kunnen vele verschillende CHD worden onderscheiden variërend van kleine, geïsoleerde, onschuldige defecten tot zeer complexe en ernstige defecten die vaak deel uitmaken van een syndroom. Vanwege deze grote variatie is het vaak lastig om een nauwkeurige geboorte prevalentie te bepalen. Daarom varieert de wereldwijde prevalentie van 4 tot 50 per 1000 levendgeborenen. Ondanks deze grote spreiding zijn CHD de meest voorkomende aangeboren afwijkingen. Ieder jaar worden wereldwijd 1 miljoen kinderen en in Nederland ongeveer 1300 kinderen geboren met een CHD. CHD zijn niet alleen de meest voorkomende aangeboren afwijkingen, ze vormen ook de voornaamste oorzaak van sterfte onder zuigelingen. Een groot aantal van deze kinderen die overleven moeten meerdere malen geopereerd worden en hebben vaak ernstige fysieke en psychologische problemen. Vanwege de steeds betere medische behandelingen, daalt de mortaliteit en worden kinderen met CHD steeds ouder, met alle problemen en beperkingen van dien. Inherent hieraan groeit de vraag naar adequate gezondheidszorg voor de volwassenen met CHD. Deze groeiende zorg brengt ook veel kosten met zich mee. De gemiddelde kosten per jaar zijn immens: in Nederland bedraagt dit ongeveer 79,6 miljoen euro en in de Verenigde Staten van Amerika ongeveer 1.258 miljoen dollar per jaar. Kortom, de hoge geboorte prevalentie, de hoge morbiditeit en mortaliteit, de grote impact op zowel de kinderen met CHD als hun familie en de hoge gezondheidszorg kosten maken dat gestreefd moet worden naar de primaire preventie van CHD.

Om primaire preventie te bereiken is kennis over de oorzaken van CHD van belang. We weten dat bij slechts 15% van de CHD een bekende oorzaak kan worden aangetoond, zoals genetische of chromosomale afwijkingen en omgevingsinvloeden. Echter, de meeste CHD worden beschouwd als complexe ziekten met een multifactoriële ontstaanswijze, waarbij interacties tussen genetische- en omgevingsfactoren een rol spelen. Recente onderzoeken hebben aangetoond dat stoornissen in de homocysteine stofwisseling mogelijk betrokken zijn bij het ontstaan van CHD. Foliumzuur speelt hierin een belangrijke rol en is ook een methylgroep-donor. Het is een van de B-vitamines die al sinds 1950 geassocieerd is met neurale buis defecten, zoals spina bifida, maar ook met uitstroomdefecten van het hart. Meerdere epidemiologische onderzoeken hebben aangetoond dat het gebruik van foliumzuur bevattende supplementen tijdens de zwangerschap het risico op het krijgen van een kindje met CHD substantieel verlaagd.

De methylstofwisseling bestaat uit twee gekoppelde cycli, namelijk de foliumzuur en de homocysteine stofwisseling. Homocysteine wordt geremethyleerd tot methionine, dat vervolgens wordt omgezet in S-adenosylmethionine (SAM). Door het afgeven van zijn methylgroep wordt SAM omgezet in S-adenosylhomocysteine (SAH). Deze methylgroep wordt door methyltransferases, zoals nicotinamide N-methyltransferase (NNMT), gebonden aan bijvoorbeeld DNA, eiwitten of vetten. Een te lage inname van B-vitamines of functionele variaties in sleutelenzymen kunnen leiden tot een hoog homocysteine gehalte in het bloed en een gestoorde methylstofwisseling. Het is niet bekend of het hoge homocysteine gehalte of juist

vatting

de gestoorde methylering een oorzaak is voor een afwijkende hartontwikkeling. Omdat de [redacted] moeder de omgeving is van het kind dat zich in haar baarmoeder ontwikkelt, zal een gestoorde methyleringsstatus bij de moeder, door zowel functionele variaties in de B-vitamine gerelateerde genen als door een verminderde B-vitamine inname, ook de methyleringsstatus in de embryonale weefsels kunnen beïnvloeden. Bovendien kunnen de genetische variaties bij moeder en kind interacties aangaan met de maternale voedingsstatus. Onze hypothese is dan ook dat de hartontwikkeling beïnvloedt kan worden door expressie van maternale en/of foetale functionele genetische variaties die betrokken zijn bij de methylstofwisseling, alleen of in interactie met de maternale voedingsstatus.

De meeste gepresenteerde resultaten zijn afkomstig uit de HAVEN-studie, wat een acroniem is voor het onderzoek naar CHD en de rol van genetische- en voedingsfactoren. Deze patiënten-controle studie wordt sinds 2003 uitgevoerd door de afdeling Verloskunde en Vrouwenziekten, subafdeling Verloskunde en Prenatale Geneeskunde van het Erasmus MC te Rotterdam. Kinderen met een CHD en hun beide ouders worden uitgenodigd om mee te doen in samenwerking met de Kindercardiologische afdelingen van het Erasmus MC, het Leids Universitair Medisch Centrum, het VU Medisch Centrum en het Academisch Medisch Centrum in Amsterdam. De gezonde controle kinderen en hun beide ouders worden uitgenodigd via de consultatiebureaus van 'Thuiszorg Nieuwe Waterweg Noord' in de omgeving van Rotterdam.

Deel I

Het eerste deel van dit proefschrift beschrijft twee methodologische vraagstukken. De studie die we in *Hoofdstuk 2* presenteren, is uitgevoerd binnen de FOLFO-studie. Dit is een prospectieve studie opgezet om de rol van voeding en leefstijl op fertiliteit te onderzoeken. In dit hoofdstuk hebben we de voedingsstatus van de moeder van vóór de zwangerschap vergeleken met de voedingsstatus van 1 jaar na de geboorte van het kind. Door middel van deze studie hebben we het gestandaardiseerde studiemoment dat we in de HAVEN-studie gebruiken kunnen valideren. Bij 30 vrouwen hebben we op beide momenten bloed afgenomen en algemene- en voedingsvragenlijsten laten invullen. In het bloedplasma werden de concentraties van totaal homocysteine (tHcy), foliumzuur, vitamine B6 en B12 gemeten. De algemene vragenlijst omvat informatie over etniciteit, educatie en leefstijl, zoals bijvoorbeeld roken en het slikken van foliumzuurbevattende vitaminesupplementen. De voedingsfrequentie vragenlijst (FFQ), die ontwikkeld is in samenwerking met de Divisie Humane Voeding van de Wageningen Universiteit in Wageningen, is speciaal gericht op het schatten van inname van energie, voedingsstoffen en B-vitamines. Uit dit onderzoek blijkt dat de concentraties van tHcy, vitamine B6 en B12 niet significant verschillen tussen beide studiemomenten. De foliumzuur concentratie daarentegen, is significant hoger vóór de zwangerschap dan 1 jaar na de geboorte. Dit is geheel te verklaren door het significant hogere gebruik van foliumzuur bevattende supplementen vóór de zwangerschap. De gerapporteerde voedingsinname is vergelijkbaar tussen beide studiemomenten, met uitzondering van alcohol, koolhydraten, vitamine B2 en retinol. Hieruit kunnen we concluderen dat de voedingsstatus van vrouwen vóór de zwangerschap vergelijkbaar is met de voedingstatus 1 jaar na de geboorte van hun kind.

In *Hoofdstuk 3* onderzoeken we bij 336 vrouwen in de vruchtbare leeftijd welke factoren bepalend zijn voor de concentratie van tHcy, SAM en SAH in het bloed. Hierbij bestuderen we leefstijl-, voedings- en genetische factoren. Naast de concentraties van tHcy, SAM en SAH hebben we ook die van folaat en vitamine B12 gemeten. Uit dit onderzoek blijkt dat de concentraties van vitamine B12 en folaat niet gecorreleerd zijn met SAM en SAH, maar wel met tHcy. Verder vinden we dat BMI de belangrijkste factor is voor het voorspellen van SAM en in mindere mate van SAH, waarvoor ook vitamine B6 een belangrijke determinant is. De factoren die de tHcy concentratie bepalen zijn de 5,10-methyleentetrahydrofolaat reductase (MTHFR) 677TT genvariatie, gebruik van vitamine B bevattende supplementen en eiwit inname. Concluderend kunnen we zeggen dat de factoren die bepalend zijn voor SAM en SAH duidelijk verschillen van de factoren die bepalend zijn voor tHcy. Dit is met

name belangrijk voor vervolg onderzoek naar de associaties tussen ziekten en SAM/SAH, en dus methylering, aan de ene kant en tHcy, of hyperhomocysteinemie, aan de andere kant.

Deel II

Dit deel beschrijft de associaties tussen CHD en de biomarkers, die een maat zijn voor de cellulaire methyleringsstatus. In *Hoofdstuk 4* hebben we de concentraties van tHcy, SAM en SAH gemeten in het bloed van 231 moeders van patiënten, de patiëntengroep, en 315 moeders van gezonde kinderen, de controlegroep. Alle analyses zijn gecorrigeerd voor het gebruik van B-vitamine bevattende supplementen. De concentratie van tHcy blijkt significant verschillend tussen de totale patiëntengroep en de controlegroep. Een verschil dat bijna geheel wordt verklaard door de patiëntengroep met een niet-geïsoleerde CHD. De subgroep van moeders met kinderen met het Down Syndroom en een CHD laat niet alleen een significant hoger tHcy zien, maar ook een hoger SAH en een lagere SAM/SAH ratio dan de controlegroep. Het lijkt er dus op dat maternale hyperhomocysteinemie een risicofactor is voor CHD, maar dat een maternale hypomethyleringsstatus geassocieerd is met een hoger risico op kinderen met Down Syndroom en een CHD.

In *Hoofdstuk 5* hebben we dezelfde biomarkers gemeten in het bloed van de kinderen en vergeleken tussen 143 kinderen met een CHD en 186 gezonde kinderen. We hebben ook de concentraties van folaat, vitamine B6 en B12 gemeten en de twee functionele genetische variaties in het MTHFR gen, namelijk C677T en A1298C. Alle analyses zijn gecorrigeerd voor leeftijd, medicatie en vitamine gebruik en CHD in de familie. De subgroep van niet-geïsoleerde CHDs liet de hoogste concentraties van SAM, SAH en folaat zien, vergeleken met de controlegroep. We zagen ook dat de SAM, SAH en folaat concentraties onderling gecorreleerd zijn. De hogere methyleringsstatus kon niet verklaard worden door dragerschap van één van de MTHFR varianten. Kortom, het lijkt erop dat een status van cellulaire hypermethylering in jonge kinderen geassocieerd is met niet-geïsoleerde CHD en voornamelijk met syndromale CHD. De hoge folaat status lijkt daarbij de belangrijkste determinant te zijn. Uiteraard moeten we verder uitzoeken of deze verhoogde methyleringsstatus geassocieerd is met CHD of met een onderliggende (epi)genetische aandoening.

Deel III

Het laatste deel van het proefschrift beschrijft de genetische- en omgevingsinteracties in relatie tot CHD. In *Hoofdstuk 6* hebben we de MTHFR C667T en A1298C varianten bij 230 patiënten en 251 gezonde kinderen met hun beide ouders vergeleken. Daarnaast hebben we getest of de maternale voedingsinname van folaat en vitamine B2 geassocieerd is met CHD en of er een interactie bestaat tussen de verschillende factoren. De MTHFR C677T variaties zijn niet significant geassocieerd met de kans op CHD. Echter, de MTHFR 1298 AA genotypen in vaders en kinderen zijn significant geassocieerd met een verhoogd risico. Bovendien wordt een tweemaal verhoogd risico op CHD gedemonstreerd in kinderen met het MTHFR 1298 AA genotype, die periconceptioneel niet zijn blootgesteld aan foliumzuur bevattende supplementen in vergelijking met kinderen die het MTHFR 1298 AC of CC genotype hadden en wél waren blootgesteld aan de supplementen. Er worden geen significante interacties aangetoond tussen de MTHFR genotypen 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 in een dataset van 230 patiënten en 251 controles. 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, maar dit wordt niet verstoord door het huidige gebruik van B-vitamine bevattende 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 op CHD. Echter, deze interacties zijn statistisch niet significant.

In *Hoofdstuk 8* hebben we naar drie gerelateerde factoren gekeken die geassocieerd zijn met de methylstofwisseling. Een genetische variatie in het nicotinamide N-methyltransferase (NNMT) gen bij zowel de kinderen, moeders en vaders, periconceptioneel gebruik van medicatie en een lage voedingsinname van vitamine B3 door de moeder. We zien geen significante verschillen voor de NNMT variaties tussen 292 patiënten, moeders en vaders en 316 controle kinderen, moeders en vaders. Daarentegen, periconceptioneel gebruik van medicijnen door de moeder geeft een anderhalf keer significant grotere kans op CHD en diezelfde significant grotere kans wordt gezien bij lage inname van vitamine B3 via de voeding (≤13,8 mg/dag). Opvallend is dat de combinatie van deze drie factoren een bijna driemaal zo hoog risico geeft als de moeder het NNMT

AG/AA genotype heeft en zelfs een bijna negenmaal zo hoog risico op CHD als het kind zelf het NNMT AG/AA genotype heeft. Deze resultaten zijn niet eerder gepubliceerd en dienen dan ook in nog grotere studies bevestigd te worden.

In de algemene discussie worden de resultaten besproken en de doelstellingen geëvalueerd (*Hoofdstuk 9*). Bovendien bevelen we toekomstig onderzoek aan en bediscussiëren de implicaties voor de kliniek en gezondheidszorg. Maternale hyperhomocysteinemie draagt bij aan de kans op CHD. Maternale hypomethylering lijkt een risico te zijn voor CHD en Down Syndroom, terwijl juist hypermethylering bij de kinderen met een niet-geïsoleerde CHD een rol lijkt te spelen. Als de hypomethyleringsstatus van de moeder ook tijdens de zwangerschap aanwezig zou zijn geweest, dan zou de hypermethyleringsstatus bij de kinderen verklaard kunnen worden door epigenetische mechanismen. Dit is een interessant concept waarbij de gedachte is dat blootstelling aan slechte voeding en metabole verstoringen tijdens de zwangerschap kunnen leiden tot stofwisselingsstoornissen in het zich ontwikkelende kind. De resultaten laten zien dat de interactie tussen de MTHFR A1298C variaties en folaat status en mogelijk ook de interactie tussen de TC C776G en MTRR C776G variaties en vitamine B12 status bijdragen aan de kans op CHD. Nog een voorbeeld van een dergelijke gen-omgevinginteractie is het onderzoek waarin we laten zien dat kinderen met een NNMT AG/AA genotype, die tijdens de zwangerschap zijn blootgesteld aan medicatie door de moeder en een lage vitamine B3 inname, een bijna negenmaal zo hoog risico hebben op CHD. Uitgaande van deze resultaten kunnen we zeggen dat genetische variaties die een rol spelen in de methylstofwisseling, samen met omgevingsinvloeden, zoals medicijngebruik en een suboptimale vitamine B status, betrokken zijn bij de ontstaanswijze van CHD. Daarom zou het gunstig kunnen zijn om vrouwen in hoog risico groepen een dieet rijk aan vitamine B3 en B12 te adviseren in de periconceptionele periode en wellicht ook een supplement dat niet alleen foliumzuur, maar ook vitamine B12 en B3 bevat. We moeten aan de andere kant wel voorzichtig zijn met te hoge doseringen, omdat onze resultaten ook suggereren dat foliumzuursupplementen samen met bepaalde genetische variaties het risico op CHD ook kunnen verhogen. De resultaten van dit proefschrift kunnen direct worden toegepast in de preconceptie zorg.

Op dit moment worden bovenstaande aanbevelingen al geïmplementeerd binnen de afdeling 'Verloskunde en Vrouwenziekten' van het Erasmus MC, Universitair Medisch Centrum in Rotterdam door middel van een speciaal spreekuur genaamd "Gezond zwanger worden'. Dit spreekuur is er speciaal op gericht om aanstaande ouders met een zwangerschapswens te begeleiden en preconceptionele zorg te verlenen in het bijzonder met betrekking tot gezonde voeding en leefstijl. Dit zal hopelijk in de toekomst bijdragen aan het verminderen van aangeboren afwijkingen.

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Het is zondagmiddag en over twee dagen gaat het manuscript naar de drukker. De rest is klaar... nu het dankwoord nog: het meest gelezen stukje tekst van een proefschrift. Het is leuk om te schrijven; leuk om terug te gaan in de tijd en alle ups en downs nog eens de revue te laten passeren.

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Na een jaar in het Hs-gebouw, werd ik 'geüpgrade' naar de 22^{e} etage in het faculteitsgebouw. Nicolette, Piet, Marianne, Els, Annelous, Fatima en Marijana: dank voor de geweldige tijd! Ik zal de gezellige lunches, koffieleutkwartiertjes, spannende weekendverhalen en love stories missen! Ook jullie heel veel succes met al jullie ambitieuze plannen. Een tweetal mensen wil ik in het bijzonder noemen. Anneke: punctueel,

woord

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Curricul

um Vitae

Lydi van Driel was born on the 7th of June 1983 in Arnhem, the Netherlands. She grew up in Brummen and passed her secondary school in 2001 at the 'Baudartius College' in Zutphen. Because she was not lucky in her first attempt to pass the nation-wide lottery to start medical school, she started working as a care assistance in an elderly home in Brummen for about 6 months. The other 6 months, she worked as an X-ray assistant in the 'Gelre Ziekenhuizen' hospital in Apeldoorn. In the summer of 2002, she passed the decentralised selection at the Erasmus University Rotterdam through which she could start her medical study. During this period she did an internship at the 'Hospital Universitario Dr. Jose Eleuterio Gonzalez' in Monterrey, Mexico,. In 2005, she was selected to start the part-time Master of Science program in 'Clinical Epidemiology' at the Netherlands Institute for Health Sciences (Nihes) in parallel with the medical curriculum. In 2007, she obtained both her 'doctoraal' degree in medicine and her Master of Science degree in Clinical Epidemiology. She performed a scientific research project for her Master of Science thesis at the Department of Obstetrics and Gynecology, Division of Obstetrics and Prenatal Medicine, in close collaboration with the Department of Paediatric Cardiology of the Erasmus MC, University Medical Center in Rotterdam. Subsequently, she was appointed as junior researcher at the abovementioned departments to continue the studies that are described in this thesis. In September 2008, she started her internships for medical school. She lives together with Jan-Wiebe Korstanje in Delft.

PhD po

rtfolio

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Copromotor: Dr. R.P.M. Steegers-Theunissen

Activity	Year	ECTS
General academic skills		
Biomedical English Writing and Communication (Erasmus MC)	2007	3.0
International conferences		
Society for Gynecologic Investigation (SGI), Reno, USA	2007	1.0
Society for Gynecologic Investigation (SGI), San Diego, USA	2008	1.0
Association for European Paediatric Cardiology (AEPC), Venice, Italy	2008	1.0
Presentations		
SGI (2 posters)	2007	0.6
Wetenschapsdag 'Ruimte voor de Toekomst', Erasmus MC (poster)	2007	0.3
Onderzoeksdag Sophia Kinderziekenhuis (oral)	2007	0.7
SGI (oral + poster)	2008	1.0
AEPC (oral + moderated poster walk)	2008	1.5
Nederlandse Vereniging voor Kindergeneeskunde (oral)	2008	0.7
Reprotox (oral)	2008	0.7
Teaching Activities		
Supervising Master's thesis, Leonie Zwolle, EUR, Rotterdam	2007	2.0
Supervising Master's thesis, Sylvia Borst, Nihes, Rotterdam	2008	2.0
Total		**15.5**

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