Congenital Heart Disease

Vascular Risk Factors and Medication

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Chapter 1

General introduction

Congenital heart disease (CHD) is among the most common congenital abnormalities and involves structural anomalies of the heart and/or related major blood vessels. Congenital heart disease arises in the first trimester of pregnancy, occurring often and in many forms. The reported CHD birth prevalence rate ranges from 6 per 1,000 newborns in the Netherlands to 9 per 1,000 newborns in the United States of America.[1,2] This percentage is much higher if fetal deaths are taken into account.[3] CHD is the leading cause of infant mortality[4] and contributes to 30% to 40% of all deaths during infancy and early childhood.[5] The morbidity varies with the severity of the CHD and can be quite serious and life threatening. The multiple surgeries needed to correct the anatomical defects can be debilitating. Furthermore, the quality of life of these patients is often compromised due to severe physical as well as psychological problems. Having a child with CHD is also a source of great concern for parents and significantly affects the quality of life in the families as well.[6] Moreover, in 1992 the estimated average lifetime cost of the most clinically important CHD ranged from $262,000 to $505,000 per new case in the United States of America.[7] Over the past decades the diagnosis, medical care and surgery have considerably improved the prospects for children with CHD and resulted in a significant decrease of CHD mortality and morbidity.[8] Nowadays approximately 85% of children with CHD survive. Therefore, the primary prevention of CHD would be a big step forward, being only possible when more insight is gained into the embryogenesis of the heart and the role of genes and environmental factors.

Embryology

Human embryonic heart development takes place between the third and eighth week of gestation.[9] Knowledge of the cardiovascular development largely originates from animals, among which the chick embryo is one of the most frequently used models. During early embryonic development, the bilateral cardiogenic plates, which are part of the left and right splanchnic mesoderm, fuse and form an initially straight heart tube. This primitive heart tube is composed of two distinct layers, the endocardium and myocardium, separated by extracellular matrix called cardiac jelly.[10] After cardiac looping, endocardial cushion swellings derived of the cardiac jelly become populated by valve precursor cells originating from a process called epithelial-mesenchymal transformation (EMT). The endocardial cushions are named according to the region in which they develop: the outflow tract cushions and atrioventricular cushions.[11] The formation of the atrial and ventricular septum together with rearrangement of

the inflow and outflow tract separates the heart into four chambers. The septum is primarily muscular but also comprises a small membranous part, which is formed by the atrioventricular cushions and the proximal end of the fused outflow cushions.[12] The outflow tract is divided into the ascending aorta and the pulmonary trunk and aligned to the left and right ventricle, respectively. Migration of extracardiac cell populations, such as neural crest cells, significantly contribute to the condensed mesenchyme of the aorticopulmonary septum and thereby the definitive outflow tract.[13,14] Precise control of processes such as neural crest cell migration, differentiation, proliferation, apoptosis and intracellular signaling are essential for correct formation and modeling of the embryonic heart.[15] Finally, the aorta with its aortic valves is continuous with the left ventricle, the pulmonary trunk with its pulmonary valves originates from the right ventricle, while the outflow tract is separated by the continuous outflow and ventricular septum.

Classification

CHDs appear in many forms and affect most parts of the heart. Cardiomyopathies, conduction-system disease and laterality defects, are inherited and sometimes present at birth, but are often not considered CHDs because of their distinct clinical presentation. CHDs are associated with other anomalies or occur as part of a syndrome, but are most frequently present as an isolated malformation. Classification for the purpose of aetiological studies is both necessary and challenging. CHDs are heterogeneous and can be classified anatomically, clinically, epidemiologically and developmentally. Ideally, an approach based on underlying developmental mechanisms can provide a rational basis for aggregating cases and preserve internal homogeneity, but the knowledge of such mechanisms, while advancing, is far from complete. In the studies described in this thesis, we aimed to identify new potentially modifiable risk factors for CHDs. Therefore, we choose to select CHD phenotypes based on experimental and epidemiologic research that showed that hyperhomocysteinaemia and related gene-environment interactions are involved in the aetiology.[16-18]

Aetiology

Since decades, CHD has been a focus of much research in embryology and genetics. However, the identification of environmental risk factors and public health strategies for

prevention has lagged far behind. Recent studies on CHD still cite that approximately 80-90% of the cases encountered at birth have a multifactorial origin.[2] This implies that for the majority no single causative mechanism or agent is known. Rather, a general interaction model of genetic susceptibility and intra-uterine exposures is proposed. A genetic component of CHD was initially implicated by CHD recurrences in families.[19] The pattern of genetic inheritance was often not clear, however, as apparently different CHD phenotypes arise within one family and mild defects are sometimes discounted or undiagnosed. Chromosomal anomalies such as Trisomy 21, 18, 13 and the 22q11 deletion are the strongest known contributors to CHD. Moreover, genetic studies in humans and knock-out embryos have identified numerous genes, such as TBX5, NKX2-5, GATA4, CX43, NOTCH1 and VEGF that are responsible for both inherited and sporadic cases of CHD.[20] Epidemiological data also point to environmental influences. Currently, identified environmental risk factors for CHD are maternal illnesses such as diabetes mellitus, phenyl ketonuria, obesity and lifestyle factors such as nutrition, exposure to toxins, i.e. nicotine and alcohol, and medication use.[21] Research on the role of nutrition in the aetiology of CHD has mainly been focused on maternal use of multivitamin supplements containing folic acid and several epidemiological studies reported a protective effect.[22-24] A low dietary intake of vitamin B12 may also be an independent risk factor for CHD.[25] The B-vitamins folate and vitamin B12 are important substrates in the homocysteine pathway (Figure 1). Animal studies have demonstrated the involvement of the homocysteine pathway in heart development. In chick embryos, homocysteine was shown to stimulate the proliferation and migration of neural crest cells and exposure to mild hyperhomocysteinaemia resulted in CHD in most of these embryos.[18,26] Also in humans, maternal hyperhomocysteinaemia has been associated with a three-to-ten fold increased risk of CHD.[16,17,27] Plasma total homocysteine concentrations are maintained within narrow ranges by efficient functioning of several genes encoding enzymes that participate in the homocysteine pathway, such as methylene tetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR) and cystathionine β-synthase. Moreover, besides folate and vitamin B12, also vitamin B2 (riboflavin) and B6 (pyridoxine) determine efficient remethylation of homocysteine into methionine because these micronutrients are cofactors or substrates in this pathway (Figure 1). Compromised concentrations of these vitamins result in mild to moderate hyperhomocysteinaemia.
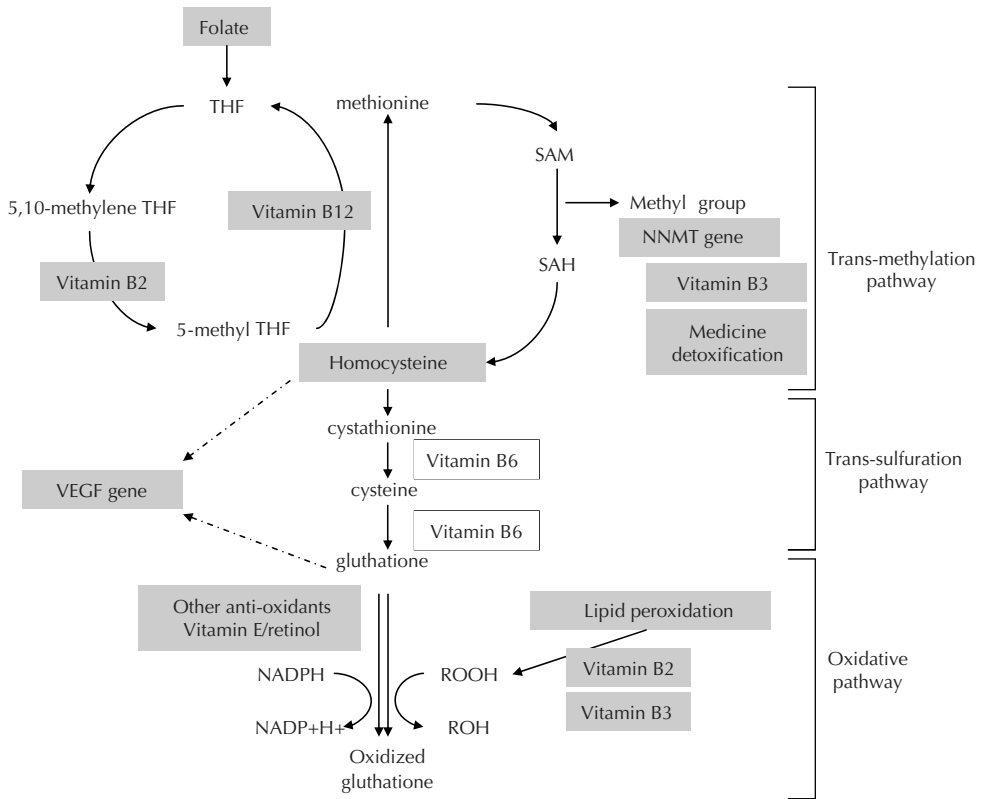
Figure 1. Homocysteine pathway and oxidative balance. THF, tetrahydrofolate. SAM, s-adenosylmethionine. SAH, s-adenosylhomocysteine. NNMT, nicotinamide N-methyl-transferase. VEGF, vascular endothelial growth factor. NADPH, nicotinamide adenine dinuc-cleotide phosphate-oxidase. ROOH, lipid hydroperoxides.

The relationship between maternal nutrition and fetal development is complex, involving numerous factors that contribute to or confound the effects of nutrition on the immediate-, intermediate- and long-term health of the human fetus. It is now widely accepted that the incidence of cardiovascular disease in adulthood can have its roots in the early stages of fetal development.[28] Recently, several studies have suggested that the atherogenic process begins during fetal life, possibly as a result of maternal and fetal hypercholesterolemia, which contributes to the development and progression of atherosclerosis during the perinatal period and increases long-term susceptibility of offspring for adult coronary heart disease.[29] It is intriguing to hypothesize that the same

maternal nutritional and metabolic derangements predispose to cardiovascular disease in adult life[30] and also affect the vascular structures of both embryonic and placental tissues, resulting in pregnancy complications such as CHD. Hyperhomocysteinaemia, hyperglycaemia and obesity are nice examples of metabolic derangements influencing both adult cardiovascular health and the development of CHD. In addition, also maternal hypercholesterolemia might result in both fetal and adult atherosclerosis and CHD in the offspring. During early embryogenesis, fatty acids play an important role in formation of membrane lipids and are involved as transcription factors in the regulation of gene expression. Oxidation of fatty acids is dependent of vitamin B2 (riboflavin), also cofactor in the homocysteine pathway, and B3 (nicotinamide). Interestingly, maternal use of the western dietary pattern, i.e., high saturated fats and low B-vitamins including riboflavin, increases the risk of orofacial clefts almost two-fold.[31] Furthermore, low dietary intakes of riboflavin and nicotinamide have been associated with an increased risk of orofacial clefts and spina bifida. [32, 33] These malformations share similarities in the pathogenesis of CHD, as all three originate from disturbances in neural crest cell behavior by hyperhomocysteinaemia and low folate. Therefore, it is conceivable that these nutritional factors play a role in the aetiology of CHD as well.

Hyperhomocysteinaemia is associated with alterations in the transsulfuration pathway resulting in lower levels of the endogenous anti-oxidant glutathione.[34] The latter is substantiated by the finding that oxidized glutathione, a marker of oxidative stress, was increased in mothers with CHD affected pregnancies.[35] Besides endogenous antioxidants, also exogenous antioxidants such as vitamin E and retinol are necessary to counteract damage from reactive oxygen species. Vitamin E and retinol are essential lipid soluble vitamins and play a role in immune mechanism, growth and cellular differentiation[36], while lipids are the building blocks of cell membranes and are involved in gene regulation. Insufficient intake of vitamin E and to a lesser extent retinol might lead to oxidative stress due to a reduced anti-oxidant defense. However, high doses can also act as pro-oxidant. Therefore, low dietary intake of antioxidants may be harmful to the developing embryo, but also excessive intakes may be associated with potential risks. In addition, also an unfavorable lipid surplus due to a high cholesterol diet disturbs the precise oxidative balance by altering lipid levels and lipid peroxidation as well as glutathione levels and antioxidant enzyme activities.

Elevated homocysteine concentrations result from inadequate intake of B-vitamins as well as from subtle variations in genes, such as methylene tetrahydrofolate reductase (MTHFR).[37] Recently, nicotinamide N-methyltransferase (NNMT) was identified as a new candidate gene for hyperhomocysteinaemia.[38] The NNMT enzyme is involved

in the conversion of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH), intermediates in the homocysteine pathway, and catalyses the N-methylation of nicotinamide and other pyridines. The NNMT enzyme is also important for the detoxification of medicines that undergo methylation via methyltransferases.

Since the thalidomide disaster, several other drugs have been recognized as a cardiac teratogen. Maternal intake of isotretinoin, a retinoid, has been shown to cause CHDs in addition to other malformations.[39] Also ACE-inhibitors, anticonvulsants, trimethoprim, non-steroid anti-inflammatory drugs and antihistamines are among the drugs that have been suggested to increase the risk of heart anomalies.[21] Antihistamines are the first choice medicines for nausea and vomiting during pregnancy, symptoms that affect 60-80% of pregnant women and that primarily occur during the sensitive period of cardiogenesis. Antihistamine medication is widely prescribed to pregnant women although its safety has not been established explicitly and studies reported conflicting results.[40,41]

Homocysteine is also involved in the regulation of several genes, of which vascular endothelial growth factor-A (VEGF) is one example. Recent data suggest a critical role for VEGF during heart development. VEGF heterozygous knockout mice showed impaired blood vessel formation and died at mid-gestation.[42] A key process in the development of heart septa and valves is the formation of the endocardial cushions in the atrioventricular canal and outflow tract. Numerous lines of research have implicated a strict spatio-temporal expression pattern of VEGF in the control of endocardial cushion development.[43] In human, VEGF expression shows a marked interindividual variability that is partly determined by single nucleotide polymorphisms (SNPs). First studies suggest associations between the VEGF SNPs -2578 C/A, -1154 G/A and -634 G/C and several CHD phenotypes, i.e., Tetralogy of Fallot, perimembranous ventricular septal defects and valvuloseptal defects.[44-46] Besides homocysteine, several other maternal exposures regulate VEGF expression during cardiogenesis such as hyperglycaemia and hypoxia. Despite potential differences with human embryology, chick embryos are a good model to study the influence of environmental exposures on VEGF expression and the development of CHDs and unravel new mechanisms in their aetiology. The stages of heart development in human and chick are largely comparable and well described and the chicken embryo is easy accessible for manipulations without the need to sacrifice the mother animal.

Objectives of the thesis

The main goal of the work presented in this thesis was to unravel the impact of periconceptional maternal lifestyle factors and genetic determinants, partly related to the homocysteine pathway, in the aetiology of CHD offspring. New information may in future contribute to improvement of the periconception and intrauterine environment thereby providing the optimal condition for reproductive performance and prevention of CHD.

Specific objectives were:
1. To assess associations between the maternal lipid status, comprising lipid concentrations in blood, dietary intake of fatty acids, related vitamins, and the risk of CHD in the offspring (Part I).
2. To study both maternal exposure to medicines, particularly antihistamines for nausea and vomiting, in the first 10 weeks of pregnancy and the interaction between medication use and a new polymorphism in the NNMT gene and the risk of CHD (Part II).
3. To investigate whether three functional polymorphisms in the VEGF gene are associated with human cardiac outflow tract defects and to study the expression of VEGF and its receptor VEGFR2 in the chicken embryonic heart (Part III).

Chapter Outline

This thesis displays the results of the HAVEN study, which is an ongoing case-control family study in the Western part of the Netherlands. This study was designed to investigate environmental and genetic determinants in the pathogenesis and prevention of CHD and was carried out from June 2003 onwards at the Department of Obstetrics and Gynaecology / Division of Obstetrics and Prenatal Medicine of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands. For the recruitment of cases and controls, we collaborated with the Departments of Paediatric Cardiology of the same hospital, Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Center in Amsterdam and with the child health centres of 'Careyn' in the surroundings of Rotterdam.

The first part of this thesis describes the maternal nutritional and biochemical lipid status and related vitamins in association with the risk of having a child with

CHD. **Chapter 2** focuses on concentrations in the maternal blood of lipids and total homocysteine because these biomarkers partly reflect the effects of the nutritional intake and lipid metabolism. Fatty acids play a central role in embryonic development, and the B-vitamins riboflavin and nicotinamide are co-enzymes in lipid metabolism. Therefore, in **Chapter 3**, we explored the association between the maternal dietary intake of fats, riboflavin and nicotinamide, and CHD risk. In **Chapter 4**, we assessed the role of dietary and supplement intake of the lipid soluble antioxidants vitamin E and retinol as a risk factor for CHD in the offspring.

Part II of this thesis addresses the maternal use of medication in the periconception period as a risk factor for CHD. In **Chapter 5**, we present data on the interactions between a new polymorphism in the nicotinamide N-methyltransferase (NNMT) gene, low maternal dietary nicotinamide intake, and medication use in the periconception period. **Chapter 6** shows an analysis of the maternal intake of antihistamines, indicated for nausea and vomiting, during the first trimester of pregnancy.

Part III concerns the vascular endothelial growth factor (VEGF) gene, which is essential for endocardial cushion formation. In **Chapter 7**, we investigated whether three functional polymorphisms in the VEGF gene are associated with cardiac outflow tract defects. In **Chapter 8**, we present results on the expression of VEGF and VEGFR2 in the chick embryonic heart by using the new Optical Projection Tomography (OPT) technique to produce high-resolution 3-dimensional images. **Chapter 9** provides a general discussion on the combined results of the studies in a broader perspective. We recommend future research and elaborate on the implications regarding preconception care.

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Part I

Maternal lipids and related vitamins

Chapter 2

A derangement of the maternal lipid profile is associated with an elevated risk of congenital heart disease in the offspring

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Abstract

Background and aims: Maternal hyperglycaemia and hyperhomocysteinaemia are risk factors for congenital heart disease (CHD). Metabolic derangements and deranged lipid levels are associated with adult cardiovascular disease. We examined whether maternal lipid levels are associated with the risk of CHD offspring.

Methods and Results: From 2003 onwards, a case-control study was conducted. Participants were mothers of children with (n=261) and without (n=325) CHD. At around 16 months after the index-pregnancy maternal lipid levels were determined. Maternal characteristics and lipid levels were compared by Student's T-test. In a multivariable logistic regression model, risk estimates were calculated for associations between CHD and lipid levels. Adjustments were made for maternal age, diabetes, ethnicity, BMI, parity, periconception folic acid use and total homocysteine levels. Outcome measures are presented in (geometric) means (P5-P95) and odds ratios (OR) with 95% confidence intervals (CI).

Case mothers showed higher cholesterol (4.9 vs. 4.7 mmol/L, $P<0.05$), LDL-cholesterol (3.2 vs. 3.0 mmol/L, $P<0.05$), apolipoprotein B (84.0 vs. 80.0 mg/dL, $P<0.01$) and homocysteine (10.8 vs. 10.2 µmol/L, $P<0.05$) than controls. LDL-cholesterol above 3.3 mmol/L (OR 1.6 (95%CI, 1.1-2.3)) and apolipoprotein B above 85.0 mg/dL were associated with an almost two-fold increased CHD risk (OR 1.8 (95%CI, 1.2-2.6)). This was supported by elevated CHD risks per unit standard deviation increase in cholesterol (OR 1.2 (95% CI 1.03-1.5)), LDL-cholesterol (OR 1.3 (95%CI, 1.1-1.6), apolipoprotein B (OR 1.3 (95% CI 1.1-1.6)). Apolipoprotein B was most strongly associated with CHD risk.

Conclusion: A mildly deranged maternal lipid profile is associated with an increased risk of CHD offspring.

Introduction

Worldwide 1 million children are born each year with congenital heart disease (CHD).[1] The majority is thought to result from complex interactions between maternal environmental exposures and genetic susceptibilities.[2] Maternal diabetes, obesity and hyperhomocysteinaemia are features of the metabolic syndrome and are individually associated with increased risks of adverse pregnancy outcome, including CHD offspring.[3-6] It is known that these metabolic derangements as well as hypercholesterolaemia are implicated in the development of adult cardiovascular disease.[7,8] High concentrations of total cholesterol and low-density lipoprotein (LDL) cholesterol in particular increase and high density lipoprotein (HDL) cholesterol reduces the risk of cardiovascular disease.[9] The rising evidence that maternal hyperlipidaemia during pregnancy affects both the fetal and adult cardiovascular system is fascinating.[10] Animal studies have suggested that in utero exposure to a maternal diet rich in fat may lead to an increased risk of cardiovascular disease in the offspring[11] and to endothelial cell dysfunction as an early feature of atherogenesis preceding plaque formation.[12] During embryogenesis and fetal growth lipids are essential for the synthesis of cell membranes. Moreover, lipids act as ligand for receptors and transcription factors involved in intracellular signalling processes and the regulation of gene expression.[13] We hypothesize that derangements in the periconception maternal lipid profile may influence the biological processes implicated in the embryogenesis of the heart.

A prospective preconception study to investigate this association with CHD is not feasible due to its relatively low birth prevalence rate of 6-8/1000, and thereby high financial costs. As second best we therefore conducted a large case-control study with a standard study moment of 16 months after pregnancy which in general reflects maternal nutritional intake, lifestyle and metabolism during the periconception period.[14,15,16] This is supported by studies showing that maternal cholesterol levels return within 12 months after pregnancy to preconception levels[17+18] and apolipoprotein A-I, apolipoprotein B and triglycerides return to the first trimester levels within 6 weeks after pregnancy.[19]

We have previously shown an association between the maternal dietary intake of saturated fats and an increased risk of a child with CHD[20]. Sofar, both animal and human studies focusing on associations between hypercholesterolaemia and CHD are lacking.

From this background, we aimed to investigate associations between maternal lipid levels and the risk of CHD offspring.

Methods

Study population

The design of the HAVEN Study has been published previously.[5] Briefly, this case-control study is conducted from 2003 onwards in the Western part of the Netherlands at the Department of Obstetrics and Gynaecology of the Erasmus MC, Rotterdam, the Netherlands. Children with CHD and both parents are recruited in collaboration with the Departments of Paediatric Cardiology of the Erasmus MC in Rotterdam, Leiden University Medical Centre in Leiden, and VU University Medical Centre and Academic Medical Centre in Amsterdam. Control children and their parents are enrolled in collaboration with the child health centres at which each child in the Netherlands is regularly checked on growth and development. Children with CHD are diagnosed and recruited in collaboration with two paediatric cardiologists and diagnoses are confirmed after birth by echocardiography and/or cardiac catheterization and/or surgery. In control children, however, recruited at the same age and without congenital malformations according to the medical records and regular health checks by the physician of the child health centre, those assessments have not been made. The selection of the CHD phenotypes is based on experimental and epidemiological studies showing that hyperhomocysteinaemia and folic acid use are associated with CHD.[21-23] Included CHD phenotypes comprised of Tetralogy of Fallot (n=38), atrioventricular septal defects (n=24), perimembranous ventricular septal defect (n=73), aortic valve stenosis (n=6), pulmonary valve stenosis (n=38), coarctation of the aorta (n=26), transposition of the great vessels (n=33), hypoplastic left heart syndrome (n=12) and miscellaneous (n=11). Control children without major congenital malformation according to the medical records and from the regular health checks by the physician at the child health centre are randomly selected.

Case and control mothers are invited at the standard study moment of 16 months after the index-pregnancy. Families are not related and are Dutch speaking. We recruited 363 mothers of a child with CHD and 418 control mothers of a healthy child. 82 case and 85 control mothers were excluded because pregnancy, breastfeeding or dieting at the study moment may influence maternal lipid levels. Mothers of whom a lipid concentration was missing due to laboratory failures or the inability to visit the hospital for blood sampling were excluded, i.e., 20 case and 8 control mothers. The levels of the fasting and non-fasting lipid concentrations were not significantly different between cases and controls, cases fasting (n=219) and non-fasting (n=42), controls fasting (n=315) and non-fasting (n=10), respectively. Exclusion of the non-fasting mothers did

not affect the differences in lipid concentrations in cases and controls nor affected the risk estimates. Therefore, we included both fasting and non-fasting case and control mothers in the analyses. This resulted in the final evaluation of 261 case mothers and 325 control mothers. The lipid profiles of a subset of the control mothers have been previously published as control values in a study of patients with polycystic ovary syndrome.[24]

Concentrations of total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides could be determined in 229 case and 320 control mothers, apolipoprotein A-I and apolipoprotein B in 258 case and 322 control mothers, total homocysteine in 210 case and 284 control mothers. The Central Committee of Human Research in The Hague and the Medical Ethical Committees of the participating hospitals reviewed and approved the study protocol. Written informed consent was obtained from all participants.

Data collection

All mothers filled out a questionnaire at home which was checked during the hospital visit by the researcher. The extracted data comprised age, parity, time after index-pregnancy, ethnicity, family history of CHD (first to third degree), pregnancy, breastfeeding and dieting at the study moment, and the use of folic acid and/or (multi)vitamins, alcohol, cigarettes and medication at the study moment and during the periconception period. Ethnicity was classified according to the definitions of Statistics Netherlands.[25] The periconception period was defined as four weeks prior to conception until eight weeks after conception. Periconception vitamin use was defined as daily intake in the whole period. Standardized anthropometric measurements were performed, including maternal height and weight (anthropometric rod and weighing scale; SECA, Hamburg, Germany). Body mass index (BMI) was defined as weight divided by the square of the height and categorized as BMI < 25, 25 ≤ BMI < 30, BMI ≥ 30.

Immediately after blood sampling, an EDTA tube was put on ice and a serum separator tube was kept at room temperature. Both tubes were centrifuged at 4,000 x *g* for ten minutes at 4°C and separated within one hour. All samples were stored at -80 °C and measured anonymously in batches within five months after collection. Serum concentrations of total cholesterol, HDL-cholesterol and triglycerides were analyzed using commercially available assays (Wako Diagnostics, Japan). HDL-cholesterol was analyzed using the direct HDL-assay from Wako diagnostics. Serum concentrations of apolipoprotein A-I and apolipoprotein B were analyzed with a commercially available immunoturbidometric assay (Wako Diagnostics, Japan). All assays were analyzed on a

Cobas Mira auto analyzer. Total plasma homocysteine concentrations were routinely measured by high performance liquid chromatography with reverse phase separation and fluorescence detection. LDL-cholesterol values were calculated using the Friedewald formula.

Statistical analyses
Maternal characteristics are compared between the case and control groups. Age, time after index-pregnancy and BMI are presented as medians and 5th and 95th percentiles, and tested using the Mann-Whitney U test due to skewness. Dichotomous variables are presented as numbers and percentages and tested using the Chi-square test. After natural log-transformation the distributions of total cholesterol, triglycerides, LDL-cholesterol, total cholesterol / HDL-cholesterol and total homocysteine are normalized. Those data are presented as (geometric) means and 5th and 95th percentiles and tested by Student's T-tests. We created upper tertiles of the lipid levels based on the control data and calculated CHD-risk in a multivariable logistic model by comparing the upper tertile to the lower tertiles as reference. A stratified analysis for BMI and periconception smoking is performed. Univariable logistic regression analyses with the lipids and total homocysteine as continuous variables are performed to estimate the relative risk of CHD by calculating odds ratios (OR) and 95% confidence intervals (CI). In the multivariable logistic regression analyses, we adjusted for maternal age, parity, BMI, ethnicity, family history of CHD, periconception smoking, folic acid or (multi)vitamins, diabetes, and separately for homocysteine. Furthermore, we added a backward stepwise logistic regression model to determine which biomarker was independently associated with CHD risk. Statistical analyses are performed using SPSS software, version 12.0.1 (SPSS Inc., Chicago, Illinois). Probability values $P<0.05$ are considered statistically significant.

Results

Tables 1 and 2 summarize the general characteristics and lipid and homocysteine concentrations in the total group of case and control mothers. Overall, maternal age is slightly higher in cases. The study moment of 16.1 months, BMI, ethnicity, CHD family history, smoking and folic acid use are comparable between the groups. 24 case mothers (9%) and 38 control mothers (12%) used cigarettes continuously during pregnancy. One case mother and two controls used insulin and none of them used lipid lowering therapy. Mean total cholesterol (4.9 vs 4.7 mmol/L), LDL-cholesterol (3.2 vs 3.0 mmol/L)

and apolipoprotein B (84.0 vs 80.0 mg/dL) are significantly higher in case mothers than in controls. Triglycerides, HDL-cholesterol and apolipoprotein A-I are comparable between the groups. Total homocysteine is higher in case mothers than in controls (10.8 vs 10.2 mmol/L), which confirms our previously demonstrated association in a subset of these mothers.[5] Furthermore, case mothers show a higher ratio of total cholesterol / HDL-cholesterol (3.5 vs 3.3) and apolipoprotein B / apolipoprotein A-I (0.59 vs 0.56) than controls.

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Table 1. General characteristics and lifestyles among mothers of a child with CHD and control mothers

Mothers	CHD group (n=261)	Control group (n=325)
Demographics		
Maternal age, y	33.1 (25.3-41.5)[1]	32.6 (24.5-39.8)
Parity		
Nullipara	104 (40)	153 (47)
Multipara	157 (60)	172 (52)
Time after index-pregnancy, m	16.1 (13.8-25.5)	16.1 (13.9-22.3)
Body Mass Index (BMI), kg/m^2		
BMI < 25	151 (58)	187 (58)
25 ≤ BMI < 30	70 (27)	98 (30)
BMI ≥ 30	40 (15)	40 (12)
Ethnicity		
Dutch Native	207 (79)	260 (80)
Western immigrant	12 (5)	12 (4)
Non-Western immigrant	42 (16)	53 (16)
Family history of CHD	23 (9)	19 (6)
Lifestyle		
Periconception use of		
Cigarettes (any)	50 (19)	72 (22)
Folic acid and/or (multi)vitamins	122 (47)	166 (51)
Medication	69 (26)	68 (21)
Current use of		
Cigarettes (any)	50 (19)	63 (19)
Folic acid and/or (multi)vitamins	50 (19)	71 (22)
Medication	53 (21)	63 (19)

CHD, congenital heart disease. Values are medians (p5-p95) or numbers (percentage). [1]$P<0.05$. Ethnicity is classified according to the definitions of Statistics Netherlands.[25]

Table 2. Biomarkers in blood from mothers of a child with CHD and control mothers

Mothers	Total CHD group	Control group	Reference values[1]
Biomarkers	(n=229)	(n=320)	
Total cholesterol, mmol/L[1,2]	4.9 (3.6-6.5)[3]	4.7 (3.5-6.4)	<5.0
Triglycerides, mmol/L[2]	0.93 (0.48-1.97)	0.93 (0.50-1.99)	<2.00
HDL-cholesterol, mmol/L	1.46 (0.93-2.15)	1.48 (0.96-2.09)	>1.55
LDL-cholesterol, mmol/L[2]	3.2 (2.1-4.8)[3]	3.0 (1.9-4.6)	<3.2
Total cholesterol / HDL cholesterol[2]	3.5 (2.2-5.4)[3]	3.3 (2.2-5.3)	
	(n=258)	(n=322)	
Apolipoprotein A-1, mg/dL	145.2 (113.1-179.9)	146.5 (114.5-184.7)	>160.0
Apolipoprotein B, mg/dL	84.0 (57.9-115.2)[4]	80.0 (56.4-114.0)	<100.0
Apolipoprotein B/ A-1	0.59 (0.38-0.84)[4]	0.56 (0.36-0.81)	
	(n=210)	(n=284)	
Total homocysteine, μmol/L[2]	10.8 (6.9-19.6)[3]	10.2 (6.4-16.8)	<15.0

CHD, congenital heart disease. [1]Reference values according to the Clinical Chemistry laboratory of the Erasmus MC in Rotterdam, The Netherlands. Values are means (p5-p95), [2]geometric means (p5-p95). [3]$P<0.05$. [4]$P<0.01$.

After adjustment for potential confounders, a lipid profile with apolipoprotein B > 85.0 mg/dL, apolipoprotein B / apolipoprotein A-I >0.6 and cholesterol / HDL-cholesterol >3.5 is associated with an almost two-fold increased CHD risk, which is independent of homocysteine (Table 3). Especially in mothers with a BMI >30, risk estimates for apolipoprotein B > 85.0 mg/dL and LDL-cholesterol >3.3 mmol/L are higher than in mothers with a BMI<30 (OR >2.5), while only total cholesterol / HDL-cholesterol ratio is significant. The confidence intervals are wide due to the low numbers (Figure 1). The associations between CHD risk, in particularly total/HDL cholesterol and apolipoprotein B / A-1 ratio, are markedly higher in mothers who smoked during the periconception period compared with non-smokers (Table 4). Univariable logistic regression analysis reveals that CHD risk increases by each standard deviation increase in total cholesterol, LDL-cholesterol, apolipoprotein B, total homocysteine, and ratio of total cholesterol / HDL-cholesterol and apolipoprotein B / apolipoprotein A-1 (Table 5). None of the covariates confounded the associations between the maternal lipids and CHD risk. Maternal triglycerides, HDL-cholesterol and apolipoprotein A-1 are not associated with CHD risk. An additional backward stepwise logistic regression model shows that apolipoprotein B is independently and most strongly associated with CHD risk, OR 1.4 (95%CI: 1.1-1.7) per standard deviation increase.

Table 3. Associations between CHD risk and maternal lipid and total homocysteine concentrations with and without adjustment for potential confounders

Biomarkers	Cases/controls	Cut-off value	OR (95%CI)	OR (95%CI)[1]	OR (95%CI)[2]	P-value[2]
	n=229/320					
Total cholesterol, mmol/L[3]	102 / 107	>5.0	1.5 (1.02-2.1)	1.5 (1.05-2.2)	1.4 (0.9-2.0)	0.11
Triglycerides, mmol/L[3]	64 / 107	>1.14	0.8 (0.5-1.1)	0.8 (0.5-1.1)	0.7 (0.5-1.1)	0.13
HDL-cholesterol, mmol/L	74 / 107	<1.28	1.0 (0.7-1.4)	0.9 (0.6-1.3)	0.9 (0.6-1.3)	0.47
LDL-cholesterol, mmol/L[3]	97 / 107	>3.3	1.5 (1.05-2.1)	1.6 (1.1-2.3)	1.5 (1.02-2.2)	0.04
Total / HDL cholesterol[3]	104 / 107	>3.5	1.8 (1.2-2.5)	1.9 (1.3-2.7)	1.8 (1.2-2.7)	0.003
	n=258/322					
Apolipoprotein A-1, mg/dL	83 / 108	<135.4	0.9 (0.7-1.3)	1.0 (0.7-1.4)	0.9 (0.6-1.3)	0.43
Apolipoprotein B, mg/dL	122 / 108	>85.0	1.8 (1.3-2.5)	1.9 (1.3-2.7)	1.8 (1.2-2.6)	0.004
Apolipoprotein B/ A-1	117 / 108	>0.6	1.6 (1.2-2.3)	1.7 (1.2-2.5)	1.7 (1.1-2.5)	0.01
	n=210/284					
Homocysteine, µmol/L[3,4]	59 / 57	>12.7	1.6 (1.02-2.4)	1.6 (1.1-2.5)	-	-

OR, odds ratio. CI, confidence interval. Cut-off values are upper or lower tertiles based on control mothers. [1]Adjusted for maternal age, parity, BMI, ethnicity, family history of CHD, periconception smoking, folic acid and/or (multi)vitamin use and diabetes. [2]Adjusted for all before mentioned covariates and total homocysteine. [3]natural log transformed values. [4]Upper quintile based on control mothers.

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Table 4. Maternal lipid profiles in association with adjusted risks of CHD stratified for periconception smoking

	Cases/ controls	Cut-off value	Periconception smoking yes	Periconception smoking no
Biomarkers	n=229/320		n=43/72	n=186/248
Total cholesterol, mmol/L[1]		>5.0	1.0 (0.4-2.4)	1.5 (0.96-2.3)
Triglycerides, mmol/L[1]		>1.14	0.7 (0.3-1.7)	0.8 (0.5-1.2)
HDL-cholesterol, mmol/L		<1.28	1.2 (0.5-2.9)	0.8 (0.5-1.3)
LDL-cholesterol, mmol/L[1]		>3.3	1.7 (0.7-4.0)	1.5 (0.96-2.3)
Total / HDL cholesterol[1]		>3.5	2.8 (1.1-6.9)	1.7 (1.1-2.6)
	n=258/322		n=49/72	n=209/250
Apolipoprotein A-1, mg/dL		<135.4	1.1 (0.4-2.7)	0.8 (0.5-1.3)
Apolipoprotein B, mg/dL		>85.0	2.4 (1.0-5.9)	1.6 (1.1-2.5)
Apolipoprotein B/ A-1		>0.6	3.4 (1.4-8.4)	1.4 (0.9-2.2)
	n=210/284		n=42/62	n=168/222
Homocysteine, μmol/L[1,2]		>12.7	0.7 (0.2-2.6)	1.6 (0.8-3.1)

OR, odds ratio. CI, confidence interval. Cut-off values are upper or lower tertiles based on control mothers. [1]natural log transformed values. [2]Upper quintile based on control mothers. Odds ratios are adjusted for maternal age, parity, BMI, ethnicity, family history of CHD, periconception folic acid and/or (multi)vitamin use and diabetes.

Table 5. Associations between maternal lipids and total homocysteine as continuous variables and CHD risk, adjusted for potential confounders

Biomarkers	Cases/controls	OR (95%CI)	OR(95%CI)[1]	OR(95%CI)[2]	P-value [2]
	n=229/320				
Total cholesterol		1.2 (1.01-1.4)	1.2 (1.03-1.5)	1.2 (1.03-1.5)	0.02
Triglycerides		1.0 (0.8-1.2)	1.0 (0.8-1.2)	1.0 (0.8-1.2)	0.63
HDL-cholesterol		1.0 (0.8-1.1)	1.0 (0.8-1.2)	1.0 (0.8-1.2)	0.92
LDL-cholesterol		1.2 (1.05-1.5)	1.3 (1.1-1.5)	1.3 (1.1-1.6)	0.01
Total/ HDL cholesterol		1.3 (1.01-1.8)	1.4 (1.02-1.9)	1.3 (0.95-1.8)	0.12
	n=258/322				
Apolipoprotein A-1		0.9 (0.8-1.1)	0.9 (0.8-1.1)	1.0 (0.8-1.2)	0.73
Apolipoprotein B		1.3 (1.1-1.5)	1.3 (1.1-1.5)	1.3 (1.1-1.6)	0.01
Apolipoprotein B/ A-1		1.3 (1.1-1.5)	1.3 (1.1-1.6)	1.3 (1.04-1.6)	0.02
	n=210/284				
Total homocysteine		1.2 (1.01-1.)	1.2 (1.01-1.5)	-	-

Odds ratio (OR) with confidence interval (CI) of CHD risk per standard deviation (SD) increase in lipids and homocysteine. One SD total cholesterol = 0.88, triglycerides = 0.47, HDL-cholesterol = 0.36, LDL-cholesterol = 0.82, total cholesterol / HDL-cholesterol = 1.64, apolipoprotein A = 21.7, apolipoprotein B = 17.5, apolipoprotein B / A-1 = 0.14, total homocysteine = 4.2. [1]Adjusted for maternal age, parity, BMI, ethnicity, family history of CHD, periconception smoking, folic acid and/or (multi)vitamin use and diabetes. [2]Adjusted for all before mentioned covariates and total homocysteine.

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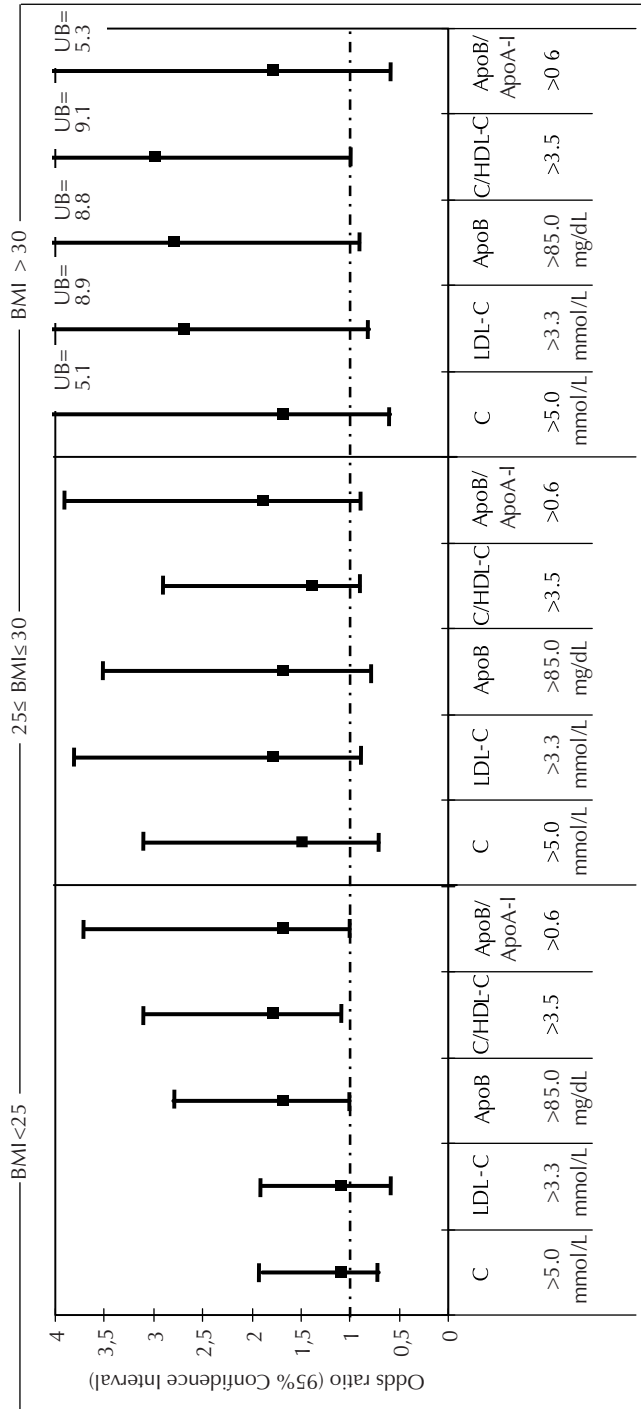
Figure 1. Maternal lipid profiles in association with adjusted risks of CHD stratified for BMI. ApoA-I, apolipoprotein A-I. ApoB, apolipoprotein B. C, total cholesterol. CI, confidence interval. HDL-C, HDL-cholesterol. LDL-C, LDL-cholesterol. OR, odds ratio. UB, upper bound. Cut-off values were based on the upper tertiles based on the control mothers. Odds ratios adjusted for maternal age, parity, BMI, ethnicity, family history of CHD, periconception smoking, folic acid and/or (multi)vitamin use and diabetes.

Discussion

A mildly deranged maternal lipid profile comprising total cholesterol, LDL-cholesterol and total cholesterol / HDL-cholesterol ratio, and in particular apolipoprotein B and apolipoprotein B / apolipoprotein A-I ratio, is associated with an almost two-fold higher risk of a child with CHD, independent of the homocysteine level. Apolipoprotein B is the strongest predictor of CHD risk. These findings are interesting because apolipoprotein B and apolipoprotein A-I are both the main structural proteins of atherogenic lipoproteins and HDL particles, respectively. Apolipoprotein B also reflects the whole spectrum of pro-atherogenic particles and therefore serves as valid biomarker for the atherogenicity of LDL-cholesterol.[26]

So far, associations between deranged maternal lipid profiles and CHD risk have not been reported. When this mildly deranged lipid profile, however, was also present in the periconception period, a causative relationship with CHD is suggested. The causality of this association needs to be further studied in national registries and in mother child cohorts conducted in several countries where blood samples for the determination of the same lipids are available. Our findings are consistent with the higher dietary intake of saturated fats in mothers of a child with CHD compared with control mothers.[20]

We determined biomarkers around the standard study moment of 16 months after the index-pregnancy. This is in contrast to other case control studies in which the study moment is not fixed and varies from 6 weeks to 24 months after birth. Therefore, differences in time after index-pregnancy cannot explain the mildly deranged maternal lipid profile in case mothers. This finding is further substantiated by the fact that we excluded mothers who were again pregnant, breastfeeding and using a diet different from the preconception period. It cannot be excluded, however, that pregnancy itself, as trigger of metabolic stress, may have provoked an underlying sensitivity of the maternal lipid metabolism.[10, 27,28] Therefore, these findings should be interpreted cautiously. Nevertheless, the observed deranged lipid profile in case mothers may be considered as proxy for the maternal risk of developing cardiovascular diseases in later life. Further prospective studies should elucidate whether lipid levels were indeed higher before conception and/or increase more rapidly during the first weeks of pregnancy in mothers of a child with CHD. This will further substantiate the need for preconception lifestyle changes, such as to quit smoking, to increase physical exercise and to use a healthier diet.

An issue inherent to case control studies on congenital malformations is potential recall bias of covariates and misclassification of cases ad controls. The standardized study

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moment relatively soon after pregnancy minimizes this recall bias. The misclassification is limited as most CHD are detected and diagnosed during the first year of life. It cannot be excluded, however, that stress or changes in physical activity in mothers of a child with CHD distort this association. Lipid lowering therapy was not used and therefore is not a confounder. As hyperhomocysteinaemia is an established risk factor for CHD, we adjusted the odds ratios for homocysteine, revealing that hyperhomocysteinaemia did not confound the observed associations between a mild 'atherogenic' lipid profile in mothers and the risk of CHD in their children. Although we cannot completely exclude residual confounding, it seems not very likely.

The clinical relevance of the changes in maternal lipid levels is dependent on the outcome measure. Although the mean differences are relatively small, for example the increase of apolipoprotein B, 17.5mg/dL (one standard deviation) was associated with a 1.3 fold increased CHD risk. An almost two-fold increased risk was demonstrated in mothers with apolipoprotein B levels in the upper tertile, above 85.0 mg/dL, which is below the adult reference value of 100mg/dL. We calculated risk estimates for the lipid levels based on the cut-off values used in our hospital to assess maternal adult cardiovascular risk. Interestingly apolipoprotein B > 120 mg/dL, 3.4% of the case and 1.5% of the control mothers are at risk for cardiovascular disease. Only 4% of case mothers and 3% of control mothers have total cholesterol levels above 6.5 mmol/L. LDL-cholesterol levels are >4.2 mmol/L in 9% of case mothers and 12% of control mothers. Data on embryonic exposure to maternal lipids are not available. It is known, however, that fetal aortas contain more and larger fatty streak lesions from mothers with total cholesterol >5.0 mmol/l compared with normocholesterolaemic mothers.[29] If the same situation is present during cardiogenesis this may suggest that only a minimal increase in lipoprotein levels might disturb normal heart development. The reference values for adult cardiovascular disease risk or congenital heart disease may therefore be different. Furthermore, genetic variations contribute to variation in lipoprotein levels and may increase the susceptibility for cardiovascular disease, even when levels are slightly increased.[30]

A direct effect of an unfavorable maternal lipid profile on embryonic heart development is biologically plausible. Dependent on the genetic background of the embryo, exposure to a slightly deranged maternal lipid profile may have differential effects on genes and tissues, i.e., neural crest, involved in cardiogenesis. Although the embryo can synthesize cholesterol, the majority of cholesterol is derived from the mother via the yolk sac and embryonic coelomic and amniotic fluids. This is substantiated by studies in mice.[31] As apolipoprotein B is abundantly expressed in the yolk sac of rodents[32]

and humans[33], it is likely that apolipoprotein B – containing lipoproteins participate in lipid transport to the embryo. In mice, a maternal diet high in saturated fats resulted in dyslipidemia in the offspring, programmed by suppression of hepatic expression of LDL-receptor mRNA.[34] Also in a mouse model, a maternal high fat diet primed offspring fatty liver disease, mediated through impaired mitochondrial metabolism and up-regulated hepatic lipogenesis, inflammatory pathways and oxidative stress.[35] It is possible that these changes are already present during cardiogenesis. In our study, oxidative stress may be increased in mothers of a child with CHD as total homocysteine, sometimes used as biomarker of oxidative stress[35], was higher in this group. This is supported by Hobbs et al. demonstrating that biomarkers of oxidative stress involved in the transsulfuration pathway were higher in mothers of a child with CHD than in controls.[37] Cell machinery crucial for normal cardiogenesis can be disturbed via complex mechanisms, including oxidation sensitive signalling pathways regulating gene expression and translation. For example, oxidized LDL-cholesterol modulates the expression of genes involved in cell differentiation and proliferation regulated by nuclear factor kappa B and influences the expression of apoptotic factors activated through Fas and TNF receptors and c-Myc-dependent transcription factors.[38,39] Moreover, oxidized LDL regulates the expression of vascular endothelial growth factor, a crucial regulator of endocardial cushion formation.[40] Thus, a window of vulnerability may exist during which slightly elevated maternal lipid levels in combination with excessive oxidative stress in the mother and embryo may result in disturbed cardiogenesis.

Here, we show that mildly elevated maternal cholesterol, LDL-cholesterol and apolipoprotein B are significantly associated with an almost two-fold increased risk of CHD offspring. Studies on subtle genetic variations in lipid pathways and prospective studies are needed to show the causality of these associations, and in specific CHD phenotypes. Moreover, relationships between mildly elevated maternal lipid levels in the reproductive period and the development of cardiovascular disease in later life warrant further investigation.

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Chapter 3

Maternal intake of fat, riboflavin and nicotinamide and the risk of having offspring with congenital heart defects

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Abstract

Background: With the exception of studies on folic acid, little evidence is available concerning other nutrients in the pathogenesis of congenital heart defects (CHDs). Fatty acids play a central role in embryonic development, and the B-vitamins riboflavin and nicotinamide are co-enzymes in lipid metabolism.

Aim of the study: To investigate associations between the maternal dietary intake of fats, riboflavin and nicotinamide, and CHD risk in the offspring.

Methods: A case-control family study was conducted in 276 mothers of a child with a CHD comprising of 190 outflow tract defects (OTD) and 86 non-outflow tract defects (non-OTD) and 324 control mothers of a non-malformed child. Mothers filled out general and food frequency questionnaires at 16 months after the index-pregnancy, as a proxy of the habitual food intake in the preconception period. Nutrient intakes (medians) were compared between cases and controls by Mann-Whitney U-test. Odds ratios (OR) for the association between CHDs and nutrient intakes were estimated in a logistic regression model.

Results: Case mothers, in particular mothers of a child with OTD, had higher dietary intakes of saturated fat, 30.9 vs 29.8 g/d; $P<0.05$. Dietary intakes of riboflavin and nicotinamide were lower in mothers of a child with an OTD than in controls (1.32 vs. 1.41 mg/d; $P<0.05$ and 14.6 vs 15.1 mg/d; $P<0.05$, respectively). Energy, unsaturated fat, cholesterol and folate intakes were comparable between the groups. Low dietary intakes of both riboflavin (<1.20 mg/d) and nicotinamide (<13.5 mg/d) increased more than two-fold the risk of a child with an OTD, especially in mothers who did not use vitamin supplements in the periconceptional period (OR 2.4, 95% CI 1.4-4.0). Increasing intakes of nicotinamide (OR 0.8, 95% CI 0.7-1.001, per unit standard deviation increase) decreased CHD risk independent of dietary folate intake.

Conclusions: A maternal diet high in saturated fats and low in riboflavin and nicotinamide seems to contribute to CHD risk, in particular OTDs.

Introduction

Cardiovascular malformations affect about six to eight infants per 1,000 live births per year and are a leading cause of infant deaths due to congenital anomalies.[1,2] Despite the increasing number of studies focusing on the aetiology of congenital heart defects (CHDs), currently a cause can be identified only in 15% of the cases. Complex interactions between genetic and lifestyle factors have been suggested in which maternal nutrition plays a significant role in the majority of cases.[1] Best known is the preventive effect of periconceptional use of folic acid containing multivitamin supplements on CHD risk.[3] Shaw et al.[4] showed that maternal use of these vitamins during the sensitive period of heart development reduced the risk of conotruncal heart defects in particular. Furthermore, our group recently demonstrated that a low dietary intake of vitamin B12 is an independent risk factor for CHDs.[5] The B-vitamins folate and vitamin B12 are involved in the remethylation of homocysteine into methionine. Insufficient folate supply impairs the methylation of DNA, lipids and proteins. Furthermore, a compromised folate and/or vitamin B12 status also results in a mild hyperhomocysteinemia.

Our group and others showed that maternal hyperhomocysteinemia increases three- to ten-fold the risk of a child with CHD.[6,7] Maternal diabetes has been associated with CHD[8] and a clear link is shown between glycemic control during organogenesis and congenital malformations.[9] A number of studies have demonstrated a positive association between maternal obesity and CHDs as well,[10,11] although others reported contradictory results. There is no doubt that obesity is a phenotype of unhealthy lifestyle factors such as an excessive consumption of energy-dense foods. Thus, we became interested in other nutrients not previously studied that may be associated with CHDs, i.e., dietary fats and related B-vitamins. During early embryogenesis, fatty acids play a crucial role in membrane lipids and act as ligands for receptors and transcription factors that regulate gene expression.[12] Oxidation of fatty acids is dependent on adequate concentrations of the B-vitamins riboflavin and nicotinamide. Besides its role in fat metabolism, riboflavin acts as a cofactor in the folate pathway.[13,14] Nicotinamide is involved in both the synthesis and oxidation of fatty acids and also plays a role in the metabolism of certain drugs and toxicants.[15] Of interest is that the strong maternal use of the western dietary pattern, e.g., high saturated fats and low B-vitamins including riboflavin, has been associated with an almost two-fold increased risk of orofacial clefts.[16] Furthermore, low dietary intakes of riboflavin and nicotinamide have been associated with an increased risk of orofacial clefts[17] and spina bifida[18], respectively. Neural tube defects and orofacial clefts share many similarities in the pathogenesis of

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CHDs as these birth defects originate from disturbances in neural crest cell behaviour by for example hyperhomocysteinemia and low folate.[19-21] Therefore, it is conceivable that nutritional factors implicated in the pathogenesis of neural tube defects and orofacial clefts also apply to CHDs.

Currently, research on dietary intake of fatty acids, related B-vitamins other than folate and vitamin B12, and CHD risk is lacking. Therefore, we aimed to investigate whether the maternal dietary intakes of fatty acids and B-vitamins riboflavin and nicotinamide differ between mothers of a child with a CHD and control mothers. This may provide new insights into the optimal balance and quality of the diet in women during the reproductive period.

Subjects and methods

Study population
The HAVEN study, a Dutch acronym for the study of heart anomalies and the role of genetic and nutritional factors, is a case-control family study designed to investigate determinants in the pathogenesis and prevention of CHDs. From June 2003 onwards, the HAVEN Study was carried out at the Department of Obstetrics and Gynaecology of Erasmus MC in Rotterdam, The Netherlands. The study design was previously described in detail.[5,7] In summary, children with a CHD were diagnosed in the Sophia Children's Hospital/Erasmus MC in Rotterdam, Leiden University Medical Centre in Leiden, VU University Medical Centre and Emma Children's hospital/AMC in Amsterdam and were recruited with both parents in collaboration with two paediatric cardiologists trained in the same hospital. Medical charts were thoroughly screened by the child health centre physicians and any control infant with a congenital anomaly was excluded. All diagnoses were confirmed by ultrasound and/or cardiac catheterisation and/or surgery. The CHD phenotypes were categorized into outflow tract defects (OTDs), comprising of tetralogy of Fallot (n=38), atrioventricular septal defects (n=27), perimembranous ventricular septal defect (n=77), aortic valve stenosis (n=6), pulmonary valve stenosis (n=42), and non-outflow tract defects (non-OTDs) including coarctation of the aorta (n=27), transposition of the great vessels (n=34), hypoplastic left heart syndrome (n=13) and miscellaneous (n=12). Case and control children were all singletons. Control children were enrolled in collaboration with the public child health care centres in the surroundings of Rotterdam and were unaffected by any major birth defect. All parents were Dutch speaking. Maternal pre-existing diabetes was present in two case

mothers and four controls. Between October 2003 and December 2006, we collected questionnaire data on 351 case mothers and 406 control mothers. Pregnant (cases n=38, controls n=32) or lactating (cases n=15, controls n=16) mothers were excluded. We also excluded mothers who reported an altered diet compared with the periconception period, e.g. slimming or vegetarian diet (cases n=22, controls n=34). This resulted in the final evaluation of 276 case mothers and 324 control mothers. The study protocol was reviewed and approved by the Central Committee of Research in Human and the Medical Ethics Committees of the participating hospitals. A written informed consent was obtained from every parent.

Data collection

We collected the dietary intake data at 16 months after the index-pregnancy. At that time, the diagnosis of the CHD phenotypes had been confirmed. Dietary intake was assessed using a modified version of the semiquantitative food frequency questionnaire (FFQ) of Feunekes et al.[22] covering the previous four weeks. This for fats validated FFQ has been updated twice based on data of Dutch national food consumption surveys in 1992 and 1998 and was also specifically validated for B vitamin intakes.[23-25] The FFQ consisted of 195 items, structured according to a meal pattern. Questions about preparation methods, portion sizes and additions were included. We used the 2001 electronic version of the Dutch food composition table to calculate average daily intake of nutrients.[26] Further details on the FFQ are described elsewhere.[22,25]

Nutritional habits are in general rather constant, with the exception of periods of dieting and increased needs during pregnancy and lactation.[27-29] During the first critical pregnancy weeks, maternofetal nutrient transfer is largely determined by the maternal dietary intake of the previous preconception weeks. Our assumption is that the nutrient data from the FFQ filled out 24 months after conception reflect the maternal nutritional status in the preconception period. Moreover, it resembles the same season of the year. The value of this approach has been demonstrated by studies before.[5,30] The FFQs were filled out at home and verified by the researcher using a standardized checklist during the hospital visit or occasionally during a telephone interview. During the hospital visit at the Erasmus MC, standardized anthropometric measurements of the mothers were performed. Weight (weighing scale, SECA, Hamburg, Germany) was measured with 0.5 kg accuracy and height (anthropometric rod, SECA, Hamburg, Germany) up to 0.1 cm accuracy. Body mass index (BMI) was defined as weight divided by height squared.

All mothers completed a questionnaire concerning lifestyle behaviours and demographic data at 16 months after the index-pregnancy and at the periconception

period, defined as four weeks prior to conception until eight weeks after conception. Extracted data included maternal age, time interval after the index-pregnancy, pre-existing diabetes, educational level, ethnicity and use of alcohol, cigarettes and vitamin supplements. Periconception vitamin supplement use was defined as daily intake in the entire periconception period. Data on vitamin supplements included information on the contents (folic acid only or multivitamin supplement) and frequency of intake. Mothers were considered to use alcohol or cigarettes when any consumption in the questioned periods was reported. Mothers were classified by educational level based on definitions used by Statistics Netherlands.[31] Primary/lower vocational/intermediate secondary level of education was defined as low, intermediate vocational/higher secondary as intermediate, and higher vocational/university level as high education. Ethnicity was categorized as Dutch natives (both parents are of European origin and born in the Netherlands), European others (one of the parents is born in a European country or is of European origin and living in the USA, Australia or Indonesia), or non-European others (one of the parents is of non-European origin).[32]

Statistical analysis

General characteristics were compared between case and control mothers. The continuous variables maternal age, time after index-pregnancy and BMI are presented as medians with 5th and 95th percentiles and tested using the Mann-Whitney U test because of skewed distributions. The categorical variables ethnicity and educational level and the dichotomous variables use of alcohol, cigarettes and folic acid containing (multi) vitamin supplements are presented as numbers with percentages and tested using the Chi-square test. The residual method was used to calculate energy adjusted nutrient intakes.[33] Except for cholesterol, folate, riboflavin and nicotinamide, the distributions of all nutrients were positively skewed and therefore log-transformed. The logarithmic transformed nutrient intakes of the individuals were regressed on their logarithmic transformed total intake of energy in MJ. This regression equation was used to calculate the predicted mean nutrient intake at the average energy intake of the total study population. By adding the predicted mean nutrient intake to the individual residuals, the nutrient intakes were energy adjusted. Energy adjusted nutrient intakes are presented as medians with 5th and 95th percentiles and compared between cases and controls by the Mann-Whitney U test. Spearman's correlation coefficients were computed to assess the correlations between the nutrients. For each nutrient, univariate and multivariate logistic regression analyses were performed. We created the 25th percentile of dietary nutrient intakes based on the distribution in control mothers and estimated the risk for a CHD

affected pregnancy by calculating odds ratios (OR) and their respective 95% confidence intervals (CI). Furthermore, multivariable logistic regression analysis with the B-vitamins folate, riboflavin and nicotinamide as continuous variables was performed to assess the independent relationship of B-vitamin intake via the diet and CHD risk. To adjust for potential confounding factors, we fitted a backward stepwise multiple logistic model. The presence or absence of CHD was the dependent variable. Covariates were maternal age, diabetes, BMI, ethnicity, educational level, periconception folic acid containing supplement, cigarette or alcohol use. The ORs were adjusted for maternal age and ethnicity, as only these predictors remained significant in the logistic model with P values of <0.05. Because mothers who used vitamin supplements in the periconception period had higher total B vitamin intakes than non-users, we performed a stratified analysis for periconception use of vitamins, supplements containing folic acid only, and non-supplement use. Probability values ≤0.05 were considered statistically significant. All analyses were performed with SPSS for Windows software (version 15.0; SPSS Inc, Chicago, IL, USA).

Results

Table 1 summarizes the general characteristics in the total group of case mothers, stratified for OTDs and non-OTDs, and control mothers. Overall, case mothers were slightly older than controls with a mean difference of 0.4 years. Mothers of offspring with non-OTDs were more often from Dutch origin than the control group (P=0.05). The median time after the index-pregnancy was around 16.1 months and comparable between case and control mothers. At 16 months after the index-pregnancy and at the periconception period all lifestyle behaviours were comparable between the groups.

The food frequency analyses showed that mothers of a child with a CHD had a significantly higher intake of saturated fat and a lower intake of nicotinamide (Table 2). These results are most pronounced in mothers of a child with an OTD. Furthermore, this group also showed significantly lower riboflavin intake. The median dietary intakes of energy, unsaturated fats, cholesterol and folate were comparable between the total CHD and stratified groups compared with controls. The dietary intakes of the fats were positively correlated. Spearman's correlation coefficients varied from 0.34 for the intake of cholesterol and saturated fat to 0.53 for the intake of monounsaturated and saturated fats. B-vitamins were correlated with Spearman's correlation coefficients varying from 0.23 for riboflavin and nicotinamide, 0.29 for folate and nicotinamide, to 0.35 for folate and riboflavin.

Table 1. General characteristics of the study population of case and control mothers

Mothers	Total CHD group	Outflow tract defects	Non-outflow tract defects	Control group
16 months after the index-pregnancy	(n=276)	(n=190)	(n=86)	(n=324)
Maternal age (years)	33.1 (25.1-41.6)[1]	33.2 (24.4-42.3)[1]	32.9 (25.5-39.0)	32.7 (24.5-39.8)
Time after index-pregnancy (months)	16.2 (13.9-26.6)	16.1 (13.8-27.3)	16.5 (13.8-25.5)	16.1 (14.0-21.9)
BMI (kg/m^2)	24.1 (19.2-34.1)	24.3 (19.1-34.2)	23.5 (19.5-32.1)	24.1 (19.6-34.6)
Ethnicity[2]				
Dutch natives	221 (80)	146 (77)	25 (87)[5]	258 (80)
European others	14 (5)	8 (4)	6 (7)	15 (5)
Non-European others	41 (15)	36 (19)	5 (6)	51 (16)
Educational level[3]				
low	74 (27)	56 (29)	18 (21)	77 (24)
intermediate	129 (47)	85 (45)	44 (51)	165 (51)
high	73 (26)	49 (26)	24 (28)	82 (25)
Use of [n (%)]				
Alcohol	139 (50)	91 (48)[4]	48 (56)	185 (57)
Cigarettes	55 (20)	37 (20)	18 (21)	63 (19)
Folic acid (multi)vitamins	55 (20)	32 (17)	23 (27)	67 (21)
PERICONCEPTION				
Use of [n (%)]				
Alcohol	94 (34)	62 (33)	32 (37)	107 (33)
Cigarettes	51 (19)	34 (18)	17 (20)	71 (22)
Folic acid (multi)vitamins	135 (49)	93 (49)	42 (49)	163 (50)

Values are given in median (P5-P95) or number (percentage). BMI: n=2 missing. [1]P=0.01, significance tested by the Mann-Whitney U test. [2]Dutch natives: Both parents are from European origin and born in the Netherlands. European others: one of the parents is born in a European country or is from European origin and living in the USA, Australia or Indonesia. Non-European others: one of the parents is from non-european origin. [3]Low (primary / lower vocational / intermediate secondary), intermediate (higher secondary / intermediate vocational) or high (higher vocational / university education). [4]P<0.05; [5]P=0.05. Significance tested by X$_2$ test.

Table 2. Energy adjusted nutrient intakes for case mothers and controls

	FCS[1]	Total CHD group (n=276)	Outflow tract defects (n=190)	Non-outflow tract defects (n=86)	Control group (n=324)
Energy (MJ)	8.5	8.7 (5.8-14.0)	8.7 (5.8-13.9)	8.8 (5.7-14.2)	8.8 (5.7-13.2)
MACRONUTRIENTS					
Total fat (g)	85	83.6 (62.8-107.9)	84.0 (62.4-108.0)	82.3 (65.1-107.7)	82.8 (63.1-103.6)
Saturated fat (g)	33	30.7 (21.8-42.3)[2]	30.9 (21.6-42.4)[3]	30.3 (22.3-42.5)	29.8 (21.2-40.5)
Monounsaturated fat (g)	30	26.4 (19.2-36.4)	26.4 (18.7-37.4)	26.5 (19.7-33.7)	26.2 (19.3-34.1)
Polyunsaturated fat (g)	15	17.7 (11.2-27.0)	17.9 (11.2-28.1)	16.9 (10.7-26.6)	17.5 (12.0-26.5)
Linoleic acid (g)	-	13.6 (8.6-22.4)	13.9 (8.6-22.8)	13.0 (8.3-21.0)	14.0 (9.2-21.9)
ALA (g)	-	1.14 (0.66-1.86)	1.13 (0.64-1.87)	1.16 (0.66-1.91)	1.07 (0.74-2.01)
EPA (g)	-	0.03 (0.00-0.19)	0.03 (0.00-0.16)	0.04 (0.00-0.25)	0.03 (0.00-0.14)
DHA (g)	-	0.06 (0.01-0.29)	0.06 (0.01-0.23)	0.07 (0.01-0.36)	0.07 (0.01-0.21)
Cholesterol (mg)	200	171 (107-267)	177 (102-283)	179 (124-262)	176 (111-281)
MICRONUTRIENTS					
Folate (µg)	153	191 (118-284)	189 (118-276)	195 (119-323)	199 (130-287)
Riboflavin (mg)	1.4	1.34 (0.74-1.08)	1.32 (0.72-2.09)[3]	1.38 (0.93-2.11)	1.41 (0.86-2.00)
Nicotinamide (mg)	-	14.7 (9.90-19.7)[3]	14.6 (9.6-20.1)[3]	14.9 (10.7-19.1)	15.1 (10.9-20.1)

ALA, α-linolenic acid. EPA, eicosapentanoic acid. DHA, docosahexanoic acid. Energy-adjusted intakes are medians (P5-P95). [1]Food Consumption Survey based on women aged 22-50 years. Significance tested by Mann-Whitney *U* test. [2]*P*=0.05; [3]*P*<0.05; Significance tested by the Mann-Whitney *U* test.

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Table 3. Energy adjusted nutrient intakes and the risk of congenital heart defects

	Cut off levels	Total group Cases/controls (n=276 / 324)	OR (95% CI)	OTDs vs controls Cases/controls (n=190 / 324)	OR (95% CI)	Non-OTDs vs controls Cases/controls (n=86 / 324)	OR (95% CI)
MACRONUTRIENTS							
Total fat	<74.9 g/d	55 / 81	0.8 (0.5-1.2)	32 / 81	0.6 (0.4-0.97)	23 / 81	1.2 (0.7-2.1)
Saturated fat	<26.9 g/d	64 / 81	1.0 (0.7-1.4)	43 / 81	0.9 (0.6-1.4)	21 / 81	1.1 (0.6-1.9)
Monounsaturated fat	<23.1 g/d	50 / 81	0.7 (0.4-0.99)	34 / 81	0.6 (0.4-0.99)	16 / 81	0.8 (0.4-1.4)
Polyunsaturated fat	<15.0 g/d	75 / 81	1.1 (0.8-1.6)	54 / 81	1.2 (0.8-1.8)	21 / 81	1.0 (0.6-1.7)
Linoleic acid	<11.9 g/d	77 / 81	1.2 (0.8-1.7)	52 / 81	1.1 (0.7-1.7)	25 / 81	1.2 (0.7-2.1)
ALA	<0.89 g/d	53 / 81	0.7 (0.5-1.1)	37 / 81	0.8 (0.5-1.2)	16 / 81	0.7 (0.4-1.2)
EPA	<0.01 g/d	68 / 81	1.1 (0.7-1.6)	49 / 81	1.2 (0.8-1.9)	19 / 81	0.9 (0.5-1.5)
DHA	<0.03 g/d	74 / 81	1.2 (0.8-1.8)	54 / 81	1.1 (0.9-2.1)	20 / 81	0.9 (0.5-1.6)
Cholesterol	<145 mg/d	60 / 81	0.9 (0.6-1.3)	48 / 81	1.1 (0.7-1.6)	12 / 81	0.5 (0.3-0.95)
MICRONUTRIENTS							
Folate	<165 µg/d	81 / 80	1.3 (0.9-1.9)	58 / 81	1.5 (0.97-2.2)	22 / 81	1.0 (0.6-1.8)
Riboflavin	<1.20 mg/d	89 / 81	1.6 (1.1-2.3)	65 / 81	1.7 (1.1-2.5)	24 / 81	1.3 (0.8-2.3)
Nicotinamide	<13.5 mg/d	89 / 81	1.6 (1.1-2.2)	62 / 81	1.6 (1.1-2.4)	27 / 81	1.5 (0.9-2.5)

OR, odds ratio. CI, confidence interval. OTD, outflow tract defect. ALA, α-linolenic acid. EPA, eicosapentanoic acid. DHA, docosahexanoic acid. Cut off values were based on the lowest quartile of intake of the control mothers. The reference category was a dietary intake above the cut off value. ORs were adjusted for maternal age and ethnicity.

Table 4. Multivariable regression analysis of the association between dietary intake of B-vitamins and the risk of congenital heart defects.

	Total group Cases/controls (n=276/324)		OTDs vs controls Cases/controls (n=190 / 324)		Non-OTDs vs controls Cases/controls (n=86 / 324)	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Model[1]						
Folate	1.0 (0.8-1.2)	0.63	1.1 (1.0-1.1)	0.23	1.1 (0.9-1.5)	0.42
Riboflavin	0.9 (0.8-1.1)	0.32	0.9 (0.7-1.1)	0.25	1.0 (0.7-1.3)	0.98
Nicotinamide	0.8 (0.7-1.001)	0.051	0.8 (0.7-1.02)	0.08	0.8 (0.6-1.1)	0.13

The results of the logistic regression analysis are presented as odds ratio (OR) of CHD risk for one-unit-standard deviation increase in B-vitamins. Folate, 1 SD = 52.3 µg/d. Riboflavin, 1 SD = 0.36 mg/d. Nicotinamide, 1 SD = 3.11 mg/d. CI, confidence interval. OTD, outflow tract defects. Non-OTD, non-outflow tract defects. ORs were adjusted for maternal age and ethnicity. [1] B-vitamins adjusted for each other.

A maternal dietary intake of monounsaturated fats below the 25th percentile of controls, i.e., 23.1 mg/d, was associated with a reduced risk of CHD, particularly OTDs (OR 0.6, 95% CI 0.4-0.99) (Table 3). A dietary intake of cholesterol below the 25th percentile of intake in controls was associated with a reduced risk of having a child with a non-OTD (OR 0.5, 95% CI 0.3-0.95). The risk of offspring with OTDs was significantly higher with dietary intakes of riboflavin and nicotinamide below the 25th percentile of intakes in control mothers (OR 1.7, 95% CI 1.1-2.5 and OR 1.6, 95% CI 1.06-2.5, respectively). A multivariable model with the three B-vitamins folate, riboflavin and nicotinamide was used to determine the independent associations with CHD risk (Table 4). We demonstrated a dose-response relationship with increasing intakes of nicotinamide and decreasing CHD risk (OR 0.8, 95% CI 0.7-1.001, per unit standard deviation increase). This association was independent of dietary folate and riboflavin intake.

Stratification for periconception use of vitamins, supplements containing folic acid only, and non-supplement use revealed that the association between a dietary intake of riboflavin below 1.20 mg/d and OTD risk was strengthened, but only significant in non-supplement users (OR 2.4, 95% CI 1.4-4.0). A maternal dietary intake of nicotinamide below the 25th percentile of controls, i.e., 13.5 mg/d, was also associated with a higher risk of having a child with an OTD but only in non-supplement users (OR 1.99, 95% CI 1.14-3.46).

Discussion

This study demonstrates that dietary intakes of saturated fat were slightly but significantly higher in mothers of a child with a CHD than in controls. On the other hand, dietary intakes of fat-related B-vitamins, i.e., riboflavin and nicotinamide, were significantly lower in case mothers, in particular mothers of a child with an OTD, compared with controls. A dietary intake of riboflavin and nicotinamide below 1.20 mg/d and 13.5 mg/d, respectively, more than 2-fold increased the risk of CHDs, especially in mothers who did not use vitamin supplements in the periconceptional period. CHD risk decreased with increasing intakes of nicotinamide, which seemed to be independent of dietary folate and riboflavin intake. Median nutrient intakes were similar to those of the Dutch National Food Consumption Survey (FCS),[24] indicating that dietary intakes of case and control mothers generally reflect the intakes of Dutch non-pregnant women aged 22-50 years. Besides this study, research on the associations between dietary fat, riboflavin and nicotinamide is lacking and the underlying mechanisms are unknown. Nevertheless, our

findings are in line with the associations demonstrated between maternal dietary intake of nicotinamide and riboflavin and the risk of a child with an orofacial cleft or spina bifida.[18,30] CHDs share similarities in the pathogenesis of spina bifida and orofacial clefts, because of the involvement of neural crest cells which are very sensitive to exposures of folate and homocysteine.[19-21] Riboflavin and nicotinamide are coenzymes in fat metabolism and are important in molecular biological processes crucial for normal embryonic heart development, such as reduction of lipid peroxides,[13,14] cholesterol and steroid synthesis, glycolysis, regulation of numerous oxidoreductases and DNA repair mechanisms.[15] Riboflavin is also a co-factor in the folate pathway, and might exert its effect via both folate and fat metabolism.

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We have shown a higher CHD risk for low dietary intakes of riboflavin and nicotinamide, particularly in mothers who did not use vitamin supplements in the periconceptional period. Thirty-six case mothers and thirty-nine controls used also a B-vitamin supplement. The dosage of the B-vitamins in supplements is much higher than in food. This may explain why we could not assess a higher CHD risk of low dietary intake of B-vitamins in this group. Periconception supplement use was defined as the daily use of vitamin supplements from four weeks before until eighth weeks after conception. Some mothers categorized as non-supplement users were in fact irregular users, which may have underestimated the OR of low dietary riboflavin and nicotinamide intake in this group. Maternal education, as a proxy for socio-economic status, was lower in mothers who did not use vitamin supplements in the periconception period. Furthermore, it is known that maternal education is correlated with periconception supplement use.[34] Therefore, the association between B-vitamin intake and CHD risk may be confounded by education. Adjustment for education, however, showed that it only marginally affected the ORs without consequences for the conclusions.

Animal studies have suggested that in utero exposure to a maternal diet rich in fat may lead to an increased risk of cardiovascular disease in the offspring.[35] Nutrition plays an important role in epigenetic modification of genes, referring to all changes in the genes other than the DNA sequence itself.[36] Mechanisms of epigenetic inheritance include methylation of DNA, modification of histones, binding of transcription factors to chromatin, and the timing of DNA replication. We hypothesize that inadequate maternal nutrition during the periconception period, e.g. excess of fatty acids and deficiencies of the B-vitamins riboflavin and nicotinamide might cause epigenetic changes in the DNA of the developing embryo resulting in an increased vulnerability for an embryo to develop a CHD.

Some strengths and limitations of this study have to be considered. Because of the relatively low prevalence rates of CHDs, we used a case-control study design with a standardized investigation at 16 months after the index-pregnancy. We consider this standardized investigation, of which the value was shown in our previous studies[5,18,30] as one of the main strengths. The investigation occurred relatively short after pregnancy to minimize recall bias regarding periconception lifestyle behaviours and to increase compliance of the participants. Furthermore, most CHD are diagnosed during the first year of life and therefore misclassification of cases and controls is avoided. Another strength is the detailed characterisation of CHD phenotypes. To further homogenize our case group in terms of pathogenesis, we clustered the OTDs, which have been reported to result from derangements in folate / homocysteine metabolism.[37,38] Finally, with regard to nutrition, the study design considers several measures to reduce bias in nutritional intake. Importantly, we used a validated FFQ[22], covering the previous four weeks whereby day-to-day variability of food intake is minimized. Moreover, the standardized investigation at 16 months after the index-pregnancy is two years after conception and equals the season of the periconception period. Thus, the seasonal influences on food intake are comparable between the groups. Devine et al.[28] support our approach and state that, in general, no difference occurs in dietary patterns between the beginning of pregnancy and at least one year postpartum. Furthermore, the famous nutrition epidemiologist Walter Willet states that dietary patterns are rather stable.[29] We excluded mothers because of conditions that affect nutritional intake, such as pregnancy, lactation or an altered diet compared with the preconception period. Thirdly, energy adjustment also minimized possible underreporting bias. The ratio of energy intake (EI) and basal metabolic rate (BMR) was comparable between cases and controls (OTDs = 1.41; non-OTDs = 1.43; controls = 1.42). In a separate analysis, the EI: BMR ratio was 1.41 for Dutch natives, 1.43 for European others and 1.32 for non-Europeans. The EI: BMI ratio of the total study population was representative of long-term habitual intake according to the cut-off value of 1.35.[39] However, there may be some underreporting in mothers from non-European origin.

A limitation of our study is the lack of biomarker data, because B-vitamin concentrations are not only determined by intake, but also by absorption, metabolism, clearance and genetic polymorphisms.

Furthermore, we cannot completely rule out the possibility that the observed associations result from residual confounding. However, the multiple logistic regression analysis revealed that maternal diabetes, BMI, educational level, periconception folic acid containing supplement use and periconception use of cigarettes or alcohol did not

confound the associations. Due to the positive correlations among the B-vitamins, the identification of one B-vitamin with a predominant role in the pathogenesis of CHD is very difficult. Nevertheless, nicotinamide intake seemed to be associated with CHD risk, independent of dietary folate and riboflavin. The different B-vitamins are frequently simultaneously present in foods, making it difficult to distinguish between the separate effects of one B-vitamin. Experimental animal studies with administration of separate B-vitamins could elucidate this issue further.

Our findings raise questions on the adequacy of the dietary intake among women of reproductive age, particularly with regard to riboflavin and nicotinamide intakes. Our study population showed adequate dietary intakes of riboflavin and nicotinamide according to the Dutch recommended dietary allowances.[40] This held even for the majority of mothers categorized in the lowest quartile of intake based on the controls. Considering our findings, one may argue whether the recommendations for riboflavin and nicotinamide intake are sufficient for women in reproductive ages and might need to be adapted. Moreover, acquiring evidence on the safety threshold for dietary fat intake is a high priority because of the increasing prevalence of a Western diet rich in fat even among women of childbearing age. In conclusion, our results suggest that a maternal diet high in saturated fat but low in riboflavin and nicotinamide contributes to a higher risk of having a child with a CHD.

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Chapter 4

High maternal vitamin E intake by diet or supplements is associated with congenital heart defects in the offspring

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Abstract

Objective: To study associations between maternal dietary and supplement intake of antioxidants vitamin E, retinol and congenital heart defects (CHD).

Design: Case-control study.

Setting: Erasmus MC, University Medical Center Rotterdam, The Netherlands.

Population: Participants were 276 case mothers of a child with CHD and 324 control mothers with their children.

Methods: Food frequency questionnaires covering the intake of the previous four weeks were filled out at 16 months after the index-pregnancy. Data were compared between cases and controls by using the Mann-Whitney U test. Risk estimates for the association between CHD and dietary intake of vitamin E and retinol were estimated in a multivariable logistic regression model.

Main outcome measures: Medians (P5-P95) and odds ratios (OR) with 95% confidence intervals (CI).

Results: Dietary vitamin E intake was higher in case mothers than in controls, 13.3 (8.1-20.4) and 12.6 (8.5-19.8) mg/d (P=0.05). CHD risk increased with rising dietary vitamin E intakes (Ptrend=0.01). Periconception use of vitamin E supplements in addition to a high dietary vitamin E intake above 14.9 mg/d up to nine-fold increased CHD risk. Retinol intakes were not significantly different between the groups and not associated with CHD risk.

Conclusions: High maternal vitamin E by diet and supplements is associated with an increased risk of CHD offspring.

Introduction

Congenital heart defects (CHDs) form a set of clinically heterogeneous birth anomalies. Worldwide, CHDs occur in 6-8 per 1,000 live births.[1] Only 15% of CHDs can be attributed to a known cause and the majority of cases are thought to result from complex interactions between genetic susceptibilities and intrauterine exposures derived from the mother.[2,3] There is information on the preventive effect of periconception use of multivitamins containing folic acid against CHDs.[4] Maternal hyperhomocysteinaemia increases the risk of CHD offspring three to ten-fold and can be treated by folic acid supplementation.[5-7] Possible mechanisms by which homocysteine exerts its embryotoxic effects include DNA hypomethylation, alterations in gene expression and oxidative stress.[8] The latter is substantiated by the finding that several markers of oxidative stress, such as oxidized glutathione, were elevated in mothers with CHD affected pregnancies.[9] As excessive oxidative stress may be a causative factor in CHD development, we hypothesize that both low and high periconception dietary intake of (anti)oxidants may affect CHD risk either directly or via the homocysteine pathway. Particularly interesting is the finding that homocysteine inhibits the synthesis of retinoic acid, which may derange intracellular signalling processes during embryogenesis resulting in CHDs and other malformations.[10] Vitamin E and retinol are essential lipid soluble vitamins and are involved in several biological processes, including immune mechanisms, growth and cellular differentiation[11] and thereby are very important in early embryogenesis. Vitamin E, and to a lesser extent retinol, has strong antioxidant capacity. Insufficient dietary intake of these vitamins might lead to oxidative stress due to a reduced antioxidant defense. On the other hand, a high vitamin A dose can act as an oxidant.[12] A high maternal intake via supplements of vitamin A and retinol, the biologically active form of vitamin A, has been associated with CHDs.[13] From this background, we hypothesize that either an inadequate or high maternal intake of vitamin E and retinol detrimentally influences embryonic heart development. Therefore, we investigated whether maternal intakes of vitamin E and retinol via the diet and supplements are associated with the occurrence of CHD.

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Methods

Study population

The Dutch HAVEN study is an ongoing study in the Western part of the Netherlands. The study design has been described in detail previously.[6] In summary, case children with both parents were identified by two paediatric cardiologists appointed at the Erasmus MC, University Medical Centre in Rotterdam, Leiden University Medical Centre in Leiden, VU University Medical Centre and Amsterdam Medical Centre in Amsterdam, The Netherlands. Diagnoses were ascertained in the first year of life by ultrasound and/or cardiac catheterization and/or surgery. At the study moment of 16 months after the index-pregnancy, cases were under regular surveillance of a paediatric cardiologist and the malformation was still present. The selection of CHD phenotypes was based on the demonstrated preventive effects of folate, the associations with hyperhomocysteinaemia and other reported gene-environment influences.[7,14] The CHD phenotypes comprised of Tetralogy of Fallot (n=38), atrioventricular septal defect (n=27), perimembranous ventricular septal defect (n=77), aortic valve stenosis (n=6), pulmonary valve stenosis (n=42), coarctation of the aorta (n=27), transposition of the great vessels (n=34), hypoplastic left heart syndrome (n=13) and miscellaneous (n=12). The total group consisted of 218 isolated and 56 non-isolated defects.

Cases were defined as non-isolated CHDs when they also had another major structural congenital anomaly besides their CHD. 40 of this 56 non-isolated cases had a genetic syndrome, i.e. Trisomy 21 (n=26), Deletion 22q11 (n=5), Noonan syndrome (n=2), Insertion 1>3 (n=1), Kabuki syndrome (n=1), Beckwith-Wiedemann syndrome (n=1), Turner syndrome (n=1), CHARGE syndrome (n=1), Saethre-Chotzen syndrome (n=1) and Alagille syndrome (n=1). 17 cases had other defects that could not be traced to a known syndrome, such as hip dysplasia, anusatresia, hydronephrosis, deafness and hydrocèle.

Eligible families are children with a CHD and healthy children at 16 months after birth with both parents living in the western part of the Netherlands. Control children with their parents were enrolled in collaboration with the public child health centres in the Rotterdam area where standard care is given with regard to the monitoring of health, growth and development of the child during the first 5 years of life. Controls were excluded if they had a congenital malformation ascertained by their physician at the child health centre. The families were Dutch speaking. Because a prospective study on associations between maternal periconception dietary intake and CHD risk is not feasible, we have chosen for a case-control design with a fixed study time. Participants

visited the hospital at the standardized study time of around 16 months post partum and at that time all mothers filled out a food frequency questionnaire (FFQ) referring to the dietary intake of the previous month. Between October 2003 and December 2006 we collected questionnaire data of 351 case and 406 control mothers. Pregnant (cases n=38, controls n=32) or lactating (cases n=15, controls n=16) mothers and those (cases n=22, controls n=34) who reported an altered diet compared with the periconception period were excluded. Data of 276 case mothers and 324 controls remained for further analysis. The study protocol was approved by the Central Committee of Research in Human in The Hague, the Netherlands, and the Medical Ethics Committees of the participating hospitals. Written informed consent was obtained from every parent.

Data collection

We used a modified version of the semi quantitative food frequency questionnaire (FFQ) of Feunekes et al.[15] to estimate habitual energy and micronutrient intake from the four weeks prior to the time of study. The FFQ had been updated twice based on data of Dutch national food consumption surveys in 1992 and 1998.[16,17] The FFQ consisted of 195 items, structured according to a meal pattern. Questions about preparation methods, portion sizes and additions were included. We used the 2001 electronic version of the Dutch food composition table to calculate average daily intake of retinol and vitamin E as alpha-tocopherol equivalents.[18] Further details on the FFQ are described elsewhere.[15,19] The FFQs were filled out at home and were verified by the researcher using a standardized checklist during the hospital visit. Standardized anthropometric measurements were performed. Weight (weighing scale, SECA, Hamburg, Germany) was measured with 0.5 kg accuracy and height (anthropometric rod, SECA, Hamburg, Germany) up to 0.1 cm accuracy. Body mass index (BMI) was defined as weight divided by the quadrate of height. All mothers completed a general questionnaire concerning lifestyle factors and demographic data referring to two different time periods. The first period was the periconception period, defined as four weeks prior to conception until eight weeks after conception. The second period was defined as four weeks before the time of study, i.e., at around 16 months after the index-pregnancy. The questionnaires were verified by the researcher during the hospital visit. Extracted data included maternal age, pre-existing diabetes, time interval after the index-pregnancy, family history of CHDs, educational level, ethnicity, use of alcohol, cigarettes and vitamin supplements. Family history of CHDs was classified as family members with a CHD in the first, second and third degree. Periconception vitamin supplement use was defined as daily intake in the complete periconception period. Data on vitamin supplements included

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information on the contents (folic acid only or multivitamin supplement containing vitamin E and/or retinol), dosage and frequency of intake. The questionnaire explicitly distinguished between folic acid only and multivitamin supplements. Use of cigarettes and alcohol was defined as any consumption in the questioned period. Educational level and ethnicity were classified as defined by Statistics Netherlands.[20] Primary/lower vocational/intermediate secondary level of education was defined as low, intermediate vocational/higher secondary as intermediate, and higher vocational/university level as high education. Ethnicity was categorized as Dutch natives (both parents are from European origin and born in the Netherlands), European others (one of the parents is born in a European country or is from European origin and living in the USA, Australia or Indonesia), or non-European others (one of the parents is from non-European origin).[21]

Statistical analyses

Sociodemographic and lifestyle characteristics as well as dietary intakes of vitamin E and retinol are presented as medians (P5-P95) and compared by the Mann-Whitney *U* test. Categorical variables are shown as frequency (range) and tested by the Chi-square test. The residual method of Willett et al.[22] was used to calculate energy adjusted nutrient intakes. The logarithmic transformed vitamin E and retinol intakes of the individuals were regressed on their logarithmic transformed total energy intakes. This regression equation was used to calculate the predicted mean nutrient intake at the average energy intake of the total study population. The nutrient intakes were energy adjusted by adding the predicted mean nutrient intake to the individual residuals. Quartiles of dietary intake were computed from the continuous variables of the control mothers. The risk for a CHD affected pregnancy was estimated by odds ratios (OR) and 95% confidence intervals (CI) for each quartile of dietary intake with the lowest quartile as a reference in an unconditional logistic regression model. We adjusted the risk estimates for potential confounders including maternal age and periconception use of folic acid containing supplements. Trends across the quartiles were evaluated, in which the quartiles were modeled after inclusion of the continuous variables in 4 categories ranging from 1 (reference) to 4 (highest quartile of dietary intake). We performed stratified analyses for periconception use of multivitamins, folic acid only supplements, and non-supplement use. Probability values ≤ 0.05 were considered statistically significant. All analyses were performed with SPSS for Windows software (version 11.0; SPSS Inc, Chicago, IL, USA).

Results

As shown in Table 1, case mothers were slightly but significantly older than controls. There were no differences in family history of CHDs, maternal diabetes, ethnicity or educational level between cases and controls. At 16 months after the index-pregnancy, there were no significant differences in BMI, use of vitamin supplement, alcohol or cigarettes. Periconception use of alcohol, cigarettes and folic acid containing vitamin supplements was similar in case and control mothers. Case mothers showed significantly higher dietary vitamin E intake than controls (12.6 vs 13.3 mg/d, P=0.05). The distribution of maternal dietary vitamin E intake is depicted in Figure 1. Total energy and retinol intakes were similar in cases and controls. To assess the adequacy of the diet, the dietary intakes of vitamin E and retinol were compared with the Dutch and U.S. recommended daily allowances (RDAs) for non-pregnant women in the reproductive age.[23,24] Median vitamin E intakes were higher than the Dutch RDA of 9.3 mg/d, for cases and controls 3.3 and 4.0 mg/d higher, respectively. Dietary intakes of vitamin E and retinol were also compared between cases and controls by computing crude ORs for each quartile of intake based on the cut-off values of the controls (Table 2). 89 (32%) out of 276 case mothers showed a vitamin E intake above 14.9 mg/d. Furthermore, a significant trend could be demonstrated towards a higher CHD risk over the quartiles of dietary vitamin E intake (Ptrend = 0.01). These distributional differences remained unchanged after adjustment for maternal age and periconception use of vitamin supplements. Mothers with dietary intakes of vitamin E above 14.9 mg/d and mothers with intakes below this level all showed comparable sociodemographic and lifestyle factors including BMI, education, ethnicity and periconception use of alcohol, cigarettes and vitamin supplements. None of the risk estimates for high dietary retinol intake were statistically significant.

Figure 2 shows the stratified analysis for maternal periconception use of a multivitamin supplement containing vitamin E, a folic acid only supplement, and no use of a supplement. In periconception users of a vitamin E containing supplement, a trend towards a higher CHD risk was demonstrated with rising dietary vitamin E intake (Ptrend = 0.008). Furthermore, the ORs at the highest quartiles of vitamin E intake were 9.1 (95% CI: 2.0-41.4) and 4.8 (95% CI: 1.1-20.2), respectively. Thus, dietary vitamin E intake above 12.6 mg/d combined with the use of a vitamin E containing supplement five- to nine-fold increased the risk of CHD. No significant associations were observed for maternal dietary retinol intake in any of the three strata.

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Table 1. Characteristics of the study populations

	Case mothers	Control mothers	*P*-value	RDA	RDA
Study moment, 16 months	n=276	n=324		NL	U.S.
Maternal age (years)	33.1 (25.1-41.6)	32.7 (24.5-39.8)	0.02		
Time after index-pregnancy (months)	16.2 (13.9-26.6)	16.1 (14.0-21.9)	0.12		
BMI (kg/m²)	24.1 (19.2-34.1)	24.1 (19.6-34.6)	0.69		
Family history of CHDs	25 (9)	19 (6)	0.14		
Maternal diabetes	2 (1)	2 (1)	0.87		
Educational level[1]			0.56		
low	74 (27)	77 (24)			
intermediate	129 (47)	165 (51)			
high	73 (26)	82 (25)			
Ethnicity[2]			0.93		
Dutch natives	221 (80)	258 (80)			
European others	14 (5)	15 (5)			
Non-European others	41 (15)	51 (16)			
Use of [n (%)]					
Alcohol	139 (50)	185 (57)	0.10		
Cigarettes	55 (20)	63 (19)	0.88		
Folic acid (multi)vitamins	55 (20)	67 (21)	0.82		
Dietary intake[3] of					
Total energy (MJ/d)	8.7 (5.8-14.0)	8.8 (5.7-13.2)	0.67	9.7-10.2	10.1
Vitamin E (mg/d)	13.3 (8.1-20.4)	12.6 (8.5-19.8)	0.050	9.3	15
Retinol (µg/d)	467 (179-1452)	493 (209-1502)	0.87	800	700
Periconception					
Use of [n (%)]					
Alcohol	94 (34)	107 (33)	0.89		
Cigarettes	51 (19)	71 (22)	0.30		
Folic acid (multi)vitamins supplement	135 (49)	163 (50)	0.73		

RDA, Recommended Dietary Allowances, NL[23] and U.S.[24] Values are given in median (P5-P95) or number (percentage). BMI: n=2 cases missing. [1]Low (primary / lower vocational / intermediate secondary), intermediate (higher secondary / intermediate vocational) or high (higher vocational / university education).[20] [2]Dutch natives: Both parents are from European origin and born in the Netherlands. European others: one of the parents is born in a European country or is from European origin and living in the USA, Australia or Indonesia. Non-European others: one of the parents is from non-European origin.[21] [3]Energy adjusted dietary intake. [22]

Table 2. Maternal dietary intake of vitamin E and retinol in association with the risk of congenital heart defects in their children

Dietary intake[1]	Cases/controls (n=276/324)	OR (95% CI)	OR (95% CI)[2]
Vitamin E (mg/d)			
4.0-10.6	54/81	1.0 (Reference)	1.0 (Reference)
10.6-12.6	55/81	1.0 (0.6-1.7)	1.0 (0.6-1.7)
12.6-14.9	78/81	1.4 (0.9-2.3)	1.4 (0.9-2.22)
14.9-33.8	89/81	1.7 (1.01-2.6)	1.6 (1.03-2.6)
Ptrend		0.01	
Retinol (µg/d)			
61-334	75/81	1.0 (Reference)	1.0 (Reference)
334-493	71/81	1.0 (0.6-1.5)	0.9 (0.6-1.5)
493-739	53/81	0.7 (0.4-1.1)	0.7 (0.4-1.1)
739-4643	77/81	1.0 (0.7-1.6)	1.0 (0.6-1.5)
Ptrend		0.82	

OR, odds ratio; CI, confidence interval. [1]Energy adjusted dietary intake [22] per quartile based on the control mothers, measured at 16 months after the index pregnancy. [2]Risk estimates adjusted for maternal age and periconception use of folic acid containing vitamin supplements.

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Figure 1. Box-and-whisker plot demonstrating the distribution of dietary vitamin E intake in case and control mothers at 16 months after the index-pregnancy
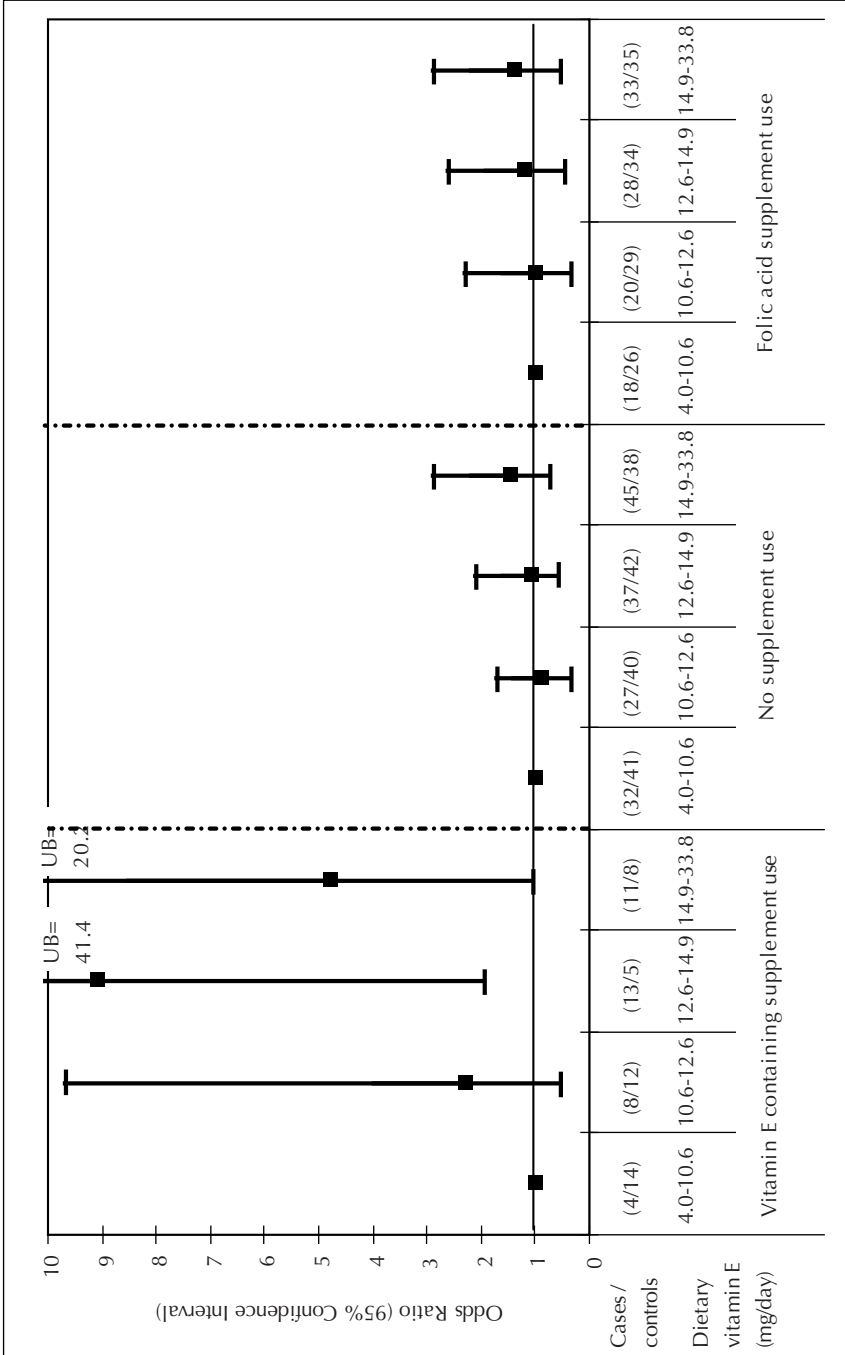
Figure 2. Quartiles of maternal dietary vitamin E intake at 16 months after the index-pregnancy, stratified for periconception vitamin E containing supplement use, no supplement use and folic acid supplement use only. UB = upper bound. Quartiles of energy adjusted dietary intake of vitamin E were calculated based on the control mothers. The first category in each panel is the reference category. P-trend <0.05 indicates a significant trend over the quartile.

Discussion

At least one year after delivery, the dietary intake has been shown to be largely comparable with the preconception period when taking breastfeeding, dieting, illnesses and pregnancy into account.[25-27] From this we infer that this study demonstrates for the first time that a high maternal intake of vitamin E via the diet or supplements in the periconception period is associated with a 1.7 to 9-fold increased CHD risk. This detrimental effect of vitamin E intake was independent of periconception folic acid supplementation. Dietary vitamin E intake was significantly higher in cases than in controls. In both groups, the mean intakes were above the Dutch RDA of 9.3 mg/d,[23] but they were lower than the more recently established American RDA of 15 mg/d.[24] Differences in dietary patterns and lifestyles between the two countries may explain the different RDAs used in those countries. Overall, the retinol intakes met the RDA[24] and were similar in the total group of case mothers and controls. Although the toxicity of vitamin E in humans is considered low, evidence on the safety of high intakes in pregnancy is limited. So far, associations between vitamin E intake and other congenital malformations than CHD in humans have not been reported. The mechanisms underlying a higher CHD risk for high maternal vitamin E intake are unknown.

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We have to consider some strengths and limitations of the study to interpret the findings. We used a standardized study time to estimate the nutritional status of the periconception period and to reduce recall bias with regard to confounders, such as periconception vitamin supplementation.[6,28] A study earlier after birth could have been influenced by maternal metabolic and endocrine changes due to lactation and recovery after pregnancy. It also would have introduced possible misclassification of cases and controls, as most congenital anomalies are diagnosed in the first year of life. Mothers were not specifically informed on a study focused on the association between vitamin E and CHDs. Therefore, it is unlikely that the data on vitamin E from diet or supplements was influenced by having a healthy or affected child. The diagnosis of CHD phenotypes is accurate, as two paediatric cardiologists trained in the same institution diagnosed all CHDs.

Our study design relies on several measures to reduce bias in nutritional intake. Firstly, the study time is around two years after conception after the index-pregnancy, which is comparable to the season of the periconception period. Devine et al. found no differences in dietary patterns between the beginning of pregnancy and at least one year postpartum.[26] The nutrition epidemiologist Walther Willet states that dietary pattern are rather stable during life.[25] Retinol and vitamin E intakes were measured with a validated

FFQ.[15] The FFQ covered the four weeks prior to the study time of 16 months after the index- pregnancy whereby the day-to-day variability of food intake is minimized. Energy adjustment also minimizes possible underreporting bias. For the total study population, the ratio of energy intake (EI) and basal metabolic rate (BMR) was 1.41. In a separate analysis, the EI: BMR ratio was 1.41 for Dutch natives, 1.43 for European others and 1.32 for non-Europeans. The EI: BMI ratio of the total study population is representative of long-term habitual intake using a cut-off value of 1.35.[29] However, there may be some underreporting in mothers of non-European origin. Vitamin E is a fat-soluble vitamin and is derived from dietary fat, which is a major source of energy. An adverse effect of high intakes of vitamin E, which may act as pro-oxidant, is biologically plausible. The oxidant / antioxidant balance is important in the first 10-12 weeks of pregnancy when the embryo and placenta develop. During this period, there is no maternal-embryonic exchange of oxygen via the placenta due to trophoblastic plugs in the spiral arteries. When these plugs dissolve too early, an imbalance in the oxidant / antioxidant activities in utero-placental tissues occurs.[30] This may not only play a pivotal role in the development of placenta-related diseases but it may also affect embryogenesis.[31] Thus, a high vitamin E intake may imbalance the oxidant/antioxidant state of the (extra) embryonic tissues. This is consistent with a recently published randomized placebo-controlled trial demonstrating that maternal vitamin C and E supplementation in pregnancy resulted in significantly more low birth weight babies.[32] However, in this trial supplementation was started only from the second trimester of pregnancy onwards. Several studies suggested a pro-oxidant effect of high concentrations of vitamin E in vivo.[33,34] Furthermore, vitamin E functions as a signalling molecule and regulator of gene expression and may, thereby, modify specific genes involved in embryonic heart development.[35] Vitamin E may also inhibit human cytosolic glutathione S-transferases that contribute to the detoxification of drugs and endogenous toxins.[36] Interestingly, periconception users of vitamin E supplements demonstrated a five- to nine-fold higher CHD risk together with dietary vitamin E intakes above 14.8 mg/d. To examine a dose-response relationship to determine a safe threshold for vitamin E intake, the total vitamin E intake from diet and supplements should be added up. However, due to the absence of brand names of the supplements used in the periconception period in 31 of 75 mothers, this was not possible for the total group. Most vitamin E containing supplements were multivitamins that also contained folic acid. Despite the potential protective effect of folic acid in these supplements, we demonstrate a higher CHD risk of high dietary intakes of vitamin E in this group. This may indicate that the harmful effect of vitamin E is not compensated by folic acid. We tested the hypothesis that also a low dietary intake

of vitamin E might affect CHD risk in an additional analysis with the highest quartile of dietary vitamin E as a reference. This analysis demonstrated a reduced risk of CHD for a dietary intake of vitamin E below 10.6 mg/d, OR 0.6 (95% CI: 0.6-0.97).

We also have to consider that the observed association results from other determinants that are related to vitamin E. It is possible that the observed association actually results from a major source of vitamin E, such as vegetable oils, nuts, vegetables, seeds and eggs. We cannot rule out that women with a high vitamin E intake also have an unhealthier dietary pattern by consuming more products that contain high amounts of fatty acids, even though the total energy intake was comparable between cases and controls. Future dietary pattern analyses may give some more information on this issue, as was previously shown by our group in mothers of a child with orofacial clefting.[37]

We can also not exclude that exposures to x-ray, maternal illnesses or medications have influenced CHD risk as we do not have data on these. Well known risk factors such as family history of CHD, maternal diabetes, obesity and lifestyle factors, such as smoking and alcohol use, were not significantly more often present in case mothers than in controls. Educational level, ethnicity and use of alcohol and folic acid supplements were not different between mothers with a dietary vitamin E intake in the upper quartile and those in the lower three quartiles (data not shown). Smoking in the periconception period, however, was more frequent in mothers with a high dietary intake of vitamin E but additional adjustment for periconception use of cigarettes did not change the risk estimates. We therefore feel that it is unlikely that a high dietary vitamin E intake is a proxy of certain maternal lifestyles.

Exclusion of non-isolated cases did not alter the median dietary vitamin E intakes and therefore we included both isolated and non-isolated cases. It is possible that CHDs related to a genetic syndrome are less sensitive to modifying factors such as vitamin E. If true, then the observed associations might be an underestimation of the actual risk. We examined the relation between the different CHD phenotypes and dietary vitamin E and retinol intake, as some specific phenotypes may be more sensitive for environmental exposures than others. No significant associations were found but our study is underpowered for such sub group analysis. Botto et al. reported an increased risk for transposition of the great arteries in children born to mothers who used more than 3000 µg/d of retinol by supplements.[13] Retinol intakes in our study were below this threshold though an unplanned subgroup analysis of mothers of children with aortic coarctation showed higher intakes of retinol (752 µg/d) compared with controls (493 µg/d). This and the dichotomy between our findings for vitamin E and the data

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supporting vitamin E as a possible treatment for the prevention of abnormalities in the infants of women with diabetes[38,39] warrant further study.

Our findings raise questions on the safety of vitamin E during pregnancy and require replication in an independent study group. With little available evidence, the Dutch Health Council has set the upper tolerable limit of vitamin E intake in pregnancy at 300 mg/d.[27] Considering our findings, one may argue whether the recommendations for vitamin E intake are safe enough for (pre)pregnant women and need to be adapted. Evidence on the safety threshold of vitamin E and retinol is important because numerous people have unhealthy dietary habits and multivitamin supplements are widely used in uncontrolled settings.

Conclusion

In conclusion, our results suggest that maternal dietary vitamin E intake at a level above the current Dutch RDA is associated with an increased CHD risk. Periconception users of a vitamin E supplement are at the highest risk. Future studies should focus on both beneficial and teratogenic effects of natural and synthetic anti-oxidants on reproductive outcome.

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Part II

Medication

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

Aims: Congenital heart defects (CHDs) have a multifactorial origin, in which subtle genetic factors and peri-conception exposures interact. 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 peri-conception period, affect CHD risk.

Methods and results: In 292 case and 316 control families, maternal peri-conception 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]. 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 peri-conception 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 (CHDs) are among the most common congenital malformations and are a leading cause of perinatal mortality. Over 85% of CHDs have a multifactorial origin, involving genetic factors, maternal nutrition and lifestyle factors during embryogenesis.[1]

One important risk factor for CHDs is maternal hyperhomocysteinaemia, 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 hyperhomocysteinaemia 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 CHDs. This hypothesis was tested in a case-control family study in an ethnically homogeneous population in the Western part of the Netherlands.

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Materials and methods

Study population

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 CHDs. From June 2003 this study has included cases from four University Medical Centres and controls in collaboration with the child health centres 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 aetiology.[12-14] Two paediatric cardiologists diagnosed the CHDs 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 centres. 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.

Measurements

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 non-users.

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.

Laboratory determinations

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

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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%.

Data 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 (ORs) and 95% confidence intervals (CIs) 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 CHDs. First, we coded separate categories for the risk of the genotype of mother or child in combination with periconception medicine use. NNMT GG carriers without 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 ORs with 95% CIs in a multivariable logistic regression model. These ORs 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 $P<0.05$ 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 ORs and P-values were adjusted for

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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.
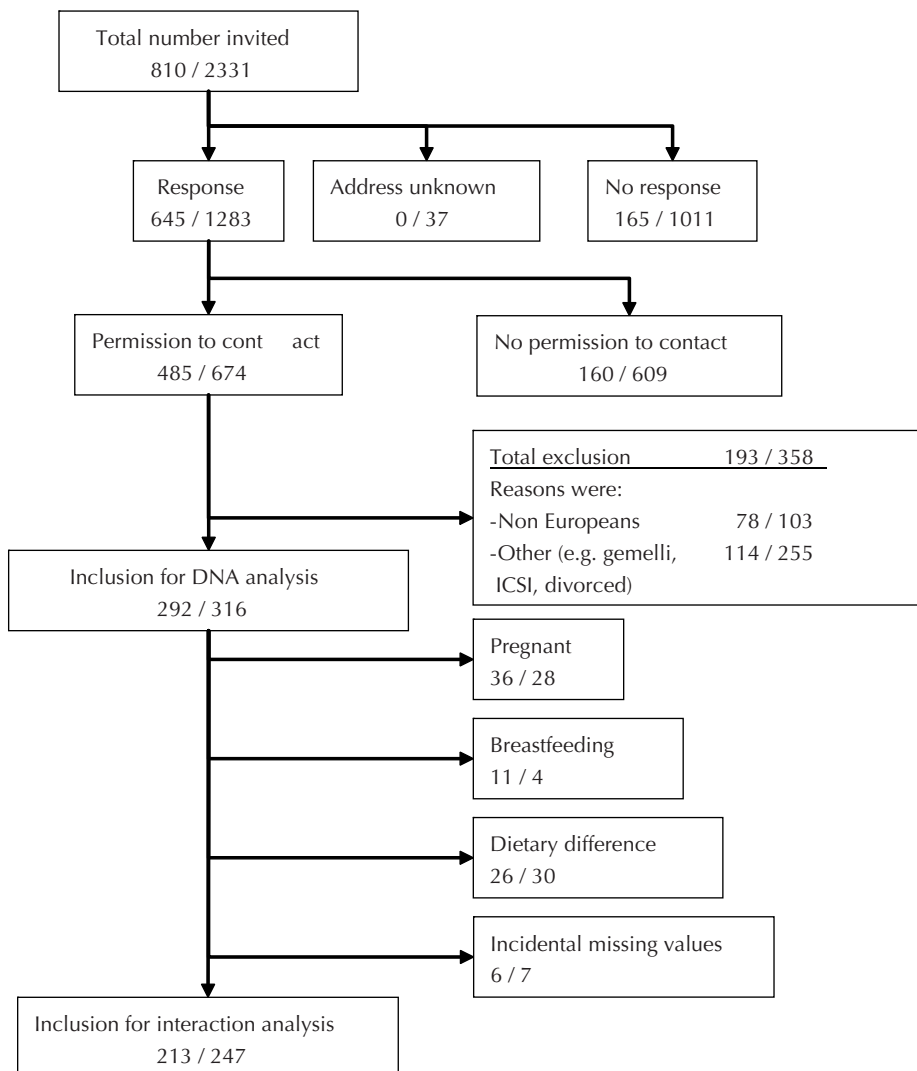
Figure 1. Study population flowchart. The numbers are the absolute numbers of case mothers / control mothers.

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.

Interaction analysis

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 = 0.006).

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

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Table 1. Sociodemographic and lifestyle characteristics

Study moment	Mothers			Fathers		
	Cases n=291	**Controls** n=313	**P-value**	**Cases** n=292	**Controls** n=314	**P-value**
Age, years	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[1]			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			
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			

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Study moment	Mothers			Fathers		
	Cases n=291	**Controls** n=313	**P-value**	**Cases** n=292	**Controls** n=314	**P-value**
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
Study moment	**Children**					
	n = 283	n = 316				
Age, months	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[2]	25 (9)	17 (5)	0.098			
Ethnicity[3]			0.991			
Dutch Natives	258 (91)	288 (91)				
European Others	25 (9)	28 (9)				

Values are medians (interquartile range) or number (percentage). [1]Categorized as low (primary/lower vocational/intermediate secondary), intermediate (higher secondary/intermediate vocational) or high education (higher vocational/university). [3] [1,2]Family members with a CHD in the first, second and third degree. [3]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]

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. OR, odds ratio. CI, confidence interval.

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

CHD phenotypes	Mothers[1]			Fathers[2]			Children[3]		
	Number (n=291)	Cases[4] AG/AA	OR(95%CI)	Number (n=292)	Cases[4] AG/AA	OR(95%CI)	Number (n=283)	Cases[4] 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)	27	6 (20)	0.6 (0.2-1.4)	27	5 (19)	0.5 (0.2-1.3)
Perimembranous ventricular septal defect	81	21 (26)	0.6 (0.3-1.02)	80	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. [1]Controls mothers NNMT AG/AA genotype: 118 (38); Total number = 313 [2]Controls fathers NNMT AG/AA genotype: 97 (31); Total number = 314 [3]Controls children NNMT AG/AA genotype: 102 (32); Total number = 316 [4]Cases are numbers (percentages) of the NNMT AG/AA genotype. The NNMT GG genotype was the reference group.

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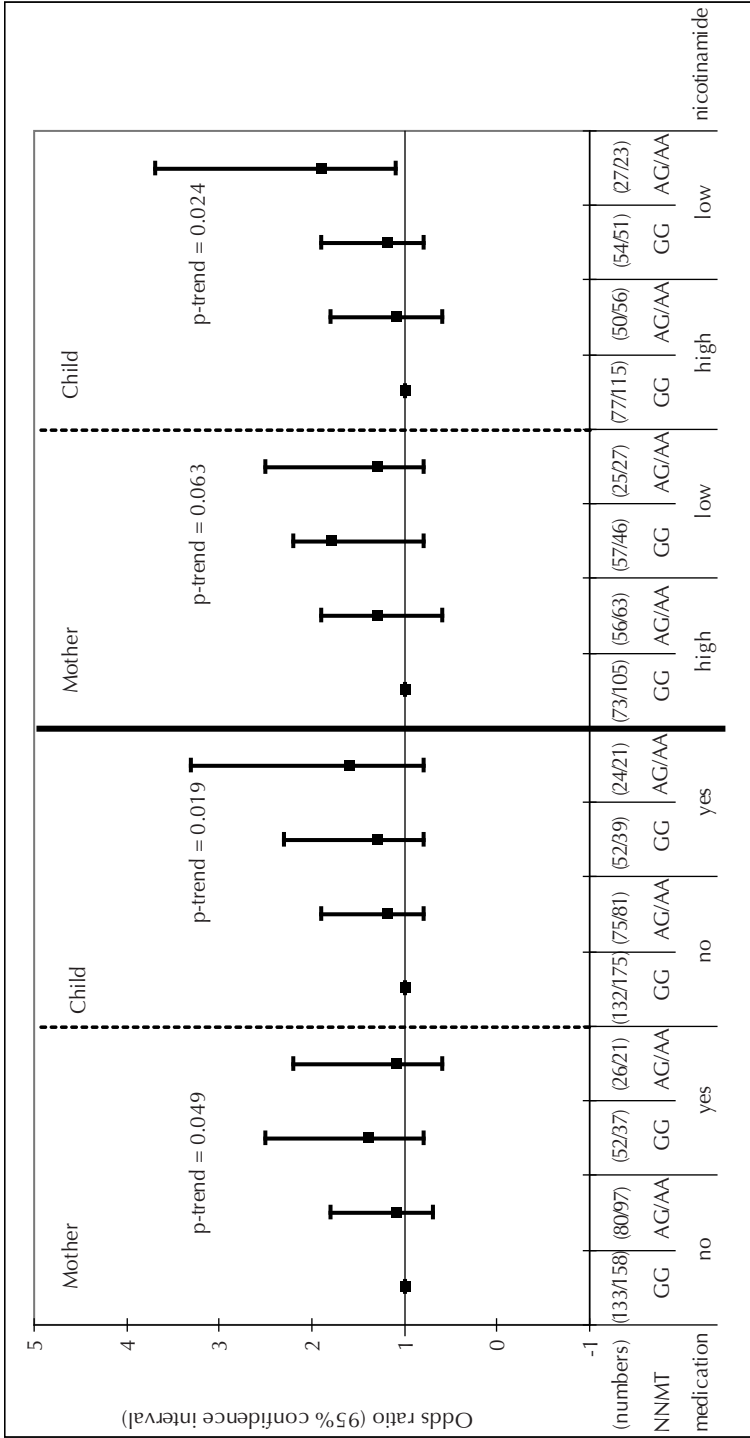
Figure 2. Periconception medicine use or dietary nicotinamide intake combined with the NNMT genotypes of the mother or the child and CHD risk. The first group in each panel represents the reference group. In the left two panels the ORs with 95% CIs are shown of the different subgroups with combinations 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 ORs with 95% CIs are shown of the combinations of maternal dietary nicotinamide intake with NNMT genotype of the mother (first panel) or the child (second panel).
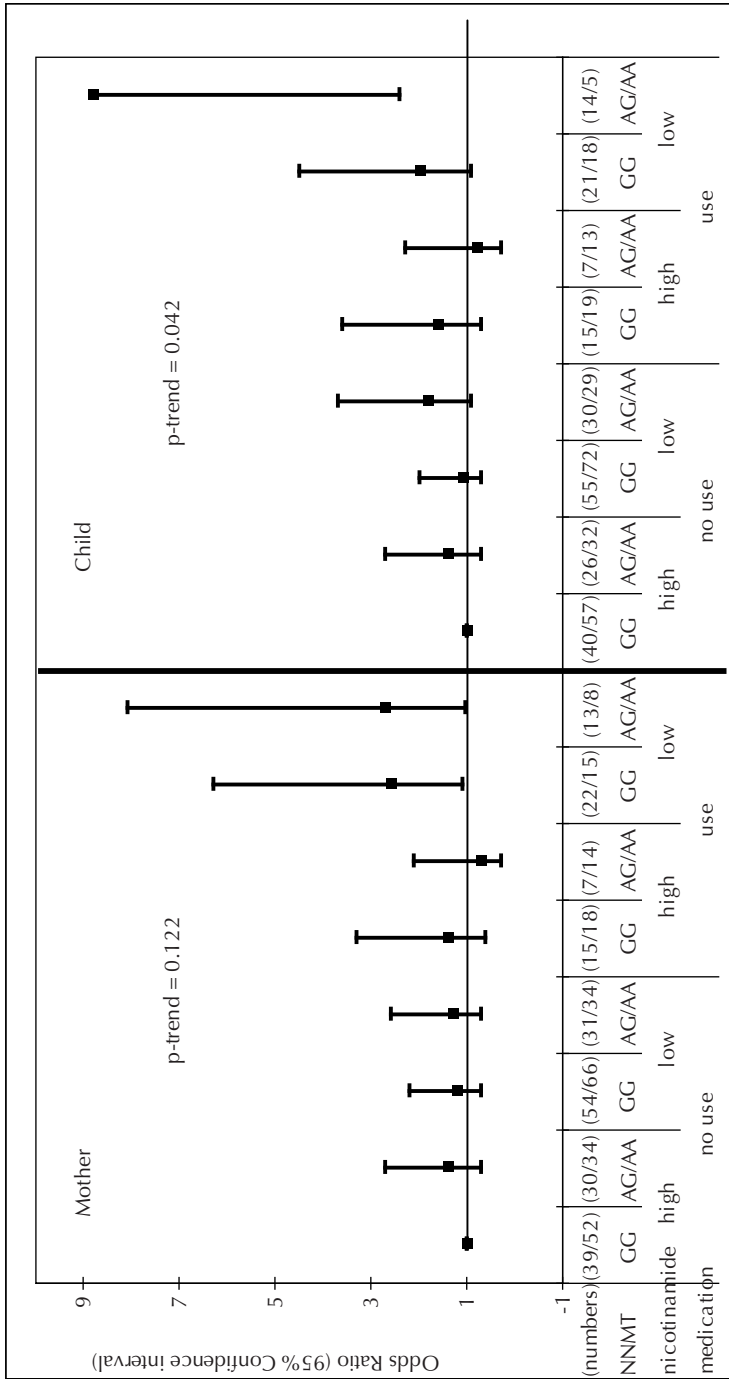
Figure 3. Periconception medicine use, dietary nicotinamide intake and the NNMT genotype. The first category in each panel is the reference category. These consist of mothers who did not use any medicines in the periconception period, who had a high dietary intake of nicotinamide based on the 30th percentile 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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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- and control-mothers (Table 1). Therefore, it is very unlikely that an accompanying low folate intake 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/

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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 hyperhomocysteinaemia 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 aetiology. Our results provide a first set of data against which future studies with larger sample sizes can be compared to.

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Early pregnancy exposure to antihistamines, pregnancy related nausea and vomiting and the risk of congenital heart disease

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Part III

Vascular Endothelial Growth Factor

Chapter 7

VEGF polymorphisms are associated with endocardial cushion defects: a family based case-control study

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Abstract

Endocardial cushion defects (ECD) of the cardiac outflow tract are among the most common congenital heart disease phenotypes. Vascular endothelial growth factor (VEGF) is essential for endocardial cushion formation and derangements in VEGF synthesis lead to ECD. Three functional single nucleotide polymorphisms (SNPs) in the VEGF gene -2578 C>A, -1154 G>A and -634 G>C play a role in cardiogenesis. In a Dutch case-control family study of triads, 190 case and 317 control children with both parents, we investigated linkage and association between these *VEGF* SNPs and ECD. Allele frequencies for the three VEGF SNPs were comparable between ECD children and controls. However, VEGF alleles -2578 C and -1154 G were transmitted more frequently to children with ECD (P=0.003 and P=0.002), in particular perimembranous ventricular septal defects (P=0.012 and P=0.006). The -2578A/-1154A/-634G haplotype was associated with a reduced risk of ECD (OR 0.7, 95% CI 0.6-1.0) and was significantly less transmitted to children with ECD (P=0.002). In a Dutch population we show that the VEGF 2578 C and -1154 G alleles and the AAG haplotype are associated with ECD. Possible VEGF gene-environment interactions exposures are discussed.

Introduction

Congenital heart diseases are the most frequent major congenital malformations with a worldwide birth prevalence rate of over one million per year.[1] Because of the high infant mortality and morbidity rates the causes of these malformations should be identified and targeted for preventive strategies in the future.[2] Most congenital heart diseases have a complex origin, in which interactions between subtle genetic variations and periconception exposures play a role.[3] Vasculogenesis and heart development are dependent on the genetic constitution of the embryo derived from both parents, and the maternal genetically controlled nutritional, endocrine and metabolic environment.[4] During cardiac development, the outflow tract is divided into the ascending aorta and the pulmonary trunk and aligned to the left and right ventricle, respectively. The formation of the endocardial cushions in the atrioventricular canal and outflow tract takes place by epithelial-mesenchymal transformation which is a key process in the development of the heart septa and valves. Numerous lines of research have implicated a strict spatio-temporal expression pattern of the vascular endothelial growth factor (VEGF*)* gene in the control of endocardial cushion development.[5] In addition to its tight regulation during cardiogenesis, VEGF gene expression is also regulated by environmental exposures. A nice example is that hypoxia induces VEGF gene expression and seems to contribute as such to the generation of major malformations in the atrioventricular-canal and outflow tract in mice.[6] Hyperglycemia inhibits epithelial-mesenchymal transformation by reducing VEGF gene expression.[7] Furthermore, hyperhomocysteinemia, which increases the risk of human congenital heart disease, was shown to up regulate VEGF mRNA in a human endothelial cell line.[8] Interestingly, vegf120/120 mutant mouse embryos, showing overexpression of the VEGF120-isoform and lacking the other VEGF-isoforms, develop endocardial cushion defects (ECDs), such as ventricular septal defects, pulmonary valve stenosis and Tetralogy of Fallot.[9,10]

In human, the VEGF gene is located on chromosome 6p21.3 and consists of 8 exons that generate different isoforms through alternative splicing. VEGF gene expression shows a marked individual variability, which is partly determined by single nucleotide polymorphisms (SNPs). The most interesting functional SNPs that differentially express VEGF in vitro are VEGF -2578 C>A and -1154 G>A, located in the promoter[11], and VEGF -634 G>C, located in the 5'untranslated region of the gene.[12] First studies suggest associations between the VEGF SNPs -2578 C>A, -1154 G>A and -634 G>C and several congenital heart disease phenotypes.[13-15]

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In a large Dutch case-control family study of triads, i.e., child, mother, father, we investigated whether three functional SNPs in the VEGF gene, i.e., -2578 C>A, -1154 G>A and -634 G>C, affect the risk of ECD, being the most common subgroup of congenital heart disease phenotypes.

Materials and methods

Study design and population

This study was embedded in the Dutch HAVEN-study, which is an ongoing case-control family study of triads in the western part of the Netherlands, initated to investigate the etiology and future prevention of congenital heart disease in which genetic and environmental factors are implicated. Details of the study design and population were described previously.[16] Briefly, the domain population comprised case and control children, between 12 and 18 months of age, living in the western part of the Netherlands. Children with a congenital heart disease were diagnosed by two pediatric cardiologists from the Erasmus MC, University Medical Center in Rotterdam, Leiden University Medical Center in Leiden, VU University Medical Center, and Amsterdam Medical Center in Amsterdam, in the Netherlands. In collaboration with the pediatric cardiologists the case children with their parents were enrolled. In the HAVEN Study we included case families of which the index child had a congenital heart defect in which gene-environment interactions are implicated in the etiology.[17-19] VEGF is reported to play a role in endocardial cushion formation. Therefore, in the current analysis of associations between SNPs in the VEGF gene and congenital heart defects we selected from the total case group of the HAVEN Study only the endocardial cushion defect (ECD) phenotypes i.e., perimembranous ventricular septal defect, pulmonary valve stenosis, Tetralogy of Fallot, aortic valve stenosis and atrioventricular septal defect. Figure 1 depicts the flow chart of the study population. Between October 2003 and January 2007, we ascertained 289 children with congenital heart disease and 317 control children with their parents. In the current analysis only Dutch native children and children from other European origin were included to increase ethnic homogeneity. Children were classified as Dutch natives when 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. If 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 we classified the child in the category European Others.[20] Non-European

children and families with Mendelian inconsistencies or unsuccessful genotyping for all three SNPs were also excluded. In total, SNP data from 190 case children with ECD and both parents and 317 control children with both parents were available for the analyses. All phenotypes were ascertained by ultrasound and/or cardiac catheterization and/or surgery and diagnosed according to the International Pediatric and Congenital Cardiac Code.[21] The ECD group consisted of 122 children with isolated and 68 with complex ECD. Complex ECD was defined as ECD with the co-occurrence of any other structural congenital anomaly. The group of complex ECD was further subdivided into 46 children of which the ECD was part of a syndrome: Trisomy 21 (n=34), deletion 22q13 (n=1), deletion 22q11 (n=2), insertion 1 > 3 (n=1), Noonan syndrome (n=1), CHARGE syndrome (n=1), Duane syndrome(n=1), Turner syndrome(n=2), VACTRL (n=1), Alagille syndrome (n=1) and Beckwith Wiedemann syndrome (n=1), and 22 children with ECD and Other anomalies that could not be traced to a syndrome, such as hydronefrosis, hip dysplasia, hypospadia, cleft lip and or palate, pyloric stenosis, clubfoot and deafness. Control children and their parents were enrolled in collaboration with public child health centers in the Rotterdam area. The Dutch health care system includes a standardized and regular check up of all newborns for health, growth and development by physicians trained in child health care. Children were eligible as controls if they did not have a major congenital malformation or chromosomal defect according to the medical records from the regular check up at the child health center. Both cases and controls were invited to participate at a fixed study moment at 16 months of age of the child. At that time, the diagnosis of congenital heart diseases is confirmed. All children originated from the western part of the Netherlands. None of the families was related, all were familiar with the Dutch language in speaking and reading and none of the children has been adopted. Potential participating parents were informed about the genetic research questions of the Haven Study. Only children of whom the parents confirmed to be both the biological parent participated. All mothers completed a general questionnaire from which we extracted for the current analyses demographic data, child's gender, family history of congenital heart disease and ethnicity. The study protocol was approved by the Central Committee of Research in Humans in The Hague, the Netherlands, 2003 and the local Medical Ethical Committees of the four participating hospitals, 2003. Written informed consent was obtained from all parents and on behalf of their child as legal representatives.
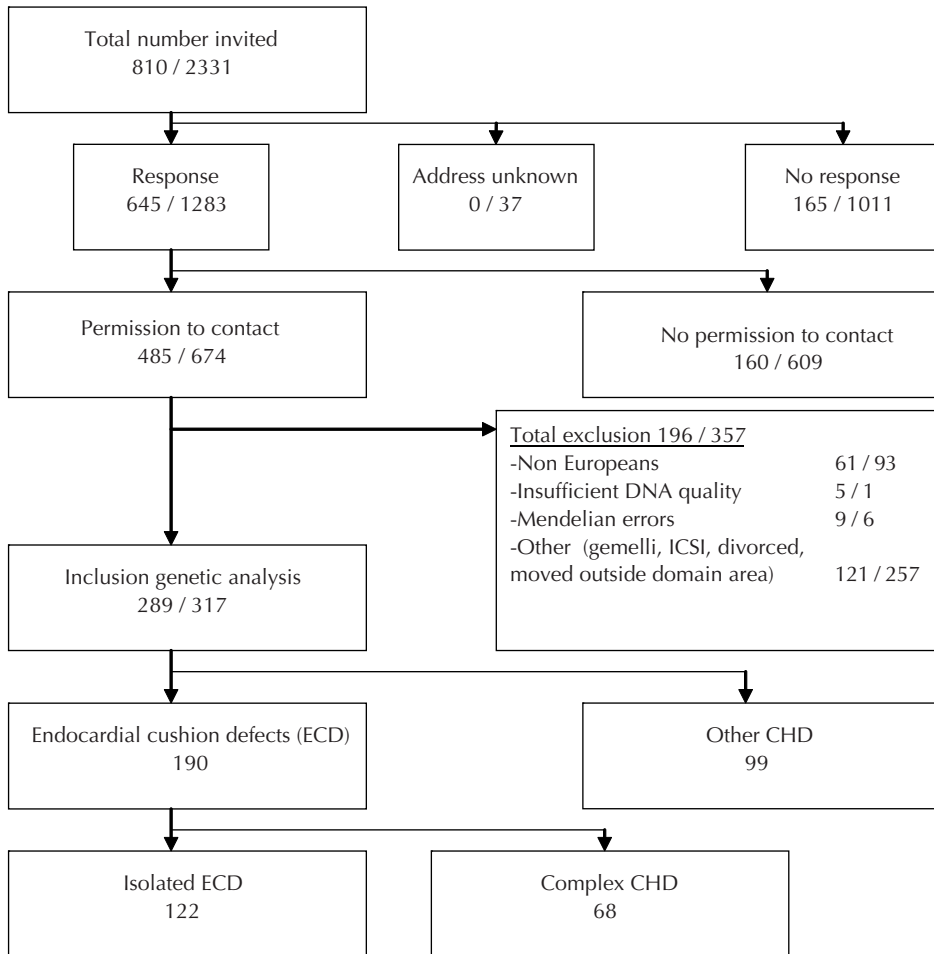
Figure 1. Study population flowchart. Numbers are the absolute numbers of case mothers / control mothers. CDH, congenital heart disease.

Genotyping

DNA was isolated from EDTA blood with a Total Nucleic Acid Extraction kit on a MagNAPure LC (Roche Molecular Biochemicals, Mannheim, Germany). DNA was isolated from buccal swabs from 4 case children, 1 case father and 1 control father, due to logistical problems or failures in blood sampling. Genotypes were determined in 1-2 ng of genomic DNA with TaqMan allelic discrimination assays and analyzed using

the software SDS 2.2.2 (Applied Biosystems, Foster City, CA). Case and control triads were genotyped for VEGF SNPs at positions -2578 C>A, -1154 G>A, and -634 G>C, numbered from the translation start site. Primers and probes of -2578 C>A (rs699947), -1154 G>A (rs1570360), and -634 G>C (rs2010963) were obtained from Applied Biosystems (http://www.appliedbiosystems.com). Each genotype plate contained no DNA template (water) controls. To check consistency, we randomly chose 5% of samples and repeated the measurements. Reproducibility was 100%.

Statistical analysis

General characteristics were compared between children with ECD and controls. The comparisons between dichotomous variables, presented as numbers and percentages, were tested by Chi-square tests. Frequencies of VEGF alleles and genotypes were calculated for children with ECD and controls, and haplotype frequencies were estimated using UNPHASED 3.0.[22] D' and r^2 values of linkage disequilibrium (LD) between SNPs were estimated using Haploview 3.3.2.[23] Deviations from Hardy-Weinberg equilibrium were tested using Chi-squared tests with one degree of freedom. Chi-squared tests were also used to evaluate differences in the distributions of alleles, genotypes and haplotypes between case and control children, and separately for each ECD phenotype. A logistic regression model was used to estimate the risk of the pooled group of ECD phenotypes. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. Statistical analysis was performed using SPSS software (version 12.0 SPSS Inc., Chicago, IL, USA). We applied the family-based association test, a transmission disequilibrium test (TDT) -like test statistic, to investigate linkage and association for VEGF alleles (FBAT) and haplotypes (HBAT) (http://www.biostat.harvard.edu/~fbat/fbat.htm).[24] FBAT analyses were performed under an additive model. The 1-POET test, which is an extension of the TDT statistic, was used to detect a possible parent-of-origin effect.[25] All tests were two-tailed and P-values were Bonferroni adjusted to account for multiple tests.

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Results

The general characteristics of children with ECD and controls are presented in Table 1. The distributions of age, gender, ethnicity and family history of congenital heart disease were comparable between the children with ECD and controls.

Moderate linkage disequilibrium was shown between VEGF -2578 C>A and -1154 G>A (r^2=0.52); between VEGF -2578 C>A and -634 G>C (r^2=0.49); and between VEGF

-1154 G>A and -634 G>C (r^2=0.26). The distributions of the VEGF alleles and genotypes are presented in Table 2. None of the genotype frequencies in the control population deviated from Hardy-Weinberg equilibrium. The genotype distributions of VEGF -2578 C>A, -1154 G>A and -634 G>C were not significantly different between the children with ECD and controls. The VEGF -2578 C and -1154 G alleles were more frequently present, albeit not significantly, in children with ECD than in controls. The distribution of the genotypes stratified per ECD phenotype is summarized in Table 3. In children with pulmonary valve stenosis the distributions of the -2578 C>A variants deviated from Hardy-Weinberg equilibrium and in these children the C-allele tended to be more frequently present than in controls (P=0.07). We inferred haplotypes from the three VEGF polymorphisms and compared the frequencies. Four of the eight possible haplotypes occurred at an appreciable frequency in both children with ECD and controls, i.e., AAG, CGC, CGG and AGG whereby each letter refers to the allele of the -2578, -1154, and -634 SNP, respectively (Table 4). Consistent with the genotype distributions, the haplotype analyses revealed that the CGG haplotype was present in 17% of children with ECD and in 13% of control children (P=0.08). The AAG haplotype was associated with a reduced risk of the ECD phenotype (OR 0.8, 95% CI 0.6-1.0).

The results of the FBAT analyses are depicted in Table 5 and point out that the -2578C and -1154G alleles were transmitted to children with an ECD more frequently than expected by Mendelian inheritance (59% of all transmissions). FBAT-o, which also incorporates information from the control triads, revealed even stronger evidence for over transmission of the -2578 C allele (P=0.003) and the -1154 G allele (P=0.002) to offspring with the ECD phenotype. These P-values survived the rather conservative Bonferroni correction for multiple testing of 5x3 independent test with α=0.0033. Stratification for five separate ECD phenotypes revealed that the -2578 C allele (P=0.012) and the -1154 G allele (P=0.006) were significantly overtransmitted to children with pVSD. Consistently, the HBAT-o results suggested that the CGC haplotype was overtransmitted to children with ECD (P=0.057) and the AAG haplotype was significantly less frequently transmitted than expected to children with ECD (41% of transmissions, which was significant at an Bonferroni α-level of 0.0025) (Table 6), in particular to children with pVSD (P=0.022). Empirical P-values obtained by permutation analyses (HBAT-p) substantiated these associations. The global P-value with four degrees of freedom for differences in the transmission of the four haplotypes to children with ECD was P=0.03. We did not find any evidence for a parent-of-origin effect of risk alleles in the VEGF candidate loci (data not shown).

Table 1. General characteristics of the case children with ECD and control children

	ECD	Controls
Child	n=190	n=317
Age at the study moment in months, median (interquartile range)	16.2 (15.1-19.2)	16.1 (15.1-18.0)
Boys, n (%)	96 (51)	170 (54)
Family history for CHD[1], n (%)	18 (10)	17(5)
Ethnicity[2], n (%)		
Dutch Natives	177 (93)	289 (91)
European Others	13 (7)	28 (9)

ECD, outflow tract defect. CHD, congenital heart disease. [1]Any congenital heart disease of family members in the first, second or third degree. [2]Children were classified as Dutch natives when 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. If 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 we classified the child in the category European Others.

Table 2. Distribution of the VEGF genotypes in case children with ECD and control children

VEGF SNPs	ECD	Controls	X^2	*Fisher's* P	OR (95% CI)
	n=185	n=312			
-2578, AA	44 (24)	88 (28)	2.52	0.28	0.8 (0.6-1.1)
CA	88 (48)	153 (49)			0.8 (0.5-1.2)
CC	53 (29)	71 (23)			1.0 (Reference)
HWE p-value	-	0.77			
C-allele	194 (52)	295 (47)	2.38	0.12	
	n=187	n=307			
-1154, AA	18 (10)	43 (14)	2.32	0.31	0.8 (0.6-1.1)
GA	79 (42)	130 (42)			0.9 (0.6-1.3)
GG	90 (48)	134 (44)			1.0 (Reference)
HWE p-value	-	0.20			
G-allele	259 (69)	398 (65)	1.55	0.21	
	n=184	n=303			
-634, CC	22 (12)	39 (13)	0.26	0.88	1.0 (0.7-1.3)
GC	85 (46)	133 (44)			1.1 (0.7-1.6)
GG	77 (42)	131 (43)			1.0 (Reference)
HWE p-value	-	0.57			
G-allele	239 (65)	395 (65)	0.00	1.00	

Values are numbers (percentage). OR, odds ratio. CI, confidence interval. ECD, outflow tract defect. HWE, Hardy-Weinberg Equilibrium.

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Table 3. Distribution of the VEGF genotypes in case children with ECD, stratified for ECD phenotypes, and control children

VEGF SNPs	ECD					Controls
	pVSD	PS	TOF	AVSD	AoS	
	n=79	n=42	n=29	n=29	n=6	n=312
-2578, AA	15 (19)	12 (29)	6 (21)	9 (31)	2 (33)	88 (28)
CA	44 (56)	14 (33)	16 (55)	11 (38)	3 (50)	153 (49)
CC	20 (25)	16 (38)	7 (24)	9 (31)	1 (17)	71 (23)
X^2; P-value[1]	0.25	0.07	0.68	0.47	0.93	
HWE p-value	-	-	-	-	-	0.77
C-allele	84 (53)	46 (55)	30 (52)	29 (50)	5 (42)	295 (47)
X^2; P-value[2]	0.16	0.18	0.49	0.65	0.72	
	n=79	n=43	n=29	n=30	n=6	n=307
-1154, AA	6 (8)	6 (14)	2 (7)	3 (10)	1 (17)	43 (14)
GA	36 (46)	13 (30)	11 (38)	15 (50)	4 (67)	130 (42)
GG	37 (47)	24 (56)	16 (55)	12 (40)	1 (17)	134 (44)
X^2; P-value[1]	0.31	0.27	0.39	0.68	0.40	
HWE p-value	-	-	-	-	-	0.20
G-allele	110 (70)	61 (71)	43 (74)	39 (65)	6 (50)	398 (65)
X^2; P-value[2]	0.24	0.28	0.16	1.00	0.36	
	n=79	n=43	n=29	n=27	n=6	n=303
-634, CC	9 (11)	6 (14)	2 (7)	4 (15)	1 (17)	39 (13)
GC	43 (54)	16 (37)	14 (48)	10 (37)	2 (33)	133 (44)
GG	27 (34)	21 (49)	13 (45)	13 (48)	3 (50)	131 (43)
X^2; P-value[1]	0.24	0.71	0.64	0.79	0.87	
HWE p-value	-	-	-	-	-	0.57
G-allele	97 (61)	58 (67)	40 (69)	36 (67)	8 (67)	395 (65)
X^2; P-value[2]	0.35	0.75	0.54	0.81	1.00	

Values are numbers (percentages), tested between case-children with ECD and controls by Chi-square tests (X^2). [1]Comparison of genotypes between cases and controls. [2]Comparison of alleles between cases and controls. ECD, outflow tract defect; pVSD, perimembranous ventricular septal defect; PS, pulmonary valve stenosis; TOF, Tetralogy of Fallot; AVSD, atrioventricular septal defect; AoS, aortic valve stenosis.

Table 4. Distribution of VEGF haplotypes in case children with ECD and control children

VEGF Haplotypes 2578/-1154/-634	ECD	Controls	X^2	*Fisher's* P	OR (95% CI)
AAG	114.4 (30)	225.1 (36)	3.92	0.05	0.76 (0.58-1.00)
CGC	133.4 (35)	220.2 (35)	0	1	-
CGG	65.0 (17)	82.4 (13)	3.07	0.08	1.37 (0.96-1.95)
AGG	65.1 (17)	104.8 (17)	0	1	-

Values are numbers (percentage). OR, odds ratio. CI, confidence interval. ECD, outflow tract defects.

Table 5. Family based association analysis of VEGF alleles transmitted from heterozygous parents to case children with the separate ECD phenotype

	Allele VEGF	Frequency	FBAT		FBAT-o	
			Informative Trios	P-value	Informative Trios	P-value
ECD	-2578 C	0.490	136	0.015	366	0.003[1]
	-1154 G	0.663	131	0.017	331	0.002[1]
	-634 G	0.648	132	0.125	345	0.066
pVSD	-2578 C	0.490	64	0.052	294	0.012
	-1154 G	0.664	59	0.039	259	0.006
	-634 G	0.639	63	0.185	276	0.099
PS	-2578 C	0.494	32	0.170	262	0.060
	-1154 G	0.668	32	0.217	232	0.063
	-634 G	0.647	28	0.398	241	0.256
TOF	-2578 C	0.489	18	0.239	248	0.109
	-1154 G	0.670	19	0.221	219	0.084
	-634 G	0.646	19	0.127	232	0.081
AVSD	-2578 C	0.490	19	0.564	249	0.300
	-1154 G	0.663	18	0.835	218	0.456
	-634 G	0.642	19	0.852	232	0.892
AoS	NT					

FBAT-o incorporates information from control trios. NT, sample size is too low. ECD, outflow tract defect. pVSD, perimembranous ventricular septal defect. PS, pulmonary valve stenosis. TOF, Tetralogy of Fallot. AVSD, atrioventricular septal defect. AoS, aortic valve stenosis. [1]P-values surviving Bonferroni correction for 5 x 3 independent tests, with X= 0.0033.

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Table 6. Family based association analysis of VEGF haplotypes transmitted from parents to case children with the separate ECD phenotype

	VEGF Haplotypes		HBAT		HBAT-o		HBAT-p
	-2578-1154-634	**Frequency**	**Informative Trios**	***P*-value**	**Informative Trios**	***P*-value**	***P*-value**
ECD	CGC	0.344	113.9	0.063	294.8	0.057	0.064
	AAG	0.330	109.9	0.008	288.8	0.002[1]	0.007
	AGG	0.173	76.0	0.612	202.9	0.492	0.611
	CGG	0.148	78.0	0.538	184.0	0.299	0.489
pVSD	CGC	0.354	56	0.056	239	0.045	0.060
	AAG	0.327	55	0.028	231	0.006	0.022
	AGG	0.172	41	0.764	169	0.629	0.699
	CGG	0.142	35	0.873	144	0.573	0.828
PS	CGC	0.352	20	0.117	191	0.090	0.098
	AAG	0.322	26	0.366	192	0.129	0.400
	AGG	0.174	15	0.819	142	0.946	0.743
	CGG	0.151	20	0.853	129	0.840	0.741
TOF	CGC	0.350	18	0.276	189	0.220	0.371
	AAG	0.320	15	0.221	181	0.090	0.322
	AGG	0.180	13	0.816	140	0.944	0.717
	CGG	0.149	14	0.590	123	0.435	0.462
AVSD	CGC	0.359	13	0.513	184	0.650	0.614
	AAG	0.326	12	0.405	178	0.227	0.449
	AGG	0.174	10	0.808	137	0.709	0.654
	CGG	0.140	11	0.166	120	0.113	0.205
AoS	NT	-	-	-	-	-	-

HBAT-o incorporates information from control trios. HBAT-p = 10,000 permutations. NT, sample size is too low. ECD, outflow tract defect. pVSD, perimembranous ventricular septal defect. PS, pulmonary valve stenosis. TOF, Tetralogy of Fallot. AVSD, atrioventricular septal defect. AoS, aortic valve stenosis. [1]P-value surviving Bonferroni correction for 5 x 4 independent tests, with α=0.0025.

Discussion

This study provides evidence for an association between the VEGF gene, in particular the VEGF -2578 C and -1154 G alleles and AAG haplotype, and ECD in human.

Recently, Vannay et al. demonstrated a higher frequency of the VEGF -634 C allele in Hungarian children with congenital heart disease.[14] They included several congenital

heart disease phenotypes with valvular and/or septal defects, which may explain the different results. In a Chinese study the VEGF -634 C allele was shown to reduce the risk of a child with the pVSD phenotype.[15] Our sample size of 79 children with pVSD and 317 controls was too limited to detect the association with this phenotype (power of 41%, OR 0.7, risk allele frequency 35%, type I error of 0.05, congenital heart disease population risk of 0.008). Lambrechts et al. reported an increased risk of the VEGF -2578/-1154/-634 AAG haplotype for both nonsyndromic and DiGeorge syndrome related TOF.[13] We show, however, that the same AAG haplotype was associated with a reduced risk for in particularly ECD. In our study the VEGF -2578 A and -1154 A alleles were less frequent in children with TOF, although not significantly different from controls. The genetic differences that may exist between our Dutch population and their mixed population of Caucasians, Afro-Americans, Hispanics and subjects from unknown origin, might explain the differences in results. Furthermore, it cannot be excluded that selective survival is confounding their results as no information was provided on the age of the children with congenital heart disease at the moment of investigation. Moreover, maternal exposures in early pregnancy that seem to influence VEGF expression may also have led to the different results.

The effect of the studied VEGF SNPs on the expression in human, particularly the VEGF isoforms, is not fully understood. In vegf120/120 mouse embryos, lacking the 164 and 188 isoforms, a spatiotemporal increase of VEGF coincides with hyperplasia of the outflow tract cushions and abnormally high levels of apoptosis. This manifests in later stages than TOF, pulmonary stenosis and ventricular septal defects.[10] In humans, higher VEGF production was observed in cells from individuals with the -2578 C and -1154 G alleles compared with -2578 A and -1154 A individuals in stimulated peripheral blood mononuclear cells from healthy volunteers.[26] Individuals homozygous for the haplotypes containing the -1154 AA and -2578 AA genotypes also showed lower circulating VEGF than heterozygous individuals, whereas the VEGF AAG haplotype resulted in lower plasma VEGF concentrations.[27] We speculate that the AAG haplotype is associated with a reduced risk for ECD by modifying the spatiotemporal VEGF expression during heart development.

Our findings suggest that the polymorphic promoter region of the *VEGF* gene, in particular the -2578 C and -1154 G alleles, contribute to a genetic predisposition to ECD. The genetic variants might cause a spatiotemporal increase in VEGF expression during endocardial cushion formation, superimposed on the developmental regulated program of expression. Hypoxia, hyperglycaemia and hyperhomocysteinemia are suggested to modify VEGF expression during cardiogenesis.[5,6,8] The interaction between VEGF and

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homocysteine is of particular interest because both epidemiological and experimental studies showed that maternal hyperhomocysteinemia increases the risk of offspring with in particular ECD.[16-18] Folate shortage results in hyperhomocysteinemia and both folic acid and homocysteine affect the behaviour of neural crest cells that are implicated in endocardial cushion formation.[28] Periconception use of folic acid supplements was shown to reduce the risk of in particular neural crest related congenital heart disease.[19] VEGF functions as an endothelial-cell specific growth factor and affects epithelial, mesenchymal and neural cells. It is unknown whether VEGF also influences neural crest cells. However, in future studies with large sample sizes these interactions should be further studied.

Some limitations and strengths of the study have to be addressed. Our dataset consisted of a relatively large set of ECD, however sample sizes of the individual ECD phenotypes were relatively small, especially that of aortic valve stenosis. Therefore, with an a priori hypothesis and to minimize type I errors possibly occurring when testing the individual phenotypes, at first we pooled the five ECD phenotypes as they share similarities in pathogenetic background. However, as there may be differential effects between the phenotypes, we also presented the FBAT and HBAT results per ECD phenotype. We acknowledge the selection of only surviving children of 16 months of age with and without a congenital heart defect which may have resulted in an over- or underestimation of the risk estimates. Only a few data have been published on the mortality rates of congenital heart defects in the first year of life. To provide some estimation of the impact on the risk estimates we calculated that the overall mortality rate of ECD in our hospital over the previous years was on average 2%. The European Association of Cardio-Thoracic Surgeons (EACTS) reported mortality rates varying between 0.76% for aortic valve stenosis to 6.98% for complete atrioventricular septal defects.[29] The overall infant mortality rate in the first year of life in the developed world is 0.8%. Therefore, it is not very likely that the selection of only surviving case and control children had a great impact on the conclusions of this study. Strength of our study is the detailed description of the ECD phenotypes and the ethnic homogeneity of case and control families. Moreover, the VEGF genotypes are reliable as the distributions in the controls are largely comparable with healthy control subjects in other European study populations.[27] Bonferroni correction is rather conservative when considering SNPs in linkage disequilibrium and may increase the risk of false negative results. However, to minimize the risk for type I errors, Bonferroni adjusted probability values were calculated for 5x3 and 5x4 independent tests for alleles and haplotypes, respectively. It is of note that the associations between the VEGF -2578 C (P=0.003) and -1154 G (P=0.002) allele

as well as the AAG haplotype (*P*=0.002) with ECD, as revealed from the FBAT-o and HBAT-o analyses, were of such magnitude that they would remain significant even after Bonferroni correction. The family based association test (FBAT) looks for distortions in the transmission frequencies of a given allele, compared to the assumption of random transmission. A TDT-like design such as FBAT is an attractive method because it is robust against population admixture or stratification.[30] Another advantage of FBAT over standard TDT is the FBAT-o option, which also incorporates information on the control triads.

The observed associations between the VEGF polymorphisms at position -2578 and -1154, the AAG haplotype and ECD phenotypes require replication in an independent study group. The rapid expansion of knowledge regarding genetic profiling will in future lead to a more detailed characterization of congenital heart disease. Large studies addressing interactions between *VEGF* and periconception environmental exposures as well as those investigating the signalling pathway upstream and downstream of VEGF during cardiac cushion formation should be encouraged to modify ECD risk in future.

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Chapter 8

Vascular Endothelial Growth Factor expression in the developing chicken embryonic heart using Optical Projection Tomography

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Chapter 9

General discussion

The aim of this thesis was to investigate periconceptional maternal nutritional and lifestyle factors and genetic determinants, partly related to the homocysteine pathway, in the aetiology of congenital heart disease (CHD). The studies described in this thesis are performed in the HAVEN-study, which is an ongoing case-control family study in the Western part of the Netherlands. We focused on three topics:

1. The maternal lipid status, comprising of lipid concentrations in blood and dietary intake of fatty acids and related vitamins.
2. The maternal use of medicines, particularly antihistamines for nausea and vomiting, in the first 10 weeks of pregnancy and the interaction with a new polymorphism in the NNMT gene.
3. Three functional polymorphisms in the VEGF gene in the family study and the expression of VEGF and its receptor VEGFR2 in the chicken embryonic heart.

A mildly deranged maternal lipid profile comprising total cholesterol, LDL-cholesterol and total cholesterol / HDL-cholesterol ratio, and in particular apolipoprotein B and apolipoprotein B / apolipoprotein A-I ratio, was associated with an almost two-fold higher risk of CHD. Consistently, mothers of a child with CHD had higher intakes of saturated fats and lower intakes of B-vitamins riboflavin and nicotinamide than control mothers. On the other hand, high maternal intake of vitamin E increased CHD risk. CHD risk was almost nine-fold increased for children carrying the *NNMT* AG/AA genotype who were exposed to both periconception medication and low nicotinamide. Early pregnancy use of antihistamines was associated with a more than 3-fold increased CHD risk, independent of its indication nausea and vomiting. Further, our study provides evidence for an association between the VEGF gene, in particular the VEGF 2578 C and 1154 G alleles and AAG haplotype, and endocardial cushion defects. In a whole mount chicken embryo model, we demonstrated expression of VEGF in the ventricular epicardium and myocardium and of its receptor VEGFR2 in the ventricular endocardium. Also, a broad saddle shaped band of VEGFR2 expression was found in the distal outflow tract.

Epidemiological research is an important tool to identify risk factors for CHD with prevention as the ultimate goal. Especially widespread exposure to 'low-level' risk factors, which must have a pathogenetic basis and thus may be considered teratogens, can be expected to have a major impact on the occurrence of congenital malformations. A small increase in risk of affected progeny may lead to a significant increase of cases if the exposure-rate is high. However, studies to identify cardiac teratogens face many challenges. Therefore, before we can make inferences on our findings, we first consider several methodological issues of the studies described in this thesis and place

our findings in the context of the literature. We end this chapter with a discussion on potential implications for future research and public health.

Methodological issues

Study design

A prospective preconception study would be the first choice to investigate associations between risk factors and CHD. The major drawback of this design, however, is the enormous number of participants needed due to the relatively low birth prevalence rate of 6-8/1000 of CHD, and thereby the high financial costs. As second best we therefore conducted a large case-control study with a standardized study moment. This is a relatively inexpensive, rapid and reliable design to investigate associations between exposures and a rare outcome.

The investigation was done around 16 months of age, which is relatively short after pregnancy, to minimize recall bias regarding periconception lifestyle behaviors and to increase compliance of the participants. Furthermore, most CHD are diagnosed during the first year of life and therefore misclassification of cases and controls is avoided. At least one year after delivery, dietary intake has been shown to be largely comparable with the preconception intake when illnesses, dieting, pregnancy and lactation are taken into account.[1,2] Moreover, the standardized investigation at 16 months after the index-pregnancy is 2 years after conception and equals the season of the periconception period. The seasonal influences on food intake are thus comparable between the groups. Also maternal metabolism, such as cholesterol and total homocysteine levels change during pregnancy and lactation and are returned to preconceptional values within 1 year after delivery.[3-5] Thus, we assume that the maternal and metabolic status determined at 16 months after pregnancy is the best estimate for those variables in the periconceptional period. A drawback of this retrospective study design is that investigation of the exact timing and dose of the exposure is very difficult, which increases the chance of type I errors.

Study population

We recruited the case families, consisting of a mother, father and child, in collaboration with the Departments of Paediatric Cardiology of the Erasmus MC in Rotterdam, Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam, The Netherlands. Case children were recruited by two paediatric

cardiologists who were trained in the same hospital. This enhances the uniformity of the CHD phenotypes diagnoses. All diagnoses were confirmed by ultrasound and/or cardiac catheterization and/or surgery. The selection of the CHD phenotypes was based on experimental and epidemiological studies showing that hyperhomocysteinaemia and folic acid use are associated with CHD.[6-8] Included CHD phenotypes comprised of Tetralogy of Fallot, atrioventricular septal defects, perimembranous ventricular septal defect, aortic valve stenosis, pulmonary valve stenosis, coarctation of the aorta, transposition of the great vessels, hypoplastic left heart syndrome and miscellaneous. CHDs can be classified anatomically, clinically, epidemiologically and developmentally, but the knowledge of these mechanisms, while advancing, is far from complete. In some of the studies described in this thesis, we classified CHDs as outflow tract defects or endocardial cushion defects and non-outflow tract defects to increase homogeneity and thereby increase the chance to detect risk factors.

We invited case children at 16 months of age, which minimized misclassification of cases and controls as nearly all CHD are diagnosed during the first postnatal year. We acknowledge that the selection of only surviving children may have resulted in an over- or underestimation of the risk estimates. The European Association of Cardio-Thoracic Surgeons (EACTS) reported mortality rates varying between 0.76% for AoS and 6.98% for complete AVSD[9]. The overall infant mortality rate in the first year of life in the developed world is 0.8%. Therefore, it is not very likely that the selection of only surviving case and control children had a great impact on the conclusions of this study. Compared with controls, the rate of close-related family members with a CHD is higher in the case group (8% and 5%, respectively, P=0.07). These data further substantiate the validity of our case group.

At the start of the HAVEN-study, we aimed to recruit the control families via the Departments of Otorhinolaryngology at the participating hospitals through which cases and controls would be from exact the same domain population. However, the Medical Ethics Committees of these hospitals did not give permission because of the burden for these children. Therefore, control children and their parents were enrolled in collaboration with the child health centres of "Thuiszorg Nieuwe Waterweg Noord" in the surroundings of Rotterdam, at which each child is regularly checked on growth and development. This increased the compliance rates of controls. The Rotterdam area is located in the Western part of the Netherlands from where the cases are derived. Also, the main group of cases is derived from the Rotterdam region. The exposures studied in cases are not expected to be different in controls. Control children without major congenital malformations according to the medical records and from the regular health

checks by the physician at the child health centre were randomly selected. Controls were not eligible to participate if there was a familial relationship with one of the cases.

Comparability of information

Response rates were higher in cases than in controls, 79 versus 53% respectively. The differences in response rated may be explained by different motivations to participate. Parents of a child affected with CHD often seek new information on the aetiology of CHD and the possibilities to prevent CHD in a new pregnancy. On the other hand, parents of control children are willing to participate because they know someone with an affected child, they seek a health check up, or because of altruism. The explanation not to participate was rather similar between case and control families, i.e. practical considerations, such as travel distance or the need for taking a day off from work, and both medical and emotional reasons such as chronic disease of a family member, a new pregnancy or a divorce. Furthermore, differential recall bias of cases and controls can not be excluded. Parents of case children actively seek an explanation for the CHD, and may thereby have a better recall. This might be the case for some exposures, but only when the exposure is well known to be associated with the disease or is socially undesirable.[10] Even for alcohol or cigarette use with a negative image regarding pregnancy, differential misclassification had only a minor effect.[11] In this thesis we studied mainly new associations between diet, lifestyle, biomarkers, single nucleotide polymorphisms (SNPs) and CHD risk and participants were not aware of the specific hypotheses of this study. We used a study moment relatively short after pregnancy compared with other studies. Furthermore, periconceptional exposures, e.g., use of vitamin supplements, alcohol and cigarettes, are equally distributed between cases and control mothers. Thus it is not likely that differential recall bias will have distorted our findings. With regard to our dietary analyses, investigating current dietary intake, reporting bias might exist. Energy adjustment minimized possible underreporting bias. Furthermore, the ratio of energy intake and basal metabolic rate (BMR) was comparable between cases and controls representative of long-term habitual dietary intake.[12]

Selection bias seems not an important issue in our study, since all general characteristics are comparable between cases and controls, except for maternal age and periconceptional use of medication. Mothers of a child with CHD are significantly but only slightly older then control mothers (33.1 and 32.7 years, respectively). Therefore, we adjusted all analyses for maternal age. The distributions of the ethnic groups were comparable between cases and controls. Furthermore, these distributions corresponded to the numbers for the Western part of the Netherlands.[13] All single nucleotide

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polymorphisms (SNP) in the control population 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.

Accuracy of the data

We measured four categories of potential risk factors in relation to CHD, i.e. dietary intake, medication use, biomarkers and genetic polymorphisms. The questionnaires have been filled out at home and were checked for completeness and consistency by the researcher during the hospital visit. We used a semi quantitative food frequency questionnaire (FFQ), validated and adjusted for the estimation of B-vitamin intake[14,15], to measure the current intake reflecting the preconception status. Median nutrient intakes were similar to those of the Dutch National food consumption survey (FCS),[16] indicating that dietary intakes of case and control mothers generally reflect the intakes of Dutch non-pregnant women aged 22-–50 years. Although random measurement errors can not be excluded, the estimated dietary intakes are adjusted for nutrient loss due to processing of the food and also energy adjusted.

Medication use during the first 10 weeks of pregnancy was obtained from the general questionnaire and coded according to the internationally accepted Anatomical Therapeutic Chemical (ATC) classification that was controlled by the World Health Organization Collaborating Centre for Drug Statistics Methodology. Medication was coded based on their pharmacological and chemical properties. The retrieval of prescribed antihistamine medication could not be checked at the pharmacies due to ethical constraints. Another limitation is the lack of detailed information on the timing, duration and dose. A child was considered to be exposed to the medicine during the first 10 weeks of pregnancy if the mother had taken the medicine at least one day during this period.

All blood samples were analysed anonymously in a standardized way in the same clinical chemistry laboratory at the Erasmus MC, University Medical Centre, Rotterdam, The Netherlands. Immediately after blood sampling, an EDTA tube was put on ice and a serum separator tube was kept at room temperature. Both tubes were centrifuged at 4,000 x g for ten minutes at 4°C and separated within one hour. All samples were stored at -80°C. This process is very important for accurate measurement of the biomarkers.[17] Possible measurement errors were random as the samples were analysed in batches. In addition, all concentrations were comparable with reference values established at the clinical chemistry laboratory (chapter 4).

Measurement errors in the genotyping are not likely to play a role in our study. We used TaqMan techniques according to the protocol provided by the manufacturer (TaqMan, Applied Biosystems, Foster City, CA, USA). Each genotype plate contained no DNA template (water) controls. To check consistency, we randomly chose 5% of samples and repeated the measurements. Reproducibility was 100%. The success rate for genotyping was 96% and the sparse inconsistent triads were excluded from analysis.

Power

We have used a population CHD risk of 0.008 and a type I error of 0.05 in the power calculations. Calculations revealed that we have identified low dietary intake of riboflavin and nicotinamide as a risk factor for CHD with a power of 71% (control prevalence of 25%, cases n=276, OR 1.6). We detected an increased CHD risk for increased apolipoprotein B with a power of 90% (control prevalence of 33%, cases n=258, OR 1.8). The sample size of 283 case families in chapter 5 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%). Although we recruited a sufficient number of case children, the power to detect risk alleles and haplotypes of the VEGF gene for the specific CHD phenotypes was still limited, e.g. perimembranous ventricular septal defects n=79, controls n=312, power of 41%, OR 0.7, risk allele frequency 35%.

Inferences on our findings

Maternal lipids and related vitamins

The window and environment in which the embryo develops is crucially influenced by nutrition, maternal lifestyles, metabolism, and physical exposures, all against the genetic background of both mother and embryo. Thus, a particular nutritional deficiency or excessive exposure may occur, even if the dietary intake of a particular nutrient is within the normal range. We demonstrate that a mildly deranged maternal lipid profile comprising total cholesterol, LDL-cholesterol and total cholesterol / HDL-cholesterol ratio, and in particular apolipoprotein B and apolipoprotein B / apolipoprotein A-I ratio, is associated with an almost two-fold higher risk of CHD, independent of the homocysteine level. Apolipoprotein B is the strongest predictor of CHD risk. Data on embryonic exposure to maternal lipids in relation to CHD are not available. It is known however, that fetal aortas contain more and larger fatty streak lesions from mothers with total cholesterol >5.0 mmol/l compared with normocholesterolaemic

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mothers.[18] If the same situation is present during cardiogenesis this may suggest that only a minimal increase in lipoprotein levels might disturb normal heart development. Complex mechanisms including endothelial cell dysfunction, up regulated lipogenesis, inflammatory pathways and oxidative stress are thought to be involved. Hyperlipidemia is a well established risk factor for adult cardiovascular incidents[19] and animal studies have suggested that in utero exposure to a maternal diet rich in fat may lead to an increased risk of adult cardiovascular disease in the offspring.[20] The rising evidence that maternal hyperlipidaemia during pregnancy affects both the fetal and adult cardiovascular system is fascinating.[21] It can not be excluded that the observed deranged lipid profiles in case mothers are a proxy for the maternal risk of developing cardiovascular diseases in later life.

Maternal nutrition plays an important role in the development of congenital malformations including CHD, and the preventive effect of folic acid containing multivitamin use in the periconception period is probably best known.[7] A positive association has been demonstrated between CHD risk and obesity,[22,23] which is a phenotype of unhealthy life style factors such as excessive intake of energy dense food. We demonstrate that mothers of a child with CHD have higher intakes of saturated fats than control mothers, which is in line with our findings on the maternal lipid concentrations in blood. Also, a low dietary intake of cholesterol was associated with a reduced risk of having a child with a non-outflow tract defect. To our knowledge, other research on the association between dietary fats and CHD risk is lacking. CHDs share many similarities in their pathogenesis with orofacial clefts and neural tube defects as these birth defects originate from disturbances in neural crest cell behavior by for example hyperhomocysteinemia and low folate.[24-26] Though, our findings are in line with the two-fold increased risk for orofacial clefts in mothers who used a western dietary pattern, i.e. high intake of saturated fats and insufficient intake of B-vitamins such as folate, riboflavin and nicotinamide.[27] We also demonstrate that a dietary intake of riboflavin and nicotinamide below 1.20 and 13.5 mg/d, respectively, more than 2-fold increased the risk of CHD, especially in mothers who did not use vitamin supplements in the periconceptional period. CHD risk decreased with increasing intakes of nicotinamide, which seemed to be independent of dietary folate and riboflavin intake. Low dietary intake of B-vitamins riboflavin and nicotinamide has been associated with spina bifida[28] and orofacial clefts[29], but other studies on CHD risk are lacking. Riboflavin and nicotinamide are coenzymes in fat metabolism[31,31] and are important in molecular biological processes crucial for normal embryonic heart development, such as reduction of lipid peroxides, cholesterol and steroid synthesis, glycolysis, regulation

of numerous oxidoreductases to reduce oxidative stress and DNA repair mechanisms.[32] Riboflavin is also a co-factor in the folate pathway, and might exert its effect via both folate and fat metabolism.

In our study, oxidative stress may be increased in mothers of a child with CHD as lipids and total homocysteine, sometimes used as biomarker of oxidative stress[33], were higher in this group. Hobbs et al. also found that several biomarkers of oxidative stress were elevated in mothers of a child with CHD.[34] We demonstrate that high maternal intake through diet or supplements of vitamin E, an essential lipid soluble vitamin with strong anti-oxidant but also pro-oxidant capacity was associated with a 1.7 fold increased CHD risk. This effect was independent of folic acid supplement use. Interestingly, periconception users of vitamin E containing supplements demonstrated a five- to nine-fold higher CHD risk together with dietary vitamin E intakes above 14.8 mg/day. We could not examine a dose–response relationship and determine a safe threshold for vitamin E intake as the total vitamin E intake from diet and supplements could not be added up due to the absence of brand names of the supplements used. Evidence on the safety of high intakes of vitamin E during pregnancy is lacking but an adverse effect is biologically plausible. Vitamin E functions as a signaling molecule and regulator of gene expression and may, thereby, modify specific genes involved in embryonic heart development.[35] Vitamin E may also inhibit human cytosolic glutathione S-transferases that contribute to the detoxification of drugs and endogenous toxins.[36] Several studies using a ferrous oxidation xylenol assay suggested a pro-oxidant effect of high concentrations of vitamin E in vivo.[37,38]

Medication use

In this thesis we demonstrate a positive association between CHD risk and nicotinamide, which is a cofactor for the *NNMT*-gene and plays a role in the detoxification of certain medicines and toxins.[39] Several medicines have been shown to be teratogens for heart development.[40] We demonstrate an almost nine fold increased CHD risk for children carrying the *NNMT* AG/AA genotype who were exposed to both periconception medication and low nicotinamide (chapter 5). Thus, children are at increased CHD risk when the detoxification of medicine exposure via the mother is compromised due to polymorphisms in the *NNMT* gene and diminished availability of nicotinamide. Unfortunately, we were not able to make a distinction between the different types of medication due to low numbers. It is suggested that the demonstrated additive risk may affect the control of the expression of specific embryonic cardiac genes. However, this hypothesis needs to be tested in experimental studies.

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During pregnancy, antihistamines for the treatment of nausea and vomiting are more often prescribed than any other medication except for vitamin and iron preparations.[41] We found that the maternal use of antihistamines in early pregnancy was associated with a more than 3-fold increased overall CHD risk and even a 6- to 12-fold increased risk of perimembranous and atrioventricular septal defects. Our results suggest that not pregnancy related nausea and vomiting, but rather early pregnancy exposure to antihistamines seems to increase the risk of CHD. Severe nausea and vomiting is associated with a reduced risk of CHD, which is consistent with a body of literature on pregnancy related nausea and several congenital malformations.[42,43] Our findings are in line with the previous studies reporting that antihistamine use was associated with a 7- to 9- fold increased risk of CHD and musculoskeletal anomalies.[44] Recent data from the large National Birth Defect Prevention Study also revealed positive associations between doxylamine, the major compound of Bendectin®, and spina bifida, cleft lip, left ventricular outflow tract obstruction defects and hypoplastic left heart syndrome.[45] On the other hand, in two prospective cohort studies, meclozine and cetirizine were not identified as risk factor for birth defects.[46-48] However, one study was hampered by a limited number of CHD cases, whereas in the other study all kind of congenital malformation were pooled which may have diluted a potential effect. Our results suggest that antihistamine medication might be a cardiac teratogen. However, an alternative hypothesis is that it is not the antihistamine medication itself, but rather the resulting reduction in nausea and vomiting that increases CHD risk. A reduction in nausea and vomiting during early pregnancy might result in increased fetal exposure to harmful agents and unknown teratogens. Our ability to distinguish between the exposure to antihistamines from nausea and vomiting is as a major strength of the study and significantly minimizes the potential for confounding by indication. When investigating medication use in a case control study, recall bias cannot be excluded. The retrieval of prescribed antihistamine medication could not be checked at the pharmacies due to ethical constraints. Another limitation is the lack of detailed information on the timing, duration and dose of medicine use.

These studies show that early pregnancy use of medication or combined with other unfavorable genetic or lifestyle factors, play a role in the aetiology of CHD. Further studies should be stimulated to confirm our original data and to shed light on underlying mechanisms.

Vascular endothelial growth factor

Previous studies have implicated a strict spatiotemporal expression of the *VEGF* gene in the control of endocardial cushion development[49] and derangements in VEGF expression can result in CHD.[50,51] *VEGF* gene expression shows a marked individual variability, which is partly determined by single nucleotide polymorphisms (SNPs).[52,53] Our study provides evidence for an association between the *VEGF* gene, in particular the *VEGF* 2578 C and 1154 G alleles and AAG haplotype, and endocardial cushion defects. For this analysis, we selected from the total case group of the Haven study, only the endocardial cushion related defects, *i.e.* perimembranous ventricular septal defect, pulmonary valve stenosis, Tetralogy of Fallot, aortic valve stenosis and atrioventricular septal defect. To date, there are four other studies on the association between VEGF SNPs and CHD risk, with conflicting results.[54-57] Griffin et al. recently published new data on the association of VEGF alleles with all types of CHD and specifically Tetralogy of Fallot (including the related conditions pulmonary stenosis/VSD and "Fallot-type" double outlet right ventricle).[57] Their study did not support the hypothesis that either common or rare genetic variation in VEGF alleles significantly predisposes to the risk of CHD. However, they do not describe which CHD phenotypes were included and both CHD and Tetralogy of Fallot group were heterogeneous, thereby significantly reducing the chance to detect an association. The genetic variants might cause a spatiotemporal increase in VEGF expression during endocardial cushion formation, superimposed by environmental exposures, such as hypoxia, hyperglycaemia, and hyperhomocysteinemia.[50,58,59] These interactions are of particular interest because of their independent associations with CHD risk.[60-63] Large human studies addressing interactions between *VEGF* and periconception environmental exposures as well as animal studies investigating the signaling pathway upstream and downstream of *VEGF* during cardiogenesis may contribute to modify CHD disease risk in future.

We have investigated the expression patterns of VEGF-A and VEGFR2 by means of mRNA in situ hybridization in whole mount chicken embryonic hearts at Hamburger and Hamilton (HH) stages 26-28.[64] These stages are crucial for endocardial cushion development and outflow tract septation. VEGF expression was detected abundantly in the ventricular walls, including epicardium and myocardium. Its receptor, VEGFR2, was also expressed in the ventricular myocardium as well as endocardium. A remarkable broad band of intense VEGFR2 signal was shown stage 26 in both the endothelium and mesenchymal compartment of the distal outflow tract and the signal gradually decreased in the ventricles and outflow tract but was still present in HH stage 28. One of the major characteristics of this study is the use of the sophisticated high resolution

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OPT microscopy[65] which enables the possibility of a new phase of 3D descriptive embryology. From the OPT-scanned images holograms were made with V-scope software on the floor, left, right and front wall of our virtual reality system (Barco I-space, Barco N.V., Kortrijk, Belgium), which allowed us to perceive depth and to interact with the whole mount labeled hearts. With a joystick, we could visualize all possible cross-cuts in every direction resulting in detailed expression data of all segments of the heart.

Future perspectives

Our data are original but as in other research it also raises new questions. We are curious about the mechanisms by which imbalances in maternal lipid and nutritional status affect fetal cardiac development. What is the extent of the differences between CHD phenotypes in response to maternal dietary changes? Both human and animal studies are needed to solve these issues. The chicken embryo is a good model to study cardiogenesis and is particularly suitable to study the impact of differences in timing and character of insults on fetal cardiac development. It also poses a useful model to broaden the horizon in investigating etiological pathways and new gene-environment interactions. For example, it would be interesting to study the occurrence of specific CHD in chicken embryos exposed to high concentrations of homocysteine, glucose or apolipoprotein B. Further investigations have to be performed on the critical balance between antioxidant activity and pro-oxidant exposures that are modifiable by genetic and/or environmental factors. Also studies focusing on epigenetic control of gene expression, particularly regarding DNA methylation, should be encouraged. For example, the link between CHD and epigenetic modification of hyperhomocysteinemia and low folate, but conceivable also low riboflavin and nicotinamide concentrations as co-factors in the methylation pathway, is an interesting field to study. As it is clear that the effect of an unhealthy diet is not just through a single nutrient, further studies should not only unravel the specific nutrients involved, but also determine dietary patterns associated with an increased CHD risk. Interesting examples are the adherence to the Mediterranean diet in association with a reduced risk of spina bifida in the offspring, the adherence to the Western diet in association with an increased risk of cleft lip and/or palate and the use of a diet rich in fish and seafood that reduced CHD risk.[23,66,69] The prospective cohort study built-in in a clinical setting, e.g., Predict Study of the department of Obstetrics and Gynaecology of Erasmus MC Rotterdam, is the best design to investigate the effects of the periconception nutritional and biomarker status on reproductive outcome. In this

design the actual periconceptional dietary intake and levels of e.g. vitamins, lipids and markers of oxidative stress can be measured. Furthermore, recall bias does not occur and multiple neonatal as well as maternal outcomes can be studied. This requires a very large cohort study over many years due to the low prevalence of CHD and it is highly time-consuming and expensive. Genome wide association studies may reveal new genetic pathways involved in the pathogenesis of CHD.

Epidemiologic studies with complete data on large series of early abortions, including karyotyping and / or genotyping, extensive post-mortem examination, clinical data on periconception exposures, i.e. nutrition, lifestyle, biomarkers, and clinical data on relatives are another potential source of information currently neglected but with great potential. A concerted action of epidemiologists, obstetricians, pediatric cardiologists, embryologists, geneticists and pharmacologists should be encouraged and may also add to the current 15% of CHD explained by a specific factor rather than by the repository of multifactorial causes. One of the most important but difficult questions to answer is how to spread the knowledge effectively to the target population, i.e. couples who are planning to become pregnant, and how to actually change their unfavorable lifestyle habits? In 2007, the "Gezond Zwanger Spreekuur", a separate consulting-hour for preconception advice on dietary habits and lifestyle, started at the outpatient clinic of the department of Obstetrics and Gynaecology of the Erasmus MC in Rotterdam.[67] The results from this outpatient clinic will provide more knowledge about targeting preconception harmful lifestyles.

Implications for public health practice

We should not only study the relationship between intrauterine exposures and the risk of CHD and publish the results in international scientific journals. The scientific community should also take responsibility in informing both health care providers, such as general practitioners and midwives, and especially the general population about new findings. Last but not least, the target population, i.e. couples planning to become pregnant, should be educated about teratogenic risks. Ideally, couples contact their general practitioner or midwife when they want to finish contraception to become pregnant. At that time, the couple can be informed about teratogenic risks and benefits. Although recent years have seen increasing attention focused on preconception care, no widespread and consequent preconception care is available yet. Since August 2006, Rotterdam was the first city in the Netherlands providing preconception care

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to future parents. Programs developed for those at highest risk are implemented and will be gradually expanded to all future parents. In 2007 The Health Council of the Netherlands advised to implement preconception care.[68] Recently, the Dutch minister of Health recognized the importance of implementing preconception care. He called up the professional groups to define preconceptional guidelines and agreed to include preconception care in the health insurance packages starting from January 1st 2011 onwards.

We were the first to demonstrate an association between mildly elevated maternal concentrations of total cholesterol, LDL-cholesterol and Apolipoprotein B and the risk of CHD in the offspring. If these findings are confirmed, standardized screening for hypercholesterolemia in preconception care programs should be considered. A cholesterol lowering diet or in some instances medication tested for safety during pregnancy might be advised to optimize the maternal periconception lipid status, thereby potentially reducing CHD risk. A maternal diet high in saturated fats, but low in riboflavin and nicotinamide seems to contribute to CHD risk. Increasing dietary nicotinamide intake was associated with a reduced CHD risk, independent of dietary folate. It may be argued whether a folic acid containing multivitamin supplement rather than a supplement containing folic acid only should be advised. A randomized controlled trial would be the exquisite method to give the answer, but is unrealistic. However, it should be stressed that multivitamin supplements cannot replace a healthy diet and also require a high compliance. Importantly, as was demonstrated by our results on vitamin E intake, vitamin supplements may also induce excessive intakes thereby posing potential risks to the developing fetus. The number of women using multivitamin supplements specifically adjusted for pregnancy requirements in our studies was too low to recommend their use. Furthermore, vitamin supplements are widely available in all different types and doses and evidence on the safety and benefit threshold for most micronutrients is lacking. Therefore, we consider it too early to advice all women who want to become pregnant to use specific nutritional supplements other than folic acid. On the contrary, these women should be educated on the importance of a healthy dietary pattern low in saturated fat and vitamin E but high in B-vitamins.

CHD risk was increased by maternal periconception medication use, particularly in combination with low nicotinamide intake and the child having the A-allele of the NNMT polymorphism. Furthermore, maternal use of antihistamines was suggested to increase CHD risk, independent of the main indication during pregnancy, which is nausea and vomiting. On the basis of our results, also considering the previous literature, it is too early to advice against use of any antihistamines in the periconceptional period,

since detailed information on the teratogenic mechanisms, dose and timing of exposure is not available. However, a more cautious attitude should be taken by health care professionals and mothers-to-be towards the prescription and intake of medicines, particularly antihistamines, during the critical period of organogenesis.

The results on the role of VEGF alleles 2578-C and 1154 G in the pathogenesis of CHD are mainly of scientific interest and my give more insight into the genetic basis of CHDs. Once the genetic basis modifying CHD risk has been elucidated, e.g., the genes involved have been recognized, it may become possible to develop diagnostic tests to identify individuals at risk. A next step may be to offer treatment to those individuals by means of gene-therapy or advice changes in lifestyle factors that interact with the genes involved.

In conclusion, studies to identify new cardiac teratogens are challenging and a fascinating research topic. Derangements in the maternal lipid status, unfavorable dietary intake, use of medication and single nucleotide polymorphisms in the VEGF gene appear as new risk factors for the development of CHD. The obtained knowledge adds to a better understanding of the aetiology of CHD and will contribute to develop strategies for primary prevention.

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Summary

Congenital heart disease (CHD) is among the most common congenital abnormalities, arises in the first trimester of pregnancy and occurs in many forms. The reported CHD birth prevalence rate ranges from 6 per 1,000 newborns in the Netherlands to 9 per 1,000 newborns in the United States of America. CHD is the leading cause of infant mortality and contributes to 30-40% of all deaths during infancy and early childhood. Quality of life of these patients and their families is often compromised due to severe physical and psychological problems. Only approximately 15% of CHD can be attributed to a known cause. For the majority, a general interaction model of genetic susceptibility and intra-uterine exposures is proposed. Therefore, when we gain more insight into the embryogenesis of the heart and the role of genes and environmental factors in the aetiology, primary prevention of CHD comes within reach. The aim of this thesis was to investigate maternal nutritional and lifestyle factors and genetic determinants, partly related to the homocysteine pathway, in the aetiology of CHD. This thesis displays the results from the HAVEN study, a case-control family study coordinated from June 2003 onwards at the Department of Obstetrics and Gynaecology, Erasmus MC, Rotterdam, The Netherlands. In the last chapter we present a chicken embryo model to assess the expression pattern of the vascular endothelial growth factor (VEGF) gene and its receptor during heart development.

A general introduction is presented in **Chapter 1**.

Part I

The first part of this thesis focuses on associations between the maternal lipid status, comprising of the dietary intake of fatty acids, related vitamins, and concentrations in blood, and the risk of CHD in the offspring. In **Chapter 2**, we examined whether maternal lipid levels are associated with CHD risk. Mildly deranged maternal lipid levels were associated with an increased CHD risk. Case mothers showed higher cholesterol, LDL-cholesterol, apolipoprotein B and homocysteine concentrations than controls. LDL-cholesterol above 3.3 mmol/L and especially apolipoprotein B above 85.0 mg/dL were associated with an almost two-fold increased CHD risk. In **Chapter 3,** we assessed whether maternal dietary intake of fats, riboflavin and nicotinamide are

associated with CHD risk. Case mothers had higher mean dietary intakes of saturated fat. We demonstrated a dose response relationship towards a reduced risk of outflow tract defects with increasing intakes of riboflavin. Low dietary intakes of both riboflavin (<1.20 mg/d) and nicotinamide (<13.5 mg/d) more than two-fold increased the risk of a child with an outflow tract defect, especially in mothers who did not use periconception vitamin supplements. In **Chapter 4**, dietary vitamin E intake was higher in case mothers than in controls and periconception use of vitamin E supplements in addition to a high dietary vitamin E intake above 14.9 mg/d up to nine-fold increased CHD risk. Our findings raise questions on the safety of vitamin E during pregnancy.

Part II

Part II of this thesis addresses the maternal use of medication in the periconception period as a risk factor for CHD. We hypothesized whether derangements in the homocysteine and detoxification pathways, due to a polymorphism in the nicotinamide N-methyltransferase (NNMT) gene, low maternal dietary nicotinamide intake, and periconception use of medicines, affect CHD risk (**Chapter 5**). Distribution of NNMT alleles was not different between case and control families. A 1.5 fold higher CHD risk was shown for both periconceptional use of medicines and low intake of nicotinamide (<13.8 mg/day). Interestingly, an almost three-fold increased risk was found if the mother was carrying the NNMT AG/AA genotype and even up to a nine-fold increased risk was found if the child was carrying the NNMT AG/AA genotype. In **Chapter 6**, we observed a 3.7-fold increased risk of CHD for early pregnancy use of antihistamines. Mothers with severe nausea and vomiting, who did not report antihistamine use, tended to have a reduced risk for CHD offspring. In contrast, in mothers who used antihistamines and reported severe nausea and vomiting CHD risk changed into a more than six-fold increased risk for particularly atrioventricular septal defects and perimembranous ventricular septal defects. However, inherent to a case-control design, the presence of selective recall bias can not completely be excluded.

Part III

The final part of this thesis concerns the VEGF gene, which is a central molecule in embryonic cardiovascular development. In **Chapter 7**, we investigated linkage and

association between three functional single nucleotide polymorphisms (SNPs) in the VEGF gene and endocardial cushion defects. VEGF alleles -2578 C and -1154 G were transmitted more frequently to case children, in particular perimembranous ventricular septal defects. The -2578A/-1154A/-634G haplotype was associated with a reduced risk of endocardial cushion defects and was significantly less transmitted to case children. In **Chapter 8**, we present 3-D whole organ imaging of VEGF-A and VEGFR2 mRNA expression during chicken embryonic heart development at Hamburger and Hamilton stages 26-28 by using the new OPT-technique and the Barco I-space virtual reality system. VEGF-A expression was detected abundantly in the ventricular epicardium and in the myocardium of the ventricles and the sites underlying the forming endocardial cushions. VEGFR2 was expressed mainly in the ventricular endocardium. Furthermore, a remarkable broad saddle shaped band of intense VEGFR2 signal was shown at the distal outflow tract, reminiscent of the location of the secondary heart field.

Chapter 9 provides a general discussion on the combined results of the studies in a broader perspective. We recommend future research and elaborate on the implications regarding preconception care.

Samenvatting

Aangeboren hartafwijkingen (CHD) behoren tot de meest voorkomende aangeboren afwijkingen. Ze ontstaan in het eerste trimester van de zwangerschap en komen in vele vormen voor. De geboorte prevalentie cijfers van CHD variëren van 6 per 1000 levendgeborenen in Nederland tot 9 per 1000 levendgeborenen in de Verenigde Staten. Aangeboren afwijkingen zijn een belangrijke oorzaak van sterfte onder pasgeborenen en 30-40% van deze sterfte wordt veroorzaakt door CHD. Door ernstige fysieke en psychologische problemen die samenhangen met CHD wordt de kwaliteit van leven van patiënten en hun families beïnvloed.

Slechts bij 15% van de CHD kan een oorzaak worden aangetoond. Op dit moment wordt verondersteld dat de meeste CHD veroorzaakt worden door complexe interacties tussen genetische aanleg en intra-uteriene blootstellingen. Primaire preventie van CHD wordt dan ook pas mogelijk als er meer inzicht verkregen in de embryogenese van het hart en de rol die genen en omgevingsfactoren hierbij spelen. Het doel van dit proefschrift was het onderzoeken van maternale voedings- en leefstijlfactoren en genetische determinanten, gedeeltelijk gerelateerd aan de homocysteine stofwisseling, in relatie tot het ontstaan van CHD. Dit proefschrift toont de resultaten van de HAVEN studie, een case controle familie studie waarvan de rekrutering heeft plaastgevonden van 2003-2010 vanuit de Afdeling Verloskunde en Vrouwenziekten van het Erasmus MC in Rotterdam in Nederland. In het laatste hoofdstuk presenteren we een kippenembryo model waarin de expressie van vascular endothelial growth factor (VEGF) en VEGF receptor 2 wordt bestudeerd tijdens de hartontwikkeling.

In Hoofdstuk 1 is de algemene introductie van het proefschrift beschreven.

Deel I

Het eerste deel van dit proefschrift beschrijft de associatie tussen de maternale lipiden status, bepaald door de inname van vetzuren via de voeding en gemeten door gerelateerde vitaminen en lipiden concentraties in bloed, en het risico op een kind met CHD.

In **Hoofdstuk 2** hebben we onderzocht of lipiden concentraties in het maternale bloed geassocieerd zijn met het risico op CHD. Licht verhoogde maternale lipiden concentraties waren geassocieerd met een toegenomen CHD risico. Moeders van

een kind met CHD hadden hogere concentraties cholesterol, LDL-cholesterol, apolipoproteine B en homocysteine dan controle moeders. LDL-cholesterol boven 3.3 mmol/L en met name ook apolipoproteine B boven 85.0mg/dL waren geassocieerd met een 2 maal verhoogd risico op CHD. In **Hoofdstuk 3** onderzochten we associaties tussen de maternale inname van vetzuren, riboflavine en nicotinamide via de voeding en het risico op CHD. Moeders van een kind met CHD hadden gemiddeld een hogere inname van verzadigde vetzuren. Hiernaast hebben we een dosis – respons relatie aangetoond waarbij de kans afneemt op uitstroomdefecten van het hart naarmate de inname van riboflavine toeneemt. Bij een lage inname van zowel riboflavine (<1.20 mg/d) en nicotinamide (<13.5 mg/d) nam het risico twee maal toe op uitstroomdefecten van het hart, met name bij moeders die geen foliumzuur bevattend vitaminesupplement gebruikten tijdens de periconceptionele periode.

De inname van vitamine E via de voeding was hoger bij case moeders dan bij controle moeders (**Chapter 4**). Bij moeders met een inname van vitamine E via de voeding boven 14.9mg/d, die daarnaast tevens een vitamine E bevattend supplement hadden gebruikt in de periconceptionele periode, was het risico op het krijgen van een kind met CHD negen maal hoger. Deze bevindingen werpen vragen op over de veiligheid van de vitamine E inname, in het bijzonder in de vorm van een tablet, tijdens de zwangerschap.

Deel II

Deel II van dit proefschrift gaat over het gebruik van medicatie in de periconceptionele periode als een mogelijke risico factor voor CHD. In **Hoofdstuk 5** hebben we de hypothese geformuleerd dat verstoringen in de homocysteine stofwisseling en detoxificatie mechanismen, door een polymorfisme in het nicotinamide N-methyltransferase (NNMT) gen, een lage maternale inname van nicotinamide, en periconceptioneel gebruik van medicijnen, het risico op CHD beïnvloeden.

De verdeling van NNMT allelen was niet verschillend tussen case en controle families. We vonden een 1.5 maal verhoogd risico op CHD bij zowel periconceptioneel medicijngebruik als een lage inname van nicotinamide (<13.8mg/d). Opvallend is dat de combinatie van deze drie factoren een bijna drie maal zo hoog risico geeft als de moeder het NNMT AG/AA genotype heeft en zelfs een bijna negen maal zo hoog risico op CHD als het kind zelf het NNMT AG/AG genotype heeft. In **Hoofdstuk 6** vonden we een 3.7 maal verhoogd risico op CHD bij het gebruik van antihistaminica in het

eerste trimester van de zwangerschap. Moeders die ernstige klachten van misselijkheid en braken in het eerste trimester rapporteerden, maar daarvoor geen antihistaminica gebruikten, leken een lager risico te lopen op het krijgen van een kind met CHD. Dit was in tegenstelling tot de moeders met ernstige klachten van misselijkheid en braken die juist wel antihistaminica hadden gebruikt, waarbij er plotseling een meer dan 6 maal verhoogd risico op CHD werd vastgesteld, in het bijzonder atrioventriculaire septum defecten en perimembraneuze ventrikel septum defecten. Hierbij moet wel in ogenschouw worden genomen dat het bestaan van selectieve recall bias ook in deze case controle studie niet geheel uitgesloten kan worden.

Deel III

Het laatste deel van dit proefschrift concentreert zich op het VEGF gen, dat een centrale rol speelt in de embryonale cardiovasculaire ontwikkeling. In **Hoofdstuk 7** hebben we de aanwezigheid van linkage en associaties bestudeerd tussen drie functionele polymorfismen (SNPs) in het VEGF gen en endocardiale kussen afwijkingen. VEGF allelen -2578 C en -1154 G bleken vaker te worden doorgegeven aan kinderen met CHD, in het bijzonder perimembraneuze ventrikel septum defecten. Het -2578A/-1154A/-634G haplotype was geassocieerd met een verlaagd risico op endocardiale kussen afwijkingen en werd significant minder vaak doorgegeven aan case kinderen. In **Hoofdstuk 8** beschrijven en presenteren we een 3-D weergave van VEGF-A en VEGFR2 mRNA expressies in complete embryonale kippenharten tijdens Hamburger en Hamilton stadia 26-28 door middel van de nieuwe OPT-techniek en het Barco I-space virtual reality systeem. VEGF-A kwam uitbundig tot expressie in zowel het epicardium en myocardium van de ventrikels als onder de zich vormende endocardiale kussens. VEGFR2 kwam met name tot expressie in het ventriculaire endocard. Opvallend was verder een zadelvormige band van een intens VEGFR2 signaal in het distale uitstroom traject, dat mogelijk een overblijfsel is van de aangroei van het tweede hart veld.

In **Hoofdstuk 9** worden de resultaten van dit proefschrift in een breder perspectief bediscussieerd. We doen aanbevelingen voor toekomstig onderzoek en bespreken de implicaties van dit onderzoek met betrekking tot preconceptie zorg.

List of abbreviations

ALA	α-linolenic acid
ATC	anatomical therapeutic chemical classification
BMI	body mass index
BMR	basal metabolic rate
CHD	congenital heart disease / congenital heart defect
CI	confidence interval
DHA	docosahexanoic acid
ECD	endocardial cushion defects
EI	energy intake
EMT	epithelial-mesenchymal transformation
EPA	eicosapentanoic acid
FBAT	family based association test
FCS	food consumption survey
FFQ	food frequency questionnaire
HBAT	haplotype based association test
HDL	high density lipoprotein
HH	Hamburger and Hamilton stage
HWE	Hardy Weinberg disequilibrium
ISH	In Situ Hybridization
LD	linkage disequilibrium
LDL	low density lipoprotein
MTHFR	methylene tetrahydrofolate reductase
MTRR	methionine synthase reductase
NNMT	nicotinamide N-methyltransferase
OPT	optical projection tomography
OR	odds ratio
OTD	outflow tract defects
pVSD	perimembranous ventricular septal defect
RDA	recommended daily allowances
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	standard deviation
SNP	single nucleotide polymorphism
TDT	transmission disequilibrium test
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2

About the author

Dineke Smedts was born on February 15th 1979 in Venray. She grew up in Nieuwegein, Hedel and Zaltbommel, together with her two sisters. After secondary school at the Stedelijk Gymnasium 's-Hertogenbosch (1991-1997), she moved to Leiden to start medical school at Leiden University. During her studies, she became interested in the field of Obstetrics and Gynaecology and did a one month internship at the Obstetrics department of the Academic Hospital Ljubljana, Slovenia. As a graduation project, from August 2002 to February 2003, she worked at the Department of Biochemistry, Harvard University, Cambridge, Massachusetts, USA, under the direct supervision of Dr. L.A. Koopman and Prof.dr. J.L. Strominger. Here she investigated chemokines in the pregnant decidua and their receptors on Natural Killer cells. After obtaining her medical degree in January 2005, she worked as a resident at the Department of Obstetrics and Gynaecology of the Bronovo Hospital in The Hague (Dr. R.A. Verweij). In October 2005, she was appointed as PhD student on the ongoing HAVEN project (>2003; project leader Prof.dr. R.P.M. Steegers-Theunissen) at the Department of Obstetrics and Gynaecology of the Erasmus MC Rotterdam (Prof.dr. R.P.M. Steegers-Theunissen and Prof.dr. E.A.P. Steegers) resulting in this thesis. During her PhD project, in the autumn of 2007, she contributed to "The Nationale DenkTank" and report "Succes op School", presenting 12 new ideas to solve problems in education as an intermediary between business and society versus student's interests. Her research activities have been rewarded by the "Society for Gynecologic Investigation" in 2008 (President's Presenter Award) and by the "Rotterdamse Gynaecologen Opleidings Cluster" in 2009 (J.W. Wladimiroff Research Award). In November 2008, she started her training in Obstetrics and Gynaecology at the Maasstad Ziekenhuis in Rotterdam (Dr. A.M. van Heusden / Dr. A. Verhoeff), and in August 2010 she continued her training at the Erasmus MC in Rotterdam (Prof.dr. C.W. Burger). Dineke is married to Christian Meerstadt.

List of publications and related awards

Articles

O. Valkenburg, R.P.M. Steegers-Theunissen, **H.P.M. Smedts**, G.M. Dallinga-Thie, B.C.J.M. Fauser, H.E. Westerveld, J.S.E. Laven. A more atherogenic serum lipoprotein profile is present in women with polycystic ovary syndrome: a case-control study. *J Clin Endocrinol Metab* 2008;93:470-6.

L.M.J.W. van Driel, **H.P.M. Smedts**, W.A. Helbing, A. Isaacs, J. Lindemans, A.G. Uitterlinden, C.M. van Duijn, J.H. de Vries, E.A.P. Steegers, R.P.M. Steegers-Theunissen. Eight-fold increased risk for congenital heart defects in children carrying the nicotinamide N-methyltransferase polymorphism and exposed to medicines and low nicotinamide. *Eur Heart J* 2008;29:1424-31.

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H.P.M. Smedts, J.H. de Vries, M. Rakshandehroo, M.F. Wildhagen, A.C. Verkleij-Hagoort, E.A.P. Steegers, R.P.M. Steegers-Theunissen. High maternal dietary intake of vitamin E is associated with congenital heart defects in the offspring. *BJOG* 2009;116:416-23.

H.P.M. Smedts, A. Isaacs , A.G. Uitterlinden, C.M. van Duijn, D. de Costa, W.A. Helbing, E.A.P. Steegers, R.P.M. Steegers-Theunissen. VEGF polymorphisms are associated with endocardial cushion defects: a family based case control study. *Ped Res* 2010;67:23-8.

H.P.M. Smedts, E.M. van Uitert, O.Valkenburg, J.S.E. Laven, M.J.C. Eijkemans, J. Lindemans, E.A.P. Steegers, R.P.M. Steegers-Theunissen. A derangement of the maternal lipid profile is associated with an elevated risk of congenital heart defects in the offspring. *Nutr Metab Cardiovasc Dis; in press.*

H.P.M. Smedts, S. Bandola, B. Stricker, E.A.P. Steegers, R.P.M. Steegers-Theunissen. Early pregnancy exposure to antihistamines, pregnancy related nausea and vomiting and the risk of congenital heart disease. *Submitted for publication.*

H.P.M. Smedts, R.P.M. Steegers-Theunissen, B. Dumee, E. de Graaff, A.H.J. Koning, L. van Iperen, A.C. Gittenberger-de Groot, P.J. van der Spek, A.J.M.H. Verkerk. Vascular Endothelial Growth Factor expression in the developing chicken embryonic heart using Optical Projection Tomography. *Submitted for publication*.

Abstracts

H.P.M. Smedts, J.H. de Vries, M. Rakshandehroo, M.F. Wildhagen, A.C. Verkleij-Hagoort, E.A.P. Steegers, R.P.M. Steegers-Theunissen. Congenital heart defects and maternal dietary vitamin A and E intake. Suppl. Reprod Sci 2007;14:216. *Oral presentation*.

H.P.M. Smedts, M. Rakhshandehroo, A. C. Verkleij-Hagoort, J.H. de Vries, E.A.P. Steegers R.P.M. Steegers-Theunissen. Low maternal dietary intake of docosahexanoic acid, riboflavin and niacin leads to congenital heart defects in human. Suppl. Reprod Sci 2007;14(1):234. *Poster presentation.*

H.P.M. Smedts, A. Isaacs , A.G. Uitterlinden, C.M. van Duijn, D. de Costa, W.A. Helbing, E.A.P. Steegers, R.P.M. Steegers-Theunissen. Associations between single nucleotide polymorphisms in the VEGF gene and congenital heart defects in human. *Suppl. Reprod Sci* 2008;15:62. *Oral presentation.*
Society for Gynecologic Investigation (SGI): President's Presenter Award 2008.

H.P.M. Smedts, E.M. van Uitert, O. Valkenburg, J.S.E. Laven, M.J.C. Eijkemans, J. Lindemans, E.A.P. Steegers, R.P.M. Steegers-Theunissen. A maternal atherogenic lipid profile increases the risk of congenital heart defects in the offspring. *Suppl. Reprod Sci* 2008;15:249. *Poster presentation.*

H.P.M. Smedts, A. Isaacs , A.G. Uitterlinden, C.M. van Duijn, D. de Costa, W.A. Helbing, E.A.P. Steegers, R.P.M. Steegers-Theunissen. Vascular endothelial growth factor polymorphisms are associated with congenital heart disease: a family based study. Gynaecongres 2008, Arnhem. *Oral presentation.*

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H.P.M. Smedts, E.M. van Uitert, O. Valkenburg, J.S.E. Laven, M.J.C. Eijkemans, J. Lindemans, E.A.P. Steegers, R.P.M. Steegers-Theunissen. An atherogenic maternal lipid profile is a risk factor for congenital heart defect offspring. *Suppl. Cardiology in the Young* 2008;18:50. *Poster presentation.*

H.P.M. Smedts, S. Bandola, B. Stricker, E.A.P. Steegers, R.P.M. Steegers-Theunissen. Periconception use of systemic antihistamines is association with a four-fold increased risk of congenital heart defects. Researchmeeting Rotterdamse Gynaecologen Opleidings Cluster, 2009, Erasmus MC, Rotterdam. *Oral presentation.*
J.W. Wladimiroff Research Award.

Non-scientific publications

Jan van Klinken. Kleine tekorten met grote gevolgen. Reformatorisch Dagblad, 15 dec 2009, nr 219, pagina 17.

Eindrapport De Nationale DenkTank 2007. Succes op school. 12 frisse ideeën voor onderwijs als intermediair tussen leerlingen, arbeidsmarkt en maatschappij.

PhD Portfolio

Name PhD candidate: Dineke H.P.M. Smedts

Erasmus MC Department: Obstetrics and Gynaecology

Promotors: Prof.dr. R.P.M. Steegers-Theunissen, Prof.dr. E.A.P. Steegers

Courses	Year	ECTS
– NIHES. Classical Methods for Data-analysis	2006	5.7
– NIHES. Principles of Research in Medicine and Epidemiology	2006	0.7
– NIHES. Genetic Analysis in Clinical Research.	2006	1.0
– Molmed. Klinische genetica: 'Hoe analyseer ik mijn gen?'	2007	0.3
– De Nationale DenkTank, summerschool Driebergen	2007	14.0
– Problem Solving		
– Structuring the problem		
– Prioritization and Work Planning		
– Creative thinking		
– Interview Skills		
– Exhibit Design and Storyline Skills		
– Presenting with impact		
– Effective top-down communication		
– Feedback model		
– Individual coaching track for team leaders		

Conferences		
– RGOC onderzoeksdag / Wladimiroff symposium. Erasmus MC, Rotterdam	2006	0.3
– Nederlands Verening voor Toxicology, Oss	2006	0.3
– RGOC onderzoeksdag / Wladimiroff symposium. Erasmus MC, Rotterdam	2007	0.3
– Society for Gynecologic Investigation (SGI), Reno, USA	2007	1.0
– Symposium Jonge Zwangerschap	2008	0.3
– Society for Gynecologic Investigation (SGI), San Diego, USA	2008	1.0
– Association for European Paediatric Cardiology (AEPC), Venice, Italy	2008	1.0

Conferences	Year	ECTS
– RGOC onderzoeksdag / Wladimiroff symposium. Erasmus MC, Rotterdam	2008	0.3
– Nederlands Vereniging vooor Obstetrie en Gynaecology, Gynaecongres Arnhem	2008	0.3
– Wetenschapsdag Arts Assistenten Vereniging Erasmus MC, Rotterdam	2008	0.3
– Nederlands Verening voor Toxicology, Rotterdam	2008	0.3
– RGOC onderzoeksdag / Wladimiroff symposium. Erasmus MC, Rotterdam	2009	0.3
– Nederlands Vereniging voor Obstetrie en Gynaecology, Gynaecongres Arnhem	2009	0.3
– Nederlands Vereniging vooor Obstetrie en Gynaecology, Gynaecongres Breda	2010	0.3

Presentations		
– Nederlands Verening voor Toxicology, Oss (oral)	2006	0.7
– Society for Gynecological Investigation (SGI), Reno, USA (2 posters)	2007	0.6
– Research meeting Rotterdamse Gynaecologen Opleidings Cluster (oral)	2007	0.7
– Society for Gynecological Investigation (SGI), San Diego, USA (1 oral, 1 poster)	2008	1.0
– Association for European Paediatric Cardiology, Venice, Italy (2 posters)	2008	0.6
– Gynaecongres, Arhem (oral)	2008	0.7
– Research meeting Rotterdamse Gynaecologen Opleidings Cluster (oral)	2008	0.7
– Wetenschapsdag Arts Assistenten Vereniging Erasmus MC, Rotterdam (poster)	2008	0.7
– Research meeting Rotterdamse Gynaecologen Opleidings Cluster (oral)	2009	0.7

Teaching activities		
– Supervising Master's thesis, Maryam Rakhshandehroo, Erasmus MC, Rotterdam	2006	2.0
– Supervising Master's thesis, Dominique de Costa, Erasmus MC, Rotterdam	2006	2.0
– Supervising Master's thesis, Evelyne van Uitert, Erasmus MC, Rotterdam	2007	2.0
– Supervising Master's thesis, Sarah Bandola, Erasmus MC, Rotterdam	2008	2.0
	42.4	

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