DEVELOPMENTAL PROGRAMMING OF CONGENITAL HEART DISEASES AND CARDIOVASCULAR RISK FACTORS

PERICONCEPTION EPIDEMIOLOGIC AND EPIGENETIC STUDIES

Kim P.J. Wijnands

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Developmental programming of congenital heart diseases and cardiovascular risk factors
Periconception epidemiologic and epigenetic studies
Thesis, Erasmus MC, University Medical Centre, Rotterdam, The Netherlands

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DEVELOPMENTAL PROGRAMMING OF CONGENITAL HEART DISEASES AND CARDIOVASCULAR RISK FACTORS

Periconception epidemiologic and epigenetic studies

Vroege programmering van congenitale hartafwijkingen en cardiovasculaire risicofactoren

Periconceptionele epidemiologische en epigenetische studies

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Chapter 1

Introduction

Rationale

Congenital heart diseases

Worldwide, birth defects are common and affect approximately 6% of total births (1). Congenital heart diseases (CHDs) are the most common birth defects with approximately 1.35 million children born each year corresponding to a birth prevalence rate around 9 per 1,000 live births (1,2). They are the leading cause of infant mortality in the United Stated and Europe, despite all medical advances over the years. As a result of novel surgical procedures and improvement in intensive care units, mortality has decreased and many children survive into adulthood (3). CHDs do not only have a serious impact on the child, but also on family, society, health care and insurances (4,5).

The most prevalent CHD is the ventricular septal defect (VSD) accounting for approximately 40% of CHDs (6). A VSD is an opening in the septum between the left and right ventricle of the heart. The perimembranous type of VSD (pVSD) is located in the membranous part of the ventricular septum and accounts for 80% of all VSDs, which corresponds to about one-third of all CHDs (Figure 1) (7).

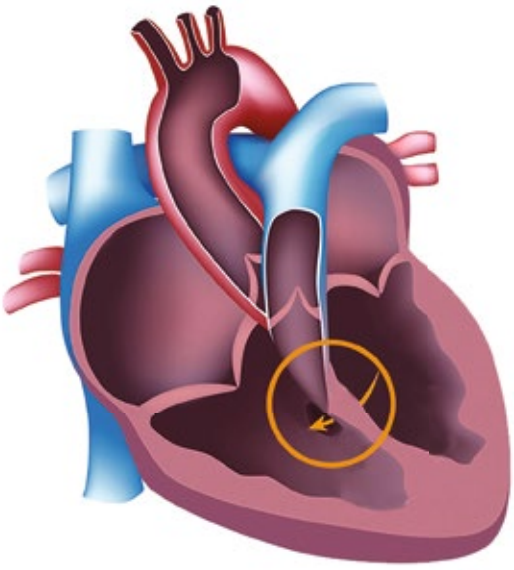

Figure 1 | Location of perimembranous ventricular septal defect in the human heart

The heart is the first organ to function within an embryo and starts to beat spontaneously around 22 days after fertilisation (8). The morphologic development of the embryonic heart is essentially completed after 7 weeks of gestation, although the heart will continue to grow throughout gestation. Since the human heart is completed so soon in gestation, CHDs are the result of abnormal development very early in pregnancy.

The pathogenesis of CHDs is complex and largely unknown with approximately only 15% than can be traced to a known cause (9). Only a small proportion of CHDs is associated with chromosomal

abnormalities (3-5%), single gene defects (3-5%) and teratogens (2-3%) (1). The majority of CHDs have a multifactorial origin and are the result of interactions between genes and environment (6). CHDs can be classified as nonsyndromic and syndromic. The prevalence of CHDs is strongly increased in individuals with Down syndrome (DS) as approximately 50% of children born with DS have a CHD.

Cardiovascular risk factors

Cardiovascular disease (CVD) has evolved into a global burden with 17.5 million people dying each year from CVD, an estimated 31% of all deaths worldwide (10). The most important CVD risk factors are smoking, high blood pressure, high cholesterol, physical inactivity, obesity, diabetes and poor nutrition (11). These CVD risk factors are also very common in women of reproductive age: physical inactivity (48.5%), obesity (31.8%), smoking (20.2%), high cholesterol (11.3%), hypertension (6.9%) and diabetes (3.1%) (12). In terms of human reproduction, these risk factors have impact on the development of pregnancy complications such as fetal growth restriction, preterm birth and congenital malformations. It has become clear that many CVDs that present in adulthood actually have their origins during fetal life, infancy and childhood (11,13).

Periconception environment

The periconception period is a highly sensitive time window during early human development. The gametes and embryo are sensitive to external exposures such as nutrition and unhealthy lifestyle like smoking and stress (14). During this stage, the embryo has the ability to adapt through various mechanisms to changing environmental conditions which may have long-term consequences that can extend into adulthood. Adverse exposures may increase the risk of congenital malformations including CHDs. Both the use of a folic acid supplement as well as a one-carbon-rich dietary pattern significantly reduces the risk of having offspring with a neural tube defect or CHD (15,16). In this thesis the periconception period is defined as 4 weeks prior to conception lasting through to 8 weeks after conception. With primary prevention as main focus, risk factors for abnormal heart development should be identified during the preconception period.

Gene-environment interactions

Like many complex diseases, CHDs are thought to be the result of complex interactions between an individual's genetic blueprint (nature) and environmental influences (nurture). Gene-environment interactions are complex since both maternal and paternal environmental exposures can interact with either the mother's genes or infant's genes and alter the risk of CHD (Figure 2) (17). One of the proposed mechanisms of gene-environment interactions is epigenetics.

Genetics vs. Epigenetics

In humans, genes make up the blueprint containing all information to build the human body. Genes are made up of pieces of DNA (deoxyribonucleic acid) and consist of four types of nucleotide bases, that is adenine (A), cytosine (C), guanine (G) and thymine (T). Genetics is based on variations in the DNA sequence. If a protein has to be synthesised, the gene has to be read first (Figure 3A). Changes in gene expression will results in different cell function or phenotype.

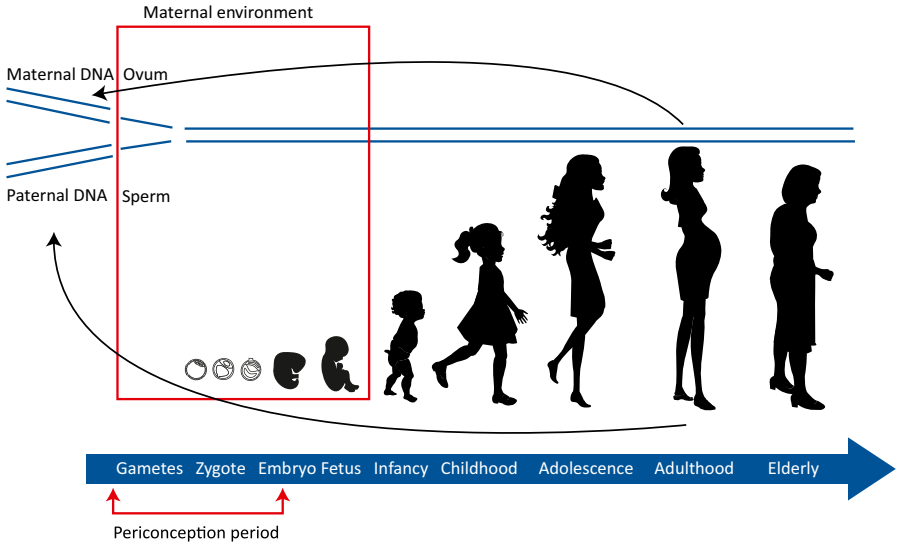

Figure 2 | Gene-environment life course epidemiology

Epigenetics is the study of heritable variations that occur without variation in the DNA sequence. Hence, the term epigenetics – from the Greek "upon" – refers to changes upon genetics. One of the best studied epigenetic mechanisms is DNA methylation which was described for the first time in 1975 (18,19). DNA methylation is a biochemical process where a methyl (CH_3) group is added to the DNA, in humans often to the fifth atom of a cytosine (Figure 4). This type of DNA methylation is also called CpG methylation, which stands for a cytosine nucleotide next to a guanine nucleotide separated by a phosphate (Cytosine-phosphate-Guanine). There are over 28 million CpG loci in the human genome of which 70-80% is methylated (20). DNA regions with a high frequency of CpG loci are called CpG islands and are often found near the promotor region of a gene (Figure 5).

The process of DNA methylation does not change the DNA sequence itself but gene expression can be altered (Figure 3B). DNA methylation is thought to influence gene expression at the level of transcription, however, other steps in the process may also be regulated epigenetically (21). DNA methylation is one of several epigenetic mechanisms that cells use to control gene expression and is very important during human embryogenesis. Since DNA methylation is sensitive to environmental changes, epigenetic mechanisms can explain how humans can respond to the environment through changes in gene expression. That is, epigenetic changes can turn on or off genes. In humans there are two important developmental time windows were methylation is reprogrammed genome-wide (22). This is in the germ cells and in preimplantation embryos.

Methyl groups for DNA methylation are primarily supplied from dietary methyl donors and as cofactors via one-carbon metabolic pathways (14). Maternal diet is an important factor that can influence the epigenome and emphasises the importance of preconceptional intake of foods such as vegetables, fruits and nuts since they are rich in one-carbon metabolites (14).

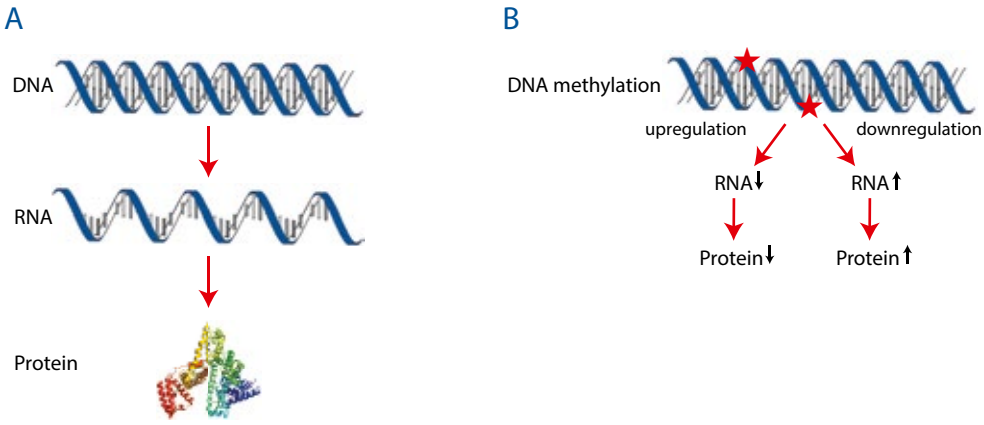

Figure 3 | Gene expression: from gene to protein

Panel A shows the process of gene expression. *Panel B* shows epigenetic up- and down regulation of gene expression.

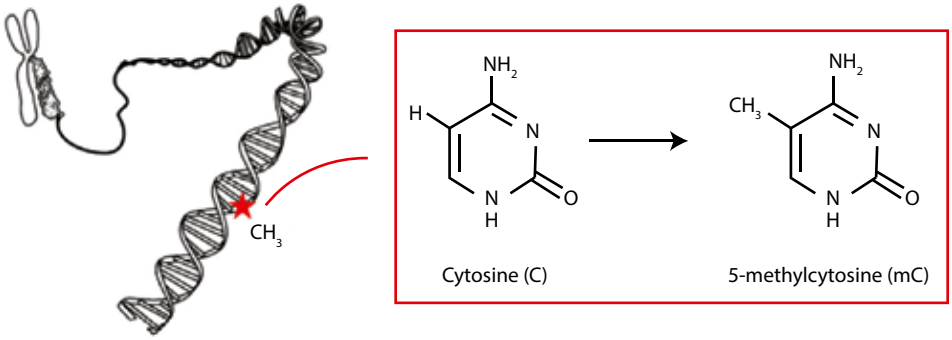

Figure 4 | DNA methylation: a methyl group (CH3) is added to the 5th atom of a cytosine

DOHaD paradigm

The developmental origins of health and disease (DOHaD) hypothesis predicts that environmental exposures experienced early in life have the potential to modify the risk of developing later-onset diseases (23). Periconception, prenatal and early postnatal life conditions may affect the vulnerability of the individual for developing cardiovascular and metabolic diseases in later life (24). One of the proposed mechanisms of DOHaD is epigenetic modification as a result of altered DNA methylation.

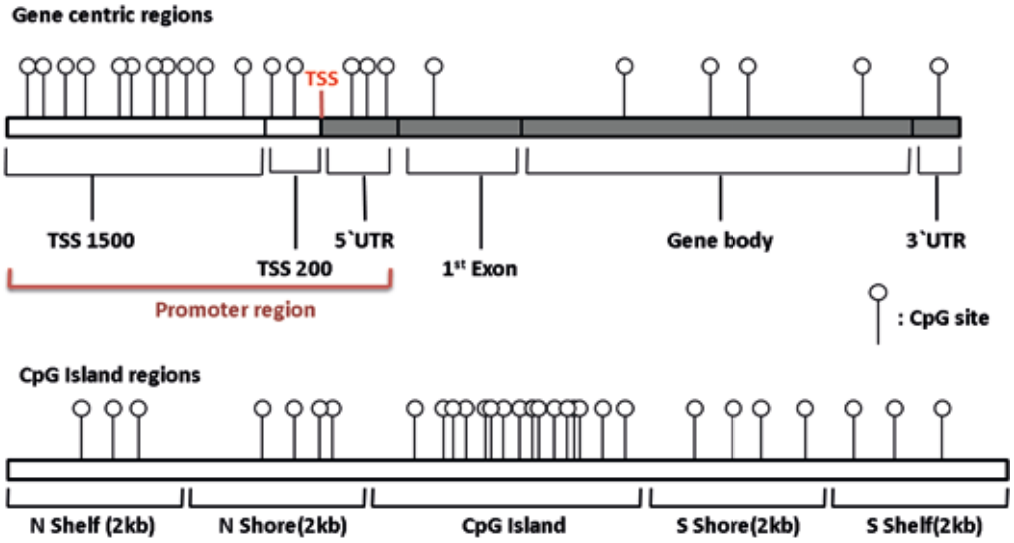

Figure 5 | Gene structure with location of CpG islands *(http://methhc.mbc.nctu.edu.tw/php/index.php)*

Methodology

Epidemiology

The word epidemiology is derived from the Greek *epi* meaning "upon", *demos* meaning "people" and *logos* meaning "study" and therefore literally means "studies upon people". Epidemiology is the science that studies the patterns, causes, and effects of health and disease conditions in a defined population (25). Epidemiologic observations mainly relate to groups of people. Statistical associations between a risk factor and a disease do not necessarily imply a causal relationship. The Bradford Hill criteria provide a way to determine if an observed association is likely to be causal (26). Inherent to the study design different types of bias and confounding are to be considered.

Epigenome-wide association studies vs. Candidate-gene studies

Epigenome-wide association studies (EWAS) aim to systematically detect the association of a disease with a CpG. Because the EWAS approach is hypothesis-free, the power is to detect new CpG loci associated with CHD. If the methylation change for a certain CpG is statistically different between two groups the CpG is associated with the disease. The level of statistical significance is calculated using Bonferroni correction. False discovery rate (FDR) control can be used as a less stringent method. In hypothesis-driven candidate-gene studies, genes are selected based on prior knowledge on its relevance in the mechanism of the disease being studied. The advantage of candidate-gene studies is that they require a smaller sample size to achieve adequate statistical power.

Epigenetic technology

In order to measure DNA methylation the isolated DNA has to be pretreated first. Different pretreatments are available, mostly used are sodium bisulphite treatment, restriction enzyme treatment and affinity enrichment. After this step, different methods can be used to study DNA methylation at a global (Alu, LINE-1, LUMA), gene-specific (pyrosequencing, Sequenom's EpiTYPER, methylation specific PCR) or genome-wide level (next generation sequencing or microarrays such as Illumina BeadChips and Tiling Array).

In this thesis all DNA has been pretreated with sodium bisulphite. After this, the Illumina Infinium® HumanMethylation450 BeadChip was used to measure genome-wide methylation (Chapter 4 and 5). For validation of the top CpG hits we used pyrosequencing (Chapter 4). For gene-specific analysis we used EpiTYPER (Chapter 7).

Core study material

Most of the aims of this thesis were addressed in the HAVEN study or in a subset of the control children of the same HAVEN study. Our study on CVD risk factors and embryonic growth trajectories is based on the Rotterdam Periconception Cohort (Predict study).

HAVEN study

The HAVEN study (Hart Afwijkingen, Vasculaire status, Erfelijkheid en Nutriënten) is a case-control family study performed in the Western part of the Netherlands. This study was designed to investigate environmental and genetic determinants in the pathogenesis and prevention of CHD. Data has been collected from June 2003 until January 2010 at the Department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands. Case children were recruited in collaboration with the Departments of Paediatric Cardiology of the Erasmus MC, University Medical Centre in Rotterdam, Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam, the Netherlands. Diagnoses were ascertained by a paediatric cardiologist during the first year of life by ultrasound and/or cardiac catheterisation and/or surgery. Control children were recruited from the public health care centres of 'Careyn' in the Rotterdam area in the Netherlands. Children were eligible as controls if they did not have a congenital or chromosomal malformation ascertained by their physician at the child health care centre. Between 2003 and 2010 387 cases and 419 controls were included.

Rotterdam Periconception Cohort

The Rotterdam Periconception Cohort (Predict study) is a prospective periconception cohort conducted at the Department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands (27). All women of at least 18 years of age with ongoing pregnancies of 6 to 8 weeks of gestation were eligible for participation and included in 2009 and 2010. A total of 259 pregnancies were included. Women received weekly transvaginal three-dimensional ultrasound scans from enrolment up to the 13th week of pregnancy. Crown-rump lengths were

measured using the BARCO I-Space (Barco N.V., Kortrijk, Belgium), a four-walled CAVE™-like (Cave Automatic Virtual Environment) virtual reality system, allowing depth perception and interaction with the projected images using stereoscopic imaging.

Blood vs. Target tissue

For the measurement of DNA methylation it is important to choose the appropriate tissue given tissue-specific gene regulation. However, it is of course not possible to obtain healthy heart tissue from healthy control children. Therefore, whole blood is collected in the HAVEN study as a proxy tissue from both case and control children to measure leukocyte DNA methylation. Blood is easily accessible and has a shared embryonic development with heart tissue since the lateral plate mesoderm in human embryos gives rise to both the heart and blood cells in the circulatory system.

Hypothesis

Data has shown that the developing cardiovascular system is sensitive to poor maternal nutrition and lifestyle conditions during the periconception period, in the womb and in early postnatal life (28). We hypothesised that CHD and CVD share variations in vascular and metabolic pathways. These gene-environment interactions may result in aberrant leukocyte DNA methylation which may be associated with increased risk of either CHD or CVD and as such can explain inter- and transgenerational effects.

Aims of the thesis

CHDs are the most common birth defects worldwide with over 1 million children born each year. Since the pathogenesis is still largely unknown we aimed to investigate environmental and epigenetic determinants associated with CHD. CVD risk factors increase with age, however, they are becoming more common at a younger age. In women of reproductive age they can have impact on pregnancy course and outcome. We examined intergenerational effects of CVD risk factors on the offspring. Epidemiologic and epigenetic studies are used to address the following aims:

Part I – Congenital heart diseases

1. To study intergenerational effects of grandparental CVD on CHD risk in the offspring.
2. To investigate the influence of parental nongenetic risk factors on CHD risk in the offspring.
3. To explore epigenome-wide DNA methylation using an hypothesis-free approach in very young children with nonsyndromic and syndromic CHD.

Part II – Cardiovascular risk factors

4. To investigate associations between maternal CVD risk factors and embryonic growth.
5. To assess gene-specific DNA methylation in young children and associations with serum lipid levels.

Outline of the thesis

In **Part I** we study CHDs in young children. **Chapter 2** describes the association between CVD in grandparents and the risk of having a grandchild with a CHD. **Chapter 3** describes associations between nongenetic parental conditions and pVSD. **Chapter 4** describes an EWAS investigating CpG loci associated with pVSD. **Chapter 5** addresses an EWAS in children with both pVSD and Down syndrome.

In **Part II** we address CVD risk factors. **Chapter 6** describes the maternal CVD risk profile in association with embryonic growth in pregnancies conceived spontaneously and after *in vitro* fertilisation with or without intracytoplasmic sperm injection. In **Chapter 7**, we investigate the early life lipid profile in relation to metabolic programming in very young children. **Chapter 8** covers the general discussion of the main findings and suggestions for future research are presented. In **Chapter 9** we provide a summary.

Part I

Congenital heart diseases

Chapter 2

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Cardiovascular diseases in grandparents and the risk of congenital heart diseases in grandchildren

Abstract

Background Hyperglycaemia, dyslipidaemia and hyperhomocysteinaemia are associated with both adult cardiovascular disease (CVD) and having a child with a congenital heart disease (CHD). We investigated associations between CVD in grandparents and the risk of CHD in grandchildren.

Methods In a case-control family study we obtained detailed questionnaire information on CVD and CHD in 247 families with a CHD child and 203 families without a CHD child.

Results Grandparents with CVD or intermittent claudication (IC) were significantly associated with an increased risk for CHD in grandchildren (OR 1.39 (95% CI 1.03-1.89) and OR 2.77 (95% CI 1.02-7.56), respectively). The risk for CHD grandchildren was particularly increased in paternal grandfathers with CVD (OR 1.85 (95% CI 1.01-3.37)). Overall, having a grandparent with CVD increased the risk of CHD in the grandchild by 1.65 (95% CI 1.12-2.41). After adjustment for potential maternal confounders this risk was 1.44 (95% CI 0.94-2.21). Having two or more grandparents with CVD was associated with an approximately threefold risk for CHD grandchildren (OR adjusted 2.72 (95% CI 1.08-6.89)).

Conclusions Our data suggest that CVD and IC in grandparents are associated with an increased risk of having a CHD grandchild. These first findings may be explained by shared causality of derangements in metabolic pathways and are in line with the fetal origins of health and disease.

Introduction

The worldwide epidemic of cardiovascular diseases (CVDs) remains the leading cause of death and represents 30% of all global deaths (29). Moreover, an estimated 1 million children are born each year with congenital heart diseases (CHDs) − the most common cause of death from congenital malformations worldwide (30). To prevent both diseases in the future, risk factors have to be identified. Both animal and human studies show that common environmental exposures, including lifestyles, can derange metabolic pathways in particular in combination with genetic susceptibilities during vulnerable periods in life and as such are implicated in the pathogenesis of both CVD and CHD (31,32).

We have previously shown a significant association between maternal dyslipidaemia and an increased risk of a child with CHD (33). This finding is in line with other metabolic risk factors for CHD offspring, such as maternal hyperhomocysteinaemia and hyperglycaemia (34,35). It is striking that the same metabolic derangements in later life are associated with adult CVD (36,37).

Therefore, we hypothesise that CVD and CHD share variations in metabolic causal pathways, owing to interactions between poor lifestyles and genetic susceptibilities clustering in families. The underlying mechanism might be a derangement of epigenetic programming, which is substantiated by the observed transgenerational effects of exposure to famine (38-40). A threefold increase in coronary heart disease and a more atherogenic lipid profile especially in women were found in the elderly whose mothers were undernourished early in pregnancy during the Dutch famine (41,42). Shared metabolic variations of CVD and CHD can affect putative mechanisms of epigenetic (re) programming of the germ line (i.e. grandparents) and embryonic cardiac tissue (i.e. grandchild) thereby mediating the risk for both diseases.

The relatively low prevalence of CHD, that is 4-8 per 1000 live births makes a prospective preconceptional cohort study unfeasible. Therefore, we conducted a case-control family study to investigate associations between CVD in grandparents and the risk of CHD offspring.

Methods

Study population

This study was part of the HAVEN study, a case-control family study designed to investigate environmental and genetic determinants in the pathogenesis and prevention of CHD, and has been described in detail before (33,35).

In addition to this study, grandparents of 806 participating families were invited to participate in the current study of which 552 families (68%) responded (Figure 1). Of these families, 91 families (11%) did not want to participate and before the analyses we excluded 11 families (1%) without any living grandparent. This resulted in 450 (56%) families for further analyses. Of these 450 families with 1800 grandparents 250 (14%) did not respond and 1550 (86%) responded by returning the questionnaire. A total of 214 grandparents from the latter group were deceased and therefore excluded which leaves 1336 grandparents for further analyses.

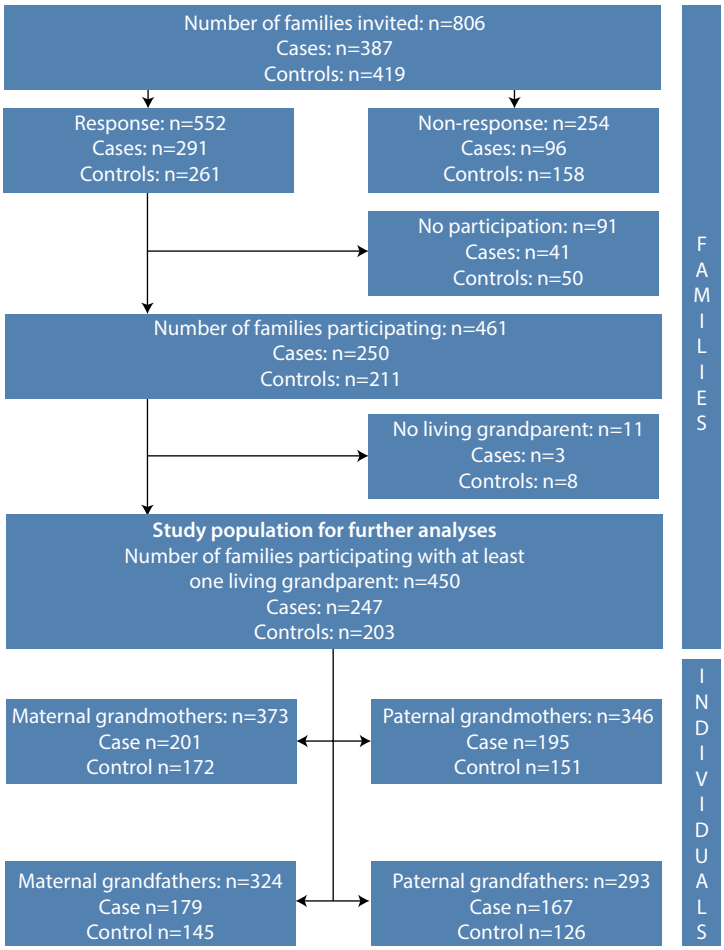

Figure 1 | Flowchart of the HAVEN study population

Questionnaires

All participating grandparents filled out a detailed paper questionnaire at home on general characteristics and their own medical history as well as that of their parents, that is great-grandparents. Questions on medical history were derived from questionnaires used on patients with heterozygous familial hypercholesterolaemia (43). From these questionnaires we extracted date of birth, medical history, medical surgery, medication use, special diet, smoking, and family medical history of CVD and CVD-related diseases.

The medical history of grandparents comprised self-reported CVD, defined as any acute, chronic, ischaemic, pulmonary or other form of heart and vascular diseases, based on the National Health Interview Survey of the Centres for Disease Control and Prevention (44). Information on the CVD-related diseases hypertension, intermittent claudication (IC), cerebrovascular accident (CVA), thrombosis, and pulmonary embolism was obtained and analysed separately. The medical history of great-grandparents comprised information on CVD only. To validate the diagnosis, the year

of diagnosis and treatment – that is medication use, special diet and or surgery – was reported. Medication use was reported as the name of the drug, date of prescription, amount prescribed and dose regimen. All drugs are coded according to the Anatomical Therapeutic Chemical (ATC) Classification System of the WHO Collaborating Centre for Drug Statistics Methodology. The use of a special diet was defined as cholesterol and or fat limited diet, low-sodium diet, a weight-reducing diet or other special diet. In addition, information was collected on the period in which the special diet was used and whether it was prescribed by a dietitian or doctor. Smoking was reported as the year when people started smoking, the amount and kind of tobacco products smoked per day, and the moment of quitting smoking. A smoker was defined as someone who smoked currently or had a history of smoking. When a first- or second-degree family member was deceased, the cause and age of death were reported.

We did not obtain data on CVD and CVD-related diseases in mothers and fathers of the case and control children, because they were too young to have already experienced these diseases. Other details on mothers, fathers and children were obtained as described previously (35,45). Periconceptional folic acid supplement use was defined as the daily intake of at least 400 µg in the entire periconception period, which was defined as 4 weeks before conception until 8 weeks after conception. Total plasma homocysteine and total serum cholesterol concentrations of venous blood samples from the mothers were obtained and published previously (33).

Statistical analyses

We compared case and control families using the Student's t-test and Mann-Whitney *U* test for continuous variables and the chi-square test for categorical variables. When the number of observations for analysis was less than five we used Fisher's exact test.

We used univariable logistic regression analyses to study the associations between different risk factors including CVD in grandparents, and the risk of CHD in grandchildren. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated.

A stratified analysis for the two maternal and two paternal grandparents was performed to investigate a significant grandparent-of-origin effect. Confounding of maternal factors was investigated using multivariable logistic regression analyses. We adjusted for the following potential maternal confounders: age at conception of the index child, educational level, total homocysteine and total cholesterol concentrations, and periconceptional folic acid supplement use. We also adjusted for CHD in the parents of the child.

Furthermore, we investigated whether the risk of CHD in grandchildren increased with more CVD affected grandparents using univariable and multivariable logistic regression analyses. In these analyses, we adjusted for the same maternal confounders and additionally for the number of grandparents that were ascertained per child.

A *P* value <0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics 20.0 for Windows software (IBM, Armonk, NY, USA).

The study protocol was approved by the Central Committee for Human Research (CCMO) in The Hague, The Netherlands, and by the Institutional Review Boards (Medical Ethics Committees) of all participating hospitals. All parents and grandparents gave their written informed consent. Mothers and their partner gave written informed consent on behalf of their participating child.

Results

A total of 247 case and 203 control families were included for analysis (Figure 1). General characteristics of the children and their parents are summarised in Table 1. CHD phenotypes of the case children comprised perimembranous ventricular septal defect (n=60), transposition of the great arteries (n=48), pulmonary valve stenosis (n=42), coarctation of the aorta (n=28), tetralogy of Fallot (n=24), atrioventricular septal defect (n=20), hypoplastic left heart syndrome (n=11), aortic valve stenosis (n=6) and miscellaneous (n=8). Case children showed a significantly lower birth weight adjusted for gestational age compared with control children. Case and control mothers were significantly different for age at birth of the index child and CHD in the family. Case and control fathers were significantly different for educational level.

The risk factors of living grandparents are presented in Table 2. The number of grandparents participating per child did not differ statistically significantly between case and control families (*P*=0.711; Supplementary Table S1). CVD and IC were more common in the grandparents of CHD children (17% versus 13%, *P*=0.034 and 2% versus 1%, *P*=0.038, respectively). Grandparents with either CVD or IC were significantly associated with an increased risk for CHD grandchildren, OR 1.39 (95% CI 1.03-1.89) and OR 2.77 (95% CI 1.02-7.56), respectively. The prevalence of CVD in the parents of these grandparents, that is great-grandparents, was not significantly different between the case and control families.

Table 3 shows the results of the stratified analysis for the four different grandparents. We observed a significant association between smoking in maternal grandmothers and the risk of a CHD grandchild (OR 1.73 (95% CI 1.02-2.93)). CVD in paternal grandfathers was associated with an increased risk for a CHD grandchild (OR 1.85 (95% CI 1.01-3.37)). No significant associations were observed in the stratified analysis for great-grandparents.

There was a 1.6-fold higher CHD risk (OR 1.65 (95% CI 1.12-2.41)) when at least one of the grandparents of the child suffered from CVD. When we adjusted the association for potential maternal confounders, the OR attenuated to 1.44 (95% CI 0.94-2.21).

Stratification for the number of CVD affected grandparents showed that children with one grandparent with CVD had a 1.5 fold increased CHD risk (OR 1.51 (95% CI 1.01-2.26), case/control children n=93/62) and with two or more CVD affected grandparents a more than 2.5 fold increased CHD risk (OR 2.58 (95% CI 1.15-5.78), case/control children n=23/9). Adjustment for maternal confounders and the number of grandparents ascertained per child attenuated the association between CHD risk and one grandparent affected by CVD (OR 1.29 (95% CI 0.83-2.02)). The association between CHD risk and two or more grandparents affected by CVD became stronger (OR adjusted 2.72 (95% CI 1.08-6.89)).

Because data were self-reported, we cross-checked CVD diagnoses in both case and control grandparents with treatment data (Supplementary Table S2). Of the grandparents with CVD, 81-100% used medication from the ATC group C (cardiovascular system) or underwent CVD-related interventions/surgery or followed a special diet for their CVD.

Table 1 | General characteristics of case families of a child with CHD and control families of a non-malformed child

	Cases (n=247)	Controls (n=203)	*P* value
Characteristics of children			
Age – months (SD)	16.6 (3.3)	15.9 (2.0)	0.088
Male gender	147 (60)	117 (58)	0.687
Birth weight adjusted for GA (median, min-max)	3390 (865-5 100)	3520 (2235-5180)	0.000
Ethnicity			0.492
Dutch native	213 (86)	180 (89)	
European Others	19 (8)	10 (5)	
Non-European	15 (6)	13 (6)	
Characteristics of mothers			
Age at birth of index child – years (SD)	35.1 (4.3)	34.0 (4.2)	0.019
Educational level			0.052
Low	45 (18)	22 (11)	
Intermediate	104 (42)	103 (51)	
High	98 (40)	78 (38)	
CHD[a]	4 (2)	0 (0)	0.131
CHD family history[b]	36 (15)	11 (5)	0.002
Periconceptional			
Folic acid supplement use[c]	42 (17)	37 (18)	0.734
Smoking	42 (17)	33 (16)	0.832
Total homocysteine, mean (SD) (n=384)	10.5 (4.4)	9.8 (3.1)	0.056
Total cholesterol, mean (SD) (n=384)	4.9 (0.9)	4.8 (0.9)	0.138
Characteristics of fathers			
Age at birth of index child – years (SD)	37.4 (4.3)	36.9 (4.7)	0.264
Educational level			0.034
Low	58 (23)	35 (17)	
Intermediate	81 (33)	90 (44)	
High	108 (44)	78 (38)	
CHD[a]	2 (1)	0 (0)	0.504
CHD family history[b]	27 (11)	14 (7)	0.139
Smoking	75 (30)	69 (34)	0.408

Values are means or numbers (percentages) unless otherwise specified.

[a] Congenital heart disease defined as perimembranous ventricular septal defect, transposition of the great arteries, pulmonary valve stenosis, coarctation of the aorta, tetralogy of Fallot, atrioventricular septal defect, hypoplastic left heart syndrome, aortic valve stenosis.

[b] Any congenital heart disease of family members in the first, second or third degree.

[c] Adequate periconceptional folic acid supplement use 4 weeks prior to conception until 8 weeks after conception.

Table 2 | Risk factors of grandparents for having a CHD grandchild

	Case grandparents (n=742)	Control grandparents (n=594)	Missing data Case/Control grandparents n (%)	OR (95% CI)
Grandparents (n=1336)				
Age – years (range)	65 (46-88)	64 (47-85)	11/5 (1/1)	1.01 (1.00-1.03)
Gender				
Male	346 (47)	270 (45)	0/0 (0/0)	1.04 (0.84-1.29)
CVD	127 (17)	77 (13)	5/2 (1/0)	1.39 (1.03-1.89)
Hypertension	217 (29)	177 (30)	5/2 (1/0)	0.98 (0.77-1.24)
IC	17 (2)	5 (1)	5/2 (1/0)	2.77 (1.02-7.56)
CVA	26 (4)	15 (3)	5/2 (1/0)	1.41 (0.74-2.68)
Thrombosis/ pulmonary embolism	20 (3)	18 (3)	5/2 (1/0)	0.89 (0.47-1.70)
Smoking	133 (18)	90 (15)	6/2 (1/0)	1.23 (0.92-1.65)
Great-grandparents				
Great-grandfather CVD (n=1237)	267 (39)	207 (37)	59/40 (9/7)	1.08 (0.85-1.36)
Great-grandmother CVD (n=1260)	206 (30)	148 (26)	45/31 (6/5)	1.18 (0.92-1.51)

CVD, cardiovascular disease. IC, intermittent claudication. CVA, cerebrovascular accident. Values are means or numbers (percentages) unless otherwise specified.

Discussion

This case-control family study suggests that both CVD and IC in grandparents are associated with an increased risk for CHD in grandchildren. Especially having two or more grandparents with CVD increases the risk for CHD in grandchildren. These findings support our hypothesis that CVD and CHD share common metabolic or other pathways. The heritability of lifestyle factors in the same way as genetic factors affecting these metabolic pathways should be considered with respect to both the high prevalence of poor nutrition, smoking and other lifestyles in the general population, and the relatively small numbers of genetic variations identified for CVD and CHD (46-48). Of interest in this regard is also the established association between maternal grandmother smoking and CHD in the grandchild. However, additional adjustment for maternal or grandparental and particularly grandmaternal smoking did not significantly affect the results (Supplementary Table S3). Smoking is a proxy measure for socioeconomic status, which is clustering in families and as such should also be considered as a risk factor for both CVD and CHD. Adjustment for maternal educational level as a proxy for socioeconomic status did not attenuate the risk for CHD in grandchildren. However, this does not rule out the contribution of poor lifestyles in families to the risk for both CVD and CHD.

Our results show the highest CHD risk in grandchildren in which the paternal grandfather suffered from CVD. This observation is in line with studies showing that the paternal diet can affect cholesterol and lipid metabolism in the offspring (49). However, underdiagnosis of CVD in women is an issue to be considered, in particular in grandmothers.

Table 3 | Risk factors of grandparents stratified for maternal and paternal grandmothers, and maternal and paternal grandfathers (n=1336) for having a CHD grandchild

	Maternal grandmothers			Maternal grandfathers			Paternal grandmothers			Paternal grandfathers		
	Case n=201	Control n=172	OR (95% CI)	Case n=179	Control n=145	OR (95% CI)	Case n=195	Control n=151	OR (95% CI)	Case n=167	Control n=126	OR (95% CI)
Age – years (range)	63 (50-84)	62 (49-80)	1.02 (0.98-1.05)	65 (46-83)	65 (54-84)	1.01 (0.97-1.04)	66 (50-87)	65 (47-85)	1.02 (0.99-1.05)	66 (49-88)	66 (52-82)	1.00 (0.97-1.04)
CVD	26 (13)	13 (8)	1.82 (0.90-3.66)	43 (24)	31 (22)	1.17 (0.69-1.98)	17 (9)	14 (9)	0.94 (0.45-1.97)	41 (25)	19 (15)	1.85 (1.01-3.37)
Hypertension	53 (26)	53 (31)	0.80 (0.51-1.26)	52 (29)	38 (26)	1.16 (0.71-1.90)	62 (32)	45 (30)	1.11 (0.70-1.76)	50 (30)	41 (33)	0.89 (0.54-1.47)
IC	1 (0)	0 (0)	N/A	7 (4)	1 (1)	5.89 (0.72-48.43)	6 (3)	2 (1)	2.38 (0.47-11.95)	3 (2)	2 (2)	1.14 (0.19-6.92)
CVA	7 (3)	2 (1)	3.07 (0.63-15.0)	6 (3)	3 (2)	1.65 (0.41-6.71)	9 (5)	6 (4)	1.18 (0.41-3.38)	4 (2)	4 (3)	0.75 (0.18-3.07)
Thrombosis/ pulmonary embolism	6 (3)	8 (5)	0.63 (0.21-1.86)	6 (3)	5 (3)	0.98 (0.29-3.26)	6 (3)	2 (1)	2.38 (0.47-11.95)	2 (1)	3 (2)	0.50 (0.08-3.03)
Smoking	47 (24)	26 (15)	1.73 (1.02-2.93)	38 (21)	24 (17)	1.38 (0.78-2.43)	20 (10)	21 (14)	0.71 (0.37-1.36)	28 (17)	19 (15)	1.14 (0.60-2.15)
Great-grandparents												
Great-grandfather CVD	81 (44)	75 (48)	0.86 (0.56-1.32)	74 (44)	48 (35)	1.41 (0.89-2.25)	58 (34)	51 (36)	0.92 (0.57-1.46)	54 (34)	33 (28)	1.35 (0.80-2.27)
Great-grandmother CVD	63 (34)	47 (28)	1.29 (0.81-2.03)	41 (25)	39 (28)	0.83 (0.50-1.39)	60 (32)	41 (29)	1.18 (0.73-1.89)	42 (27)	21 (18)	1.65 (0.91-2.98)

CVD, cardiovascular disease. IC, intermittent claudication. CVA, cerebrovascular accident, N/A, not applicable. Because of missing values, numbers in the table may not add up. Individuals can have more than one disease and can follow more than one diet. Values are means or numbers (percentages).

Besides lifestyle risk factors it should be emphasised that other environmental factors may also be involved, such as psychological stresses and pollution, especially those linked to the socioeconomic status. After fertilisation, the somatic cell line is reprogrammed among others into the cardiac tissues, which will form the embryonic heart (50). After that, the cardiac cells will be fixed in tissue-specific patterns of gene expression through subsequent generations of cell division. During this critical window epigenetic alterations in the programming of embryonic growth and/or specific cardiovascular growth genes caused by poor environmental exposures may contribute to CHD and increase the susceptibility of developing CVD in later life. The cardiovascular system consists of many different cell types from which both CVD and CHD originate. All differentiated cell types have their own unique epigenetic mark, which reflects not only the developmental history and previous environmental exposures, supported by our observed association with maternal grandmother smoking, but also its phenotype (50). In this context, the concept of the fetal origins of CVD is an attractive hypothesis (51). This is in line with findings in patients with CHD showing both a higher susceptibility to develop ischaemic heart disease and a higher prevalence of CVD risk factors (52). However, this study is a first epidemiological attempt to determine an association between CVD and CHD and should be interpreted with caution. Nevertheless, potentially modifiable risk factors, such as nutrition and smoking, are of great importance and should be further investigated.

Within families ethnic and cultural factors determine environment. In our study population, 94% of the families are from European origin (87% Dutch natives, 6% European others). Therefore our results are unlikely to be caused by differences in ethnicity or culture. However, within families classic genetic factors cannot be ignored. Familial transgenerational risks are not only determined by shared environment, but also by shared alleles. Therefore, familial transgenerational risks are the result of a combined effect of susceptibility genes, that is risk alleles, and environmental risk factors clustering in families (53). It cannot be excluded that within the case families some major alleles are being selected with a dominant inheritance, which increases risks within these families compared with the general population. Therefore, a genetic contribution by the transfer of risk alleles should be considered as well.

It has been shown that maternal environmental factors during early pregnancy modify the risk for CHD in the offspring. A well-known example is the periconceptional use of a folic acid supplement to reduce the risk of having a child with CHD (31,54). After adjustment in our analyses for several maternal factors, the risk of CHD in the child still remained significant for the highest risk group. Therefore, it is not very likely that the association between CVD and CHD is owing to confounding of the investigated maternal factors. However, residual confounding of unobserved risk factors cannot be completely excluded. We selected as potential confounders a subset of maternal environmental factors, although, it can be assumed that paternal risk factors contribute as well (55). Owing to small sample sizes in the high risk groups and the potential of overadjustment we had to limit the number of potential confounders. Moreover, there are several contradictory studies on CHD risk factors and especially paternal risk factors which make a valid selection of true confounders very difficult.

The questions about self-reported CVD diagnoses are based on the National Health Interview Survey of the Centres for Disease Control and Prevention. These data were crosschecked using self-reported CVD treatment information. Of all the grandparents with CVD, 81-100% used medication from the

ATC group C (cardiovascular system), a special diet related to CVD, and/or underwent a CVD related intervention/surgery (Supplementary Table S2). CVD was significantly correlated with these medical data (data not shown). The use of medication could not be confirmed with pharmacy data. However, because of the good agreement of self-reported CVD and medical data we do not expect recall bias between case and control grandparents.

We did not study CVD in parents because they were too young to have already experienced CVD (Table 1). Our results are based on 1336 grandparents of 450 children which is a complete data set for 74% grandparents with a comparable number of cases and controls (Supplementary Table S1). This means that data of 464 grandparents were not analysed, that is 250 alive or deceased non-responders and 214 excluded deceased grandparents. The strength of the study is that in the excluded group of deceased grandparents the distribution of cases and controls were comparable (cases n=124 and controls n=90, *P*=0.511), as well as the number of CVD-related deaths (cases n=34 and controls n=23, *P*=0.761). Data on other diseases in this group were incomplete (49%) and therefore not further analysed. A sensitivity analysis restricted only to children with data on all four grandparents attenuated the ORs to close to 1.0 although with wide confidence intervals because of small sample sizes. Another strength of our study is that the participants were not aware of the diseases of interest as an extensive questionnaire on multiple diseases was completed. Therefore, we conclude that it is not very likely that selection and recall bias have confounded our results although bias because of missing grandparents cannot be ruled out.

Here we show that families in which grandparents are affected by CVD and IC have an increased risk for having grandchildren with CHD. These interesting findings may stimulate further research into modifiable environmental risk factors of underlying common metabolic pathways to prevent both CHD and CVD in future generations.

Supplementary material

Table S1 | Number of living grandparents participating per child

Number of grandparents participating per child	Case children n=247	Control children n=203	Total children n=450
1 grandparent	14 (6%)	17 (8%)	31 (7%)
2 grandparents	73 (30%)	61 (30%)	134 (30%)
3 grandparents	58 (23%)	45 (22%)	103 (23%)
4 grandparents	102 (41%)	80 (39%)	182 (40%)

Table S2 | Validation of self-reported CVD (n=204) in grandparents by treatment

	Maternal				Paternal			
	Grandmother		Grandfather		Grandmother		Grandfather	
	Cases n=26	Controls n=13	Cases n=43	Controls n=31	Cases n=17	Controls n=14	Cases n=41	Controls n=19
Medication use, n (%):								
Cardiovascular system[a]	20 (77)	11 (85)	37 (86)	28 (90)	13 (76)	10 (71)	38 (93)	18 (95)
C01 Cardiac therapy	6 (23)	3 (23)	9 (21)	10 (32)	5 (29)	3 (21)	8 (20)	4 (21)
C02 Antihypertensives	–	–	1 (2)	–	1 (6)	–	1 (2)	1 (5)
C03 Diuretics	2 (8)	5 (39)	4 (9)	6 (19)	3 (18)	6 (43)	6 (15)	3 (16)
C07 Beta blocking agents	15 (58)	9 (69)	21 (49)	17 (55)	7 (41)	6 (43)	27 (66)	10 (53)
C08 Calcium channel blockers	4 (15)	2 (15)	8 (19)	8 (26)	3 (18)	1 (7)	11 (27)	3 (16)
C09 Agents acting on the renin-angiotensin system	12 (46)	6 (46)	22 (51)	20 (65)	6 (35)	6 (43)	26 (63)	14 (74)
C10 Lipid modifying agents	14 (54)	4 (31)	26 (61)	18 (58)	6 (35)	4 (29)	24 (59)	14 (74)
Interventions / Surgeries, n (%):								
CVD-related	8 (31)	2 (15)	20 (47)	17 (55)	3 (18)	5 (36)	19 (46)	9 (47)
Coronary catheterisation	5 (19)	2 (15)	4 (9)	2 (6)	1 (6)	1 (7)	6 (15)	3 (16)
Coronary artery bypass graft	1 (4)	–	3 (7)	4 (13)	–	1 (7)	7 (17)	2 (11)
Cardiac ablation	–	–	1 (2)	1 (3)	–	–	–	–
Heart valve repair or replacement surgery	1 (4)	–	5 (12)	–	1 (6)	–	2 (5)	–
Heart surgery	–	–	1 (2)	2 (6)	–	–	–	1 (5)
Aneurysm	–	–	1 (2)	–	–	–	–	1 (5)
Cardiac pacemaker insertion	–	–	1 (2)	2 (6)	–	1 (7)	1 (2)	–
Carotid artery stenting	–	–	–	1 (3)	–	–	–	–
ICD placement	–	–	1 (2)	1 (3)	–	1 (7)	–	–
Percutaneous intervention for lower extremity peripheral vascular disease	1 (4)	–	–	1 (3)	–	–	–	–
Multiple interventions/surgeries; combination of the above	–	–	3 (7	3 (10)	1 (6)	1 (7)	3 (7)	2 (11)
Special diet use, n (%):	6 (23)	4 (31)	6 (14)	11 (35)	8 (47)	6 (43)	15 (37)	8 (42)
Any CVD-related medication/ intervention/special diet use, n (%)	21 (81)	12 (92)	40 (93)	29 (94)	16 (94)	13 (93)	40 (98)	19 (100)

[a] ATC Group C: 1 or more medicaments per person.
None of the grandparents used medication from ATC groups 'C04 Peripheral vasodilators' or 'C05 Vasoprotectives'

Table S3 | CVD in grandparents and the risk of having a CHD grandchild, adjusted for maternal and grandparental smoking

Maternal smoking				
	n=450 Unadjusted		n=450 Adjusted for maternal smoking	
Grandparents	OR	*P* value	Adjusted OR	*P* value
CVD	1.6 (1.1-2.4)	0.010	1.6 (1.1-2.4)	0.011

	n=450 Unadjusted		n=450 Adjusted for maternal smoking	
Grandparents	OR	*P* value	Adjusted OR	*P* value
No CVD	1.0 (ref)		1.0 (ref)	
1 CVD	1.5 (1.0-2.3)	0.044	1.5 (1.0-2.3)	0.045
>1 CVD	2.6 (1.2-5.8)	0.022	2.6 (1.1-5.8)	0.022

Grandparental smoking						
	n=1336 Unadjusted		n=1336 Adjusted for grandparental smoking		n=1336 Adjusted for maternal grandmother smoking	
Grandparents	OR	*P* value	Adjusted OR	*P* value	Adjusted OR	*P* value
CVD	1.4 (1.0-1.9)	0.034	1.4 (1.0-1.9)	0.024	1.4 (1.0-1.9)	0.028

Chapter 3

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Periconceptional parental conditions and perimembranous ventricular septal defects in the offspring

Abstract

Background The perimembranous ventricular septal (pVSD) defect is the most common congenital heart disease (CHD) phenotype. Several parental factors are associated with pVSD risk in the offspring. To contribute to the future prevention of pVSDs, we investigated associations with nongenetic parental conditions.

Methods In a case-control study with standardised data collection at 17 months after birth, 115 parents of a child with pVSD and 484 parents of a healthy child completed questionnaires about periconceptional nongenetic conditions. Univariable and multivariable logistic regression analyses were used to estimate odds ratios (OR) with 95% confidence intervals (95% CI).

Results Complete data were available for 588 families (98%). Maternal risk conditions associated with pVSD offspring were a positive family history of CHD (OR 2.61 (95% CI 0.98-6.91)), medication use (OR 1.80 (95% CI 1.13-2.85)) and advanced age (OR 1.07 (95% CI 1.02-1.12)). Exposure to phthalates (OR 1.93 (95% CI 1.05-3.54)) was the only paternal risk condition associated with pVSD offspring.

Conclusions Four periconceptional parental conditions contributed to pVSD risk in the offspring. Couples planning pregnancy should be counselled on these risk conditions which are partially modifiable to contribute to the future prevention of pVSDs.

Introduction

Congenital heart diseases (CHD) are the most frequent birth defects with a worldwide birth prevalence rate of 0.6 to 1.9 in 100 newborns per year (56,57). CHD remains the leading cause of morbidity and mortality among infants (58). The most prevalent CHD phenotype is the ventricular septal defect (VSD) accounting for approximately 40% of CHD (6). The perimembranous ventricular septal defect (pVSD) is a more severe type than the muscular ventricular septum defect, which often spontaneously closes during life (7).

The pVSD is an outflow tract defect originating in early pregnancy with a multifactorial aetiology in which only 15% can be attributed to a known maternal and sometimes paternal cause (9). Derangements in migration of cardiac neural crest cells to the endocardial ridges of the proximal outflow tract are suggested to be involved (59). The one-carbon pathway is important during embryogenesis and influenced by both genetic variations and environmental conditions, such as nutrition – in particular folate intake, lifestyle factors, and medication use. We and others showed in epidemiological and experimental embryo studies that a derangement of this pathway during the periconceptional period leading to a mild to moderate hyperhomocysteinaemia and global hypomethylation, contributes to the development of pVSD due to an altered behaviour of the neural crest cells (35,60-62). Moreover, it has been shown that strong adherence to a dietary pattern rich in folate, methionine and choline, and folic acid supplement use reduces the risk of CHDs including pVSD, which can be explained in part by a restoration of a deranged one-carbon metabolism (16,63). Environmental conditions associated with the pVSD phenotype comprise maternal pregestational diabetes mellitus, the use of assisted reproductive technology and exposure to medication, alcohol, drugs and fever (64).

To predict risks for any outcome, a cohort study is the gold standard. However, because pVSD is a rare disease, a cohort study is not feasible and a case-control study is the accepted alternative.

From this background, our aim was to further improve preconceptional care by identifying in a case-control study a risk profile of periconceptional parental conditions in association with pVSD in the offspring.

Methods

Study population

This study was part of the HAVEN study, a Dutch case-control family study designed to identify risk factors in the pathogenesis and prevention of CHDs. The study design has previously been described in detail (35,65). In summary, the study has been conducted from 2003 onward at the Department of Obstetrics and Gynaecology of the Erasmus University Medical Centre, in Rotterdam, the Netherlands. Case children and both parents were recruited from four university medical centres in the Netherlands. Diagnoses were ascertained by a paediatric cardiologist during the first year of life by ultrasound and/or cardiac catheterisation and/or surgery. Cases were defined as isolated CHD when they did not have another major structural congenital malformation besides their CHD. In line

with the role of the one-carbon pathway and to create homogeneity of the CHD phenotype, we included for the present study only parents and their child with pVSD .

Control children and their parents were enrolled in collaboration with the child health centres at which each child in the Netherlands is regularly checked on growth and development. Children were eligible as controls if they did not have a congenital or chromosomal malformation ascertained by their physician at the child health care centre.

Both case and control children were eligible for inclusion if they were singletons, their parents were familiar with the Dutch language in reading and writing, and there was no familial relationship between cases and controls. Participants visited the hospital at the standardised study moment of around 17 months after delivery.

Data collection

All case and control parents completed a general questionnaire at home. During the hospital visit these questionnaires were checked by the researcher for completeness and consistency. The periconception period as window of exposure to parental conditions was defined as 4 weeks before conception until 8 weeks after conception and covers the critical period of embryonic cardiogenesis. Educational level and ethnicity were classified according to the definitions of Statistics Netherlands (66,67). Family history of CHD was considered positive if a relative in the third degree or closer was diagnosed with pVSD, transposition of the great arteries, coarctation of the aorta, atrioventricular septal defect, tetralogy of Fallot, aortic valve stenosis, pulmonary valve stenosis, or hypoplastic left heart syndrome. We defined alcohol, smoking and medication use as any use during the periconception period. A medication subgroup was made for the use of folate antagonists. Folic acid supplement use was defined as daily intake of at least 400 µg during the entire periconception period, either in a multivitamin preparation or as a single tablet. Inconsistent users were classified as nonusers. Standardised anthropometric measurements were performed during the hospital visit, including weight, height and blood pressure. We assessed occupational exposures to chemicals by applying a job-exposure matrix with a focus on endocrine disrupting chemicals, as published previously (65).

Statistical analysis

Baseline characteristics were compared between case and control parents and children. Categorical variables were analysed using the chi-square test and Fisher exact test. Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test after which we used either the parametric Student's t-test or the nonparametric Mann-Whitney *U* test.

Univariable logistic regression analysis was used to determine the risk conditions significantly associated with pVSD. Odds ratios (OR) with 95% confidence intervals (95% CI) were estimated. The selection of risk conditions to estimate the risk of having a pVSD child was based on our studies within the HAVEN study and those of others.

The potential maternal risk conditions that were investigated are: age, parity (primipara and multipara), educational level, BMI, diabetes mellitus, ethnicity, positive family history of CHD, blood pressure, smoking, folic acid supplement, alcohol and medication use, and occupational

exposures to pesticides, phthalates, alkylphenolic compounds, polychlorinated compounds, and heavy metals. The potential paternal risk conditions that were investigated are: age, educational level, ethnicity, positive family history of CHD, smoking, alcohol use, and occupational exposure to pesticides, phthalates, alkylphenolic compounds, polychlorinated compounds and heavy metals. The dichotomous dependent variable was the case/control indicator. Discrete variables were treated categorically and continuous variables were treated continuous.

The final multivariable logistic regression model was derived after hierarchical backward elimination of all covariates with *P* value <0.10. Possible interactions were tested and interactions with *P* value <0.10 were entered in the model. The area under the receiver operating curves (AUC) was used to estimate how well the model discriminates between case and control families.

The analysis was repeated for isolated pVSD only, that is excluding syndromal and chromosomal pVSD. Because less than 2% of the data was missing we did not impute missing values. This led to the exclusion of 11 individuals (eight controls and three cases) in the multivariable analysis. A *P* value <0.05 was considered statistically significant and all tests were two-tailed. Analyses were performed with IBM SPSS Statistics Version 20.0 for Windows software (IBM, Armonk, NY, USA).

Results

A total of 115 case children with pVSD and 484 control children and both parents were analysed (data from three fathers was missing). The total group of pVSDs consisted of 90 isolated and 25 nonisolated defects. Of the 25 nonisolated pVSDs, 15 children suffered from one of the following syndromes: Down syndrome (n=10), 22q11 deletion (n=2), Noonan (n=1), 22q13 duplication (n=1) and miscellaneous (n=1). The remaining 10 nonisolated pVSDs had other defects that could not be traced to a known syndrome, such as hip dysplasia, club foot, craniosynostosis and fourth nerve palsy.

Case children were compared with control children significantly older at the study moment (16.9 (3.7) vs. 16.1 (2.3) months; *P*=0.024) and had a lower birth weight adjusted for gestational age at birth (3,214 (675) vs. 3,531 (657) grams; *P*=<0.001). Gender was comparable between case and control children (n=54 (47%) and 273 (56%) males, respectively; *P*=0.067).

The baseline characteristics of the parents are shown in Table 1. In the case group, mean maternal age was significantly higher compared with controls (31.5 vs. 30.4 years respectively (*P*=0.028)). Case mothers had a significantly shorter duration of pregnancy with a median of 39 weeks versus 40 weeks (*P*=0.004) and used more often medication, 33% versus 21% respectively (*P*=0.005). Folate antagonists were more used by case mothers compared with control mothers, 7% versus 3% (*P*=0.015).

Univariable logistic regression analysis revealed that maternal family history of CHD, medication use, alcohol and age, and paternal exposure to alkylphenolic compounds and phthalates were significantly associated with pVSD in the child (Table 2).

Table 1 | Periconceptional parental conditions of children with a perimembranous ventricular septal defect and healthy control children

Variables	Cases n=115	Controls n=484	*P* value
Mothers			
Age at birth of index child in years (range)[a]	32 (19-46)	30 (18-45)	0.028
Educational level[b]			0.423
Low	28 (24)	106 (22)	
Intermediate	49 (43)	239 (49)	
High	38 (33)	139 (29)	
Ethnicity[b]			0.477
Dutch natives	92 (81)	378 (78)	
European others	8 (7)	29 (6)	
Non-European	13 (12)	77 (16)	
Parity[b]			0.257
Primipara	48 (42)	241 (50)	
Multipara	67 (58)	242 (50)	
Pregnancy[b]			
Spontaneous pregnancy	109 (95)	460 (95)	0.941
Previous abortion	24 (21)	126 (26)	0.251
BMI (kg/m^2) median (range)[c]	24 (17-41)	24 (16-52)	0.796
Diabetes mellitus[d]	2 (2)	5 (1)	0.624
Blood pressure[a]			
Systolic median (range)	118 (95-145)	115 (80-155)	0.350
Diastolic median (range)	74 (55-94)	74 (50-104)	0.987
Positive family history of CHD[b]	7 (6)	13 (3)	0.068
Periconception			
Medication[b]	38 (33)	101 (21)	0.005
Folate antagonist[b]	8 (7)	16 (3)	0.015
Alcohol[b]	46 (41)	154 (32)	0.092
Smoking[b]	17 (15)	101 (21)	0.146
Adequate folic acid supplement intake (yes)[b]	15 (13)	65 (13)	0.940
Fathers			
Age at birth of index child in years (range)[a]	34 (23-47)	34 (20-56)	0.793
Positive family history of CHD[b]	6 (5)	16 (3)	0.322
Educational level[b]			0.231
Low	27 (24)	128 (27)	
Intermediate	40 (35)	196 (41)	
High	47 (41)	158 (33)	

Table 1 | *(Continued)*

Variables	Cases n=115	Controls n=484	*P* value
Ethnicity[b]			0.484
Dutch natives	96 (84)	381 (79)	
European others	6 (5)	33 (7)	
Non-European	12 (11)	67 (14)	
Periconception			
Alcohol[b]	87 (77)	373 (78)	0.899
Smoking[b]	36 (32)	178 (37)	0.305

CHD, congenital heart disease. Values are n (%) unless stated otherwise. [a] Student's t-test [b] Chi-square test [c] Mann-Whitney *U* test [d] Fisher Exact test.

Table 2 | Periconceptional parental conditions and associations with perimembranous ventricular septal defects in the offspring

Variable	Unadjusted odds ratio (95% CI)	*P* value
Maternal		
Age (years)	1.05 (1.01-1.10)	0.028
Parity		
Nulliparous	reference group	
Multiparous	1.39 (0.92-2.10)	0.117
Educational level		
Intermediate	reference group	
Low	1.29 (0.77-2.16)	0.337
High	1.33 (0.83-2.14)	0.233
BMI (kg/m^2)	0.99 (0.95-1.04)	0.796
Diabetes mellitus	1.70 (0.32-8.85)	0.531
Ethnicity		0.530
Dutch native	reference group	
European other	1.13 (0.50-2.56)	0.763
Non-European	0.69 (0.37-1.30)	0.255
Family history of CHD	2.35 (0.92-6.03)	0.076
Systolic blood pressure (mmHg)	1.01 (0.99-1.03)	0.350
Diastolic blood pressure (mmHg)	1.00 (0.98-1.03)	0.987
Lifestyle; preconception use of		
Folic acid supplement	0.97 (0.54-1.75)	0.924
Alcohol	1.44 (0.94-2.19)	0.093
Smoking	0.66 (0.38-1.16)	0.148
Medication use	1.87 (1.20-2.92)	0.006

Table 2 | *(Continued)*

Variable	Unadjusted odds ratio (95% CI)	*P* value
Exposure to		
Phthalates	1.06 (0.22-5.07)	0.941
Alkylphenolic compounds	0.34 (0.08-1.47)	0.149
Paternal		
Age (years)	1.01 (0.97-1.05)	0.793
Educational level		
Low	reference group	
Intermediate	0.97 (0.57-1.66)	0.904
High	1.41 (0.83-2.39)	0.202
Ethnicity		
Dutch natives	reference group	
European others	0.72 (0.29-1.78)	0.476
Non-European	0.71 (0.37-1.67)	0.306
Positive family history of CHD	1.62 (0.62-4.23)	0.327
Smoking	1.26 (0.81-1.95)	0.306
Alcohol	1.03 (0.63-1.68)	0.899
Exposure to		
Pesticides	1.85 (0.79-4.34)	0.157
Phthalates	1.96 (1.09-3.50)	0.024
Alkylphenolic compounds	2.08 (1.01-4.26)	0.046
Heavy metals	4.28 (0.27-68.94)	0.305
Polychlorinated compounds	1.37 (0.67-2.79)	0.383

No mothers were exposed to pesticides, heavy metals or polychlorinated compounds.

The results of the multivariable analysis are shown in Table 3. Maternal family history of CHD (OR 2.61; 95% CI 0.98-6.91; *P*=0.054), medication use (OR 1.80; 95% CI 1.13-2.85; *P*=0.013) and age (OR 1.07; 95% CI 1.02-1.12; *P*=0.007), and paternal phthalates exposure (OR 1.93; 95% CI 1.05-3.54; *P*=0.034) were associated with the risk of pVSD offspring. Figure 1 shows the identified parental risk profile by the receiver operating characteristic curve obtained from the final multivariable logistic regression model (AUC=62.7%; 95% CI 57.0-68.5; *P*=0.000). The Nagelkerke R^2 was 0.057.

The same multivariable analysis for isolated pVSDs only, showed comparable results and included maternal family history of CHD (OR 3.14; 95% CI 1.13-8.73; *P*=0.028), medication use (OR 1.76; 95% CI 1.06-2.93; *P*=0.030), age (OR 1.06; 95% CI 1.01-1.12; *P*=0.016), and paternal phthalates exposure (OR 2.24; 95% CI 1.20-4.29; *P*=0.015). The accuracy of the model was comparable (AUC=64.1%; 95% CI 57.7-70.5; *P*=0.000).

Table 3 | Multivariable logistic regression results

	β	S.E.	Sig.	Odds ratio	95% CI
Maternal					
Positive family history of CHD	0.958	0.497	0.054	2.61	0.98-6.91
Medication use	0.586	0.235	0.013	1.80	1.13-2.85
Age	0.063	0.023	0.007	1.07	1.02-1.12
Paternal					
Phthalates	0.657	0.310	0.034	1.93	1.05-3.54

Hosmer-Lemeshow goodness-of-fit χ^2 2.209; *P* 0.974, Nagelkerke R^2 0.057

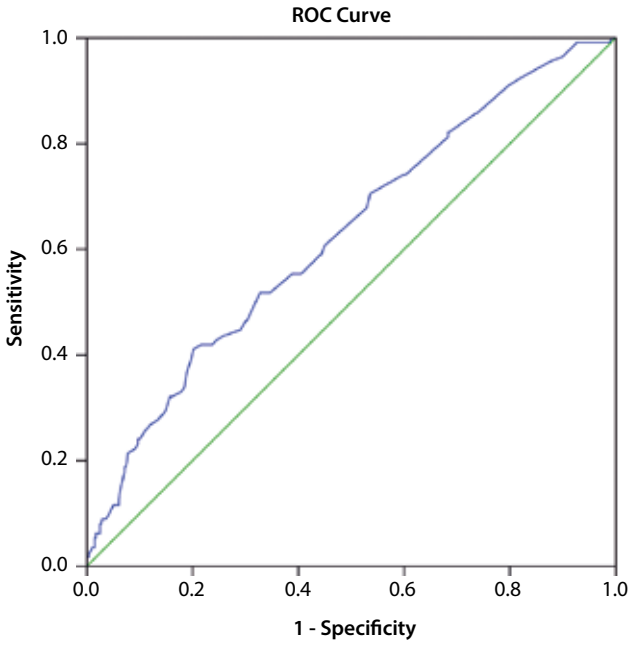

Figure 1 | Receiver operating characteristic curve of the model for perimembranous ventricular septal defects, calculated with multivariable analysis. The area under the curve for this model was 62.7% (95% CI 57.0-68.5)

Discussion

This study shows that maternal positive family history of CHD, medication use and age, and paternal exposure to phthalates contribute to the risk of pVSD and isolated pVSD offspring only. These risk factors – except a positive family history – have been associated with derangements of one-carbon metabolism which is an underlying mechanism of CHD. Advanced maternal age can alter one-carbon metabolism and lead to moderate to severe hyperhomocysteinaemia by reducing the bioavailability of substrates, cofactors and intermediates. Hyperhomocysteinaemia influences

oxidative, vascular, apoptotic, inflammatory and methylation pathways, and protein, lipid and DNA synthesis. Generic medication use can antagonise enzymes in folic acid metabolism, or influence folic acid absorption or degradation and inhibit the folic acid methylation cycle. Paternal phthalates exposure is epigenetically toxic and can cause epigenetic modifications.

The main strengths of our study are the homogeneity of the pVSD phenotype and the standardised data collection in cases and controls at the fixed moment of 17 months after birth, which equates to approximately two years after the periconception period. Other studies have also observed associations but mostly of a heterogeneous group of CHD phenotypes often due to the limited case numbers, which makes it difficult to observe true associations. Furthermore, the risk conditions are studied in a defined exposure window for this phenotype. This is helpful to reduce recall bias and seasonal influences. This strict approach also minimises the chance of misclassification of cases and controls because most malformations are diagnosed in the first year of life.

Some methodological issues have to be considered. We were not able to estimate maternal diabetes mellitus as risk factor because this health condition was rare in our study group (n=7). Rare risk factors are hard to study in a case-control design. Medication data included both prescribed and over-the-counter medication. Medications most frequently used were amoxicillin, carbamazepine, clarithromycin, clomifene, fluoxetine, fluticasone, ibuprofen, levothyroxine, meclozine, methyldopa, paracetamol, paroxetine and valproic acid. These medication data could not be verified in pharmacy records. Therefore, we consider medication use as measure of health more than a direct effect of the medication itself. In case mothers 33% and in control mothers 21% used any medication (*P*=0.005), of which the latter proportion was lower than the 37% reported in the general population in the Netherlands in 2004 (68). Therefore, possible recall bias and type II errors cannot be excluded which makes caution in place especially due to the case-control design of this study.

The explained variance of the four risk conditions for pVSD is 5.7% from which we conclude that based on our model of parental conditions not all risk conditions are identified. However, so far only 15% of pVSD can be attributed to a known maternal or paternal cause. Therefore, a conclusive AUC is not feasible at this moment. The AUC could be improved by including genetic risk conditions and diseases such as maternal diabetes mellitus, phenylketonuria and rubella. Unfortunately, these conditions are relatively rare and therefore hard to study. However, to explain the aetiology of the remaining 85% of pVSD cases it is imperative that more research on pVSD has to be performed because it is the most frequent birth defect with lifelong health consequences. Nevertheless, our model is useful for preconception counselling of parents-to-be. Risk identification makes it possible to inform couples about the risks of modifiable conditions − in particular lifestyles − and to offer possibilities for modification.

Results from logistic regression analyses show that the risk of pVSD increases significantly with advanced maternal age. There has been little consistency in studies concerning maternal age and the risk of having a child with CHD and pVSD, and increased risk has been described for both advanced and younger age (64,69,70). Because maternal age at the time of first birth has increased with almost four years during the last decades, it is important to inform future parents about their higher risk of having a child with pVSD (71).

Positive family history of CHD revealed the strongest maternal risk factor for pVSD offspring. We defined a positive family history as a relative in the third degree or closer with one of our eight included CHD phenotypes. The relevance of this choice has been described by others where they

found that within different degrees of relatives the odds ratios ranged from 1.18 (95% CI 1.05-1.32) for a third-degree relative up to 9.25 (95% CI 7.63-11.2) for twins of the same sex (72). Although this risk condition is supportive for a genetic contribution, it should be emphasised that familial risk is not only determined by genetic factors but also by the accumulation of nongenetic risk conditions through a shared environment. To minimise possible confounding of CHD clustering in families due to single-gene or chromosomal defects, we repeated the analysis for isolated pVSD without any other structural congenital anomaly or chromosomal defect. This analysis showed comparable results suggesting that nongenetic conditions might indeed contribute to the risk of pVSD offspring. The association between pVSD and paternal exposure to phthalates was described earlier by our group in the total group of cardiac outflow defects (65). Occupations probably exposed to phthalates and therefore at risk are plastic workers, painters, electricians, hairdressers and printers. Electricians should avoid exposure to phthalates from fumes when soldering and hairdressers should avoid exposure to cosmetic products such as hair sprays with phthalates. Unfortunately, other studies on paternal occupational exposures and pVSD are not available.

We conclude that preconception counselling should address the increased risks with advanced maternal age and paternal phthalates exposures. Women should not unnecessarily delay pregnancy. Maternal prescription and over-the-counter medication should be evaluated by a health care giver and the option to quit should be considered. Also CHD in the family should be evaluated thoroughly and provides, if positive, opportunities for early detection. The implementation of the findings of this study in preconception care can contribute to the future prevention of pVSDs.

Supplementary material

Table S1 | Specification folate antagonists

Antibacterials	trimethoprim, sulfonamides
Antidepressants	fluoxetine, fluvoxamine, paroxetine, citalopram, sertraline, imipramine, desipramine, amitriptyline, nortriptyline, clomipramine, bupropion, trazodone, mirtazapine, maprotiline, reboxetine, venlafaxine
Antiepileptics	clobazam, clonazepam, ethosuximide, phenobarbital, primidone, gabapentin, pregabalin, lacosamide, lamotrigine, topiramate, vigabatrin, carbamazepine, phenytoin, levetiracetam, oxcarbazepine, valproic acid
Antimalarials	atovaquone/ proguanil, proguanil, pyrimethamine
Antivirals for HIV infections	zidovudine, abacavir, lamivudine, didanosine, zalcitabine, stavudine, efavirenz, nevirapine, saquinavir, nelfinavir, indinavir, ritonavir, amprenavir
Chemotherapy	methotrexate, pemetrexed, raltitrexed, pralatrexate
Drugs for obstructive airway disease	salbutamol, salmeterol, terbutaline, indacaterol, formoterol, ephedrine, betamethasone, cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone, fludrocortisone
Lipid modifying agents	colestyramine

Chapter 4

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Genome-wide methylation analysis identifies novel CpG loci for perimembranous ventricular septal defects in human

Abstract

Background Congenital heart diseases (CHDs) are the most common birth defects worldwide and leading cause of infant mortality in the Western world. The perimembranous ventricular septal defect (pVSD) is the most prevalent CHD, however its pathogenesis is largely unknown. Epigenetics may provide an underlying mechanism of the gene-environment interactions involved explaining tissue-specific derangements in embryogenesis.

Methods We performed an epigenome-wide association study in peripheral white blood cells of young children to identify novel 5'-cytosine-phosphoguanosine (CpG) loci in association with pVSD. We investigated 455,741 CpG loci in 84 case children with pVSD and 196 healthy control children at 17 months of age using the Illumina Infinium® HumanMethylation450 BeadChip (450k array).

Results Using Bonferroni correction, we identified one CpG locus on chromosome 1 within the gene body of *PRDM16* (PR domain containing 16) that was higher methylated in pVSD children (+4.8% in cases, *P*=9.17E-08). This finding was validated by bisulphite pyrosequencing (+4.5% in cases, *P*=0.007). At 15% FDR (false discovery rate), we identified seven additional CpG loci mapped to eight genes on five chromosomes. Absolute methylation differences were observed in CpG loci mapped to *ESAM* (-2.2% in cases, *P*=8.09E-07), *PPFIA4* (+2.4% in cases, P=8.46E-07), *NFIA* (-0.8% in cases, *P*=9.13E-07), *HNRNPU* (-0.7% in cases, *P*=1.87E-06), *HIF3A* (-2.8% in cases, *P*=2.08E-06), *CDK4* (-0.4% in cases, *P*=2.11E-06) and *MOAP1* and *C14orf109* (-0.4% in cases, *P*=2.42E-06).

Conclusions We demonstrate an association between one higher methylated CpG locus within *PRDM16* and increased risk of pVSD. *PRDM16* functions as a repressor of *TGFβ* signaling controlling tissue morphogenesis crucial during cardiogenesis. Moreover, differential methylation of CpG loci mapped to eight genes involved in cardiogenesis, embryogenesis or related to severe congenital heart defects was shown. These findings provide novel insights in understanding the pathogenesis of pVSD which is of interest for future prediction and prevention.

Introduction

Worldwide approximately 1 million children are born each year with a congenital heart disease (CHD), which is not only the most prevalent birth defect but also the leading cause of infant mortality in the Western world (1). The most prevalent CHD phenotype is the ventricular septal defect (VSD) accounting for approximately 40% of CHD (6). The perimembranous type of VSD (pVSD) accounts for 80% of all VSDs (7). pVSD originates from the endocardial outflow tract cushions failing to fuse with the atrioventricular cushion mass on top of the muscular interventricular septum.

The pathogenesis of pVSD is complex and largely unknown. Mendelian transmission of CHDs in families is described, however, genetic research including genome-wide association studies revealed only a small number of genes implicated in pVSD pathogenesis (73). Also chromosomal anomalies – in which Down syndrome is most common – or teratogens contribute little to pVSD risk. In 80% of cases there is no identifiable cause for pVSD and it is assumed that they are the result of a combination of subtle genetic variations and environmental exposures (74,75).

Poor periconceptional maternal nutrition, characterised by low intakes of food groups rich in folate and other B-vitamins, antioxidants and choline significantly contribute to CHD risk in the offspring (16,76-79). Folate, but also vitamin B12, vitamin B6, methionine and choline, are important in one-carbon metabolism (1-C) providing methyl moieties for the synthesis of proteins and DNA, and methylation of DNA (14). Evidence for derangements in epigenetic programming by DNA methylation as an underlying mechanism for birth defects – including CHDs – is accumulating since derangements particular in 1-C metabolism result in disturbed embryogenesis (14). Maternal global hypomethylation has been associated with an increased risk of CHD (80). In a previous study we demonstrated that complex CHD in children is associated with higher concentrations of the methylation biomarkers S-adenosylmethionine, S-adenosylhomocysteine and folate in blood (81).

Since DNA methylation is important for human embryogenesis, we hypothesise that tissue specific derangements in DNA methylation contribute to pVSD. So far, a small number of CpG loci have been associated with pVSD. The introduction of the Illumina Infinium® HumanMethylation450 BeadChip (450k array) (Illumina, Inc) enables the simple and rapid measurement of 482,421 CpG loci and 3,091 CpH loci in the human genome across large numbers of samples (82). We performed an epigenome-wide association study (EWAS) in DNA derived from peripheral white blood cells (PWBC) – derived from the same mesodermal layer as the heart – of very young children to identify novel loci associated with pVSD.

Methods

Study population

Sampling of the participants for this case-control study has been previously described in detail (35). In summary, participants were non-related case and control children born between January 2002 and June 2007 in the Western part of the Netherlands. All case children with pVSDs were diagnosed by two paediatric cardiologists using echocardiography and/or cardiac catheterisation and/or

surgery data. For the present study only isolated pVSD were included to eliminate any chromosomal or syndromal effect. Control children had no major congenital malformations or chromosomal abnormalities according to the medical records and regular health checks by physicians of the public child health centres. During a standardised hospital visit around 17 months of age, venous blood samples were drawn from the children and the questionnaires filled out by the mother at home were checked for completeness and consistency. The study was approved by the Central Committee for Human Research (CCMO) in The Hague, The Netherlands, and by the Institutional Review Boards (Medical Ethics Committees) of the Erasmus MC, University Medical Centre in Rotterdam, Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam, the Netherlands. All parents gave written informed consent on behalf of their participating child.

DNA isolation and methylation protocol

Genomic DNA was isolated from 0.2 mL EDTA-whole blood using the Total Nucleic Acid Extraction kit on a MagNA PureLC (Roche Molecular Biochemicals). Isolated genomic DNA (500 ng per sample) was sodium bisulphite treated using the EZ-96 DNA methylation kit (Shallow) according to Illumina's protocol (Zymo Research). The Illumina Infinium® HumanMethylation450 BeadChips were processed according to the manufacturer's protocol.

Preprocessing of data

A quality control protocol was conducted in Illumina GenomeStudio software using the methylation module. It included a sample call rate check, colour balance check and control dashboard check. DNA methylation levels of probes on the sex chromosomes were used to match the gender of the child in order to identify sample mix-ups. The *dasen* procedure, which combines background adjustment and between-array normalisation applied to Type I and Type II probes separately, was used to normalise the methylation data (Bioconductor package *wateRmelon*) (83). Probes with detection *P* values >0.01 in more than 5% of the samples (n=1,721), probes with single-nucleotide polymorphisms (MAF>0.05, European population, 1000 Genomes Project) (n=17,196), and probes on the sex chromosomes (n=11,648) were excluded from analysis.

Statistical analysis

Methylation β-values were converted to M-values using: M-value=$\log_2$ (β-value/(1-β-value)) (84). To test for the association between disease status and CpG methylation, a multiple linear regression model was fitted using the methylation M-value as the outcome and disease status as the independent variable, adjusting for gender and age of the child and maternal smoking status and the potential confounders bisulphite plate and blood cell mixtures (R package 'CpGassoc') (85). The proportions of leukocyte subtypes were estimated using Houseman's method with the model trained on the data set of purified leukocytes from Reinius et al. (86,87). Top 500 cell type-informative CpGs were used for estimation. We then included the proportions of the differential subtypes as covariates in the regression model.

Bonferroni correction for 455,741 tests was used to adjust the level of significance and associations were considered epigenome-wide significant where *P* values were less than 1.1e-7. Benjamini-Hochberg false discovery rate (FDR) control procedure was also applied as a less stringent method for selecting top candidates. One-sided Kolmogorov-Smirnov (KS) test was used to test for enrichment of differential CpGs in a particular type of region based on the distribution of regional *P* values in comparison to the overall *P* value distribution. To reduce the effect of correlation between CpGs, we only sampled one CpG from a sub-region based on the annotation file provided by Illumina (HumanMethylation450 v1.2 Manifest File).

Gene Ontology (GO) term enrichment analysis was performed using Elim-KS method as implemented in the Bioconductor package TopGO (88). The minimum *P* value of the CpGs from a given gene is considered as the score for that gene for enrichment analysis. We focused on GO terms of biological processes. GO terms with less than 10 genes were excluded for testing. Benjamini-Hochberg FDR control procedure was used to select significant GO terms.

Mediation analysis was conducted to test the hypothesis that the effects of the prenatal conditions and exposures (E) maternal age at conception of the index-pregnancy, gestational age and birth weight (adjusted for gestational age) on disease outcome are mediated by methylation (M) adjusting for covariates (**X**) including the gender and age of the child, maternal smoking, bisulphite plate membership and leukocyte proportions. Mediation analysis based on structural equation modelling (SEM) was carried out using the R package 'lavaan'. Specifically, two models were fitted (1) $\text{logit}[\Pr(\text{outcome})] = \alpha 0 + \alpha 1\, E + \alpha 2\, M + \alpha 3' \mathbf{X}$ and (2) $M = \gamma 0 + \gamma 1 E + \gamma 2' \mathbf{X} + \varepsilon$. Then $\alpha 2\, \gamma 1$ measures the mediation (indirect) effect of prenatal exposures through methylation and $\alpha 2\, \gamma 1 + \alpha 1$ measures the total effect. A Wald test was performed to test for the direct effect and the indirect mediation effect of methylation. We constructed M_{PC}, the first principle component (PC) of the methylation of the top CpGs adjusted for covariate effects (**X**), to represent the overall methylation variation due to sources other than the covariates. Mediation analysis was then carried out on M_{PC}. By using M_{PC}, we can pool individually weak signals as well as reduce multiple testing burden.

To place Bonferroni significant CpGs and corresponding genes in the context of their molecular and functional interactions we used Ingenuity Pathway Analysis (IPA) tools (Ingenuity Systems®, www.ingenuity.com). Only downstream targets of the genes were used in the visualisation of networks. Statistical analyses were performed in the R environment for statistical computing (R 3.0.1).

Bisulphite pyrosequencing validation

The top three candidate CpG loci identified by the 450k array were validated in the same samples (cases n=31, controls n=61) using bisulphite pyrosequencing. After bisulphite conversion (250 ng per sample) using the EZ-96 DNA Methylation kit (Deep) (Zymo Research), the regions of interest including the exact same single CpGs were PCR amplified using the PyroMark PCR kit. Bisulphite-specific forward, reverse and sequence primers were designed using PyroMark Assay Design 2.0 software (Supplementary Table S1). Human methylated genomic control DNA (EpigenDx) was used to validate the assay with samples methylated respectively 0%, 25%, 50%, 75% and 100%. Pyrosequencing was performed using the PyroMark Q24 platform (Qiagen). PCR and subsequent steps were performed in duplicate. Mean DNA methylation of the two duplicate measurements was used to compare cases and controls using the Student's t-test.

Results

Characteristics of the children

From a total of 296 samples we excluded 4 technical samples with 50% methylated DNA and 6 duplicate samples. Furthermore, 3 samples were excluded because they failed the quality control protocol and 3 samples due to mismatch of the gender which indicates sample mix-ups. In total, 84 case children and 196 control children were included for analyses. Characteristics of the children and their mothers are shown in Table 1.

Table 1 | Characteristics of children and mothers

	Cases (n=84)	Controls (n=196)	*P* value
Child			
Age at study moment, months	17.0	16.4	0.22
Female gender	46 (55)	108 (55)	1.00
Mother			
Age at conception, years	31.4	30.3	0.11
Smoking	16 (19)	36 (18)	0.87
Periconceptional folic acid use	46 (55)	109 (56)	0.90
Body mass index, kg/m^2	25.0	24.7	0.70

Values are means or number (percentage).

Epigenome-wide association analysis

Leukocyte proportions predicted by Houseman's method showed differentiation of CD8+ T cells and natural killer (NK) cells between case and control samples, hence the proportions of CD8+ T and NK cells were adjusted for confounding due to cell mixtures (Supplementary Table S2). We then tested for association between methylation and disease outcome using linear regression adjusting for relevant covariates. The test statistic inflation was well controlled with a genomic control inflation factor of 1.02, indicating that potential confounding due to cell mixtures was effectively controlled (Figure 1).

Using conservative Bonferroni correction for 455,741 tests, we observed one epigenome-wide significant CpG locus on chromosome 1 (Figure 2*)*. This CpG (cg17001566 / chr1:2990490) is within the gene body of *PRDM16* (PR domain containing 16) and higher methylated in pVSD children (+4.8% in cases, *P* = 9.17E-08). Using the less stringent Benjamini-Hochberg procedure for controlling FDR at 15%, we also identified seven epigenome-wide significant CpG loci mapped to eight genes on five chromosomes (Table 2, Supplementary Figure S1). Differential methylation was most pronounced in CpG islands, TSS 200, 5′ UTR and 1st exon regions based on the KS test (Supplementary Table S3).

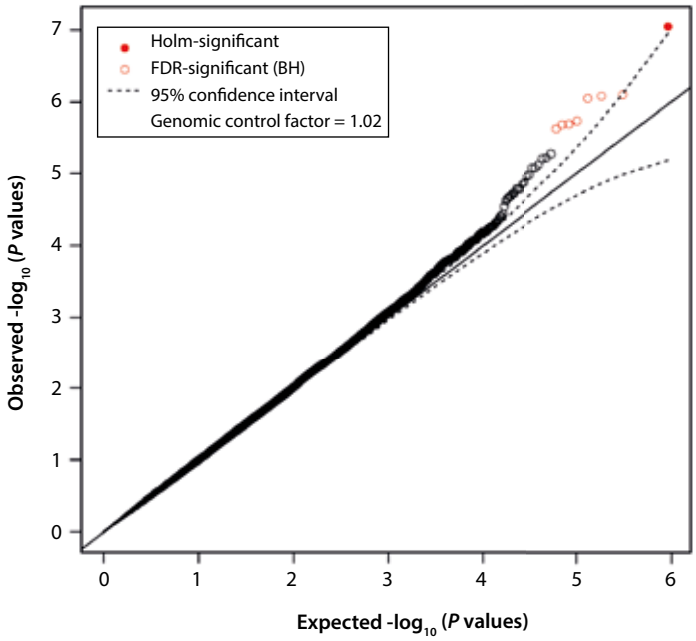


Figure 1 | Quantile-quantile plot of linear regression test for case-control status

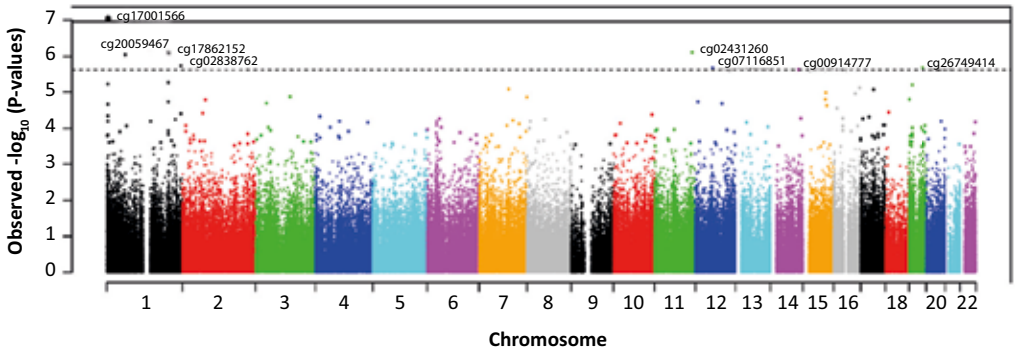


Figure 2 | Manhattan plot of epigenome-wide associations with perimembranous ventricular septal defects

Table 2 | Summary of the CpG loci associated with pVSD

Probe ID	Chromo-some	Mapinfo (bp)*	UCSC RefGene name	UCSC RefGene group	Relation to UCSC CpG island	Control methylation	Case methylation	Mean absolute difference	Mean relative difference	*P* value	FDR-adjusted *P* value
cg17001566	1	2990490	*PRDM16*	Body	Island	32.68	37.52	+4.84	+14.81	9.17E-08	0.04
cg02431260	11	124628888	*ESAM*	Body	Island	43.21	41.01	-2.20	-5.09	8.09E-07	0.10
cg17862152	1	203044930	*PPFIA4*	Body	Island	6.40	8.78	+2.38	+37.19	8.46E-07	0.10
cg20059467	1	61548115	*NFIA*	Body/5'UTR/1st Exon	N Shore	13.46	12.69	-0.77	-5.72	9.13E-07	0.10
cg02838762	1	245026729	*HNRNPU*	Body	Island	12.84	12.17	-0.67	-5.22	1.87E-06	0.14
cg26749414	19	46807272	*HIF3A*	5'UTR/Body	Island	86.39	83.60	-2.79	-3.23	2.08E-06	0.14
cg07116851	12	58146173	*CDK4*	TSS200	Island	9.73	9.29	-0.44	-4.52	2.11E-06	0.14
cg00914777	14	93651123	*MOAP1* *C14orf109*	5'UTR TSS1500/TSS200	Island	8.20	7.83	-0.37	-4.51	2.42E-06	0.14

*Mapinfo refers to Genome Research Consortium human genome build 37 (GRCh37)/UCSC human genome 19 (hg19). The coordinates correspond to the cytosine on the forward strand, regardless of whether the probe was designed on the forward or reverse strand. Bonferroni significance: *P*=1.1e-7

Functional analysis

GO term enrichment analysis is used for functional analysis of large-scale epigenome-wide data. In our data set, a total of 4599 GO terms in biological processes were scored. At 5% FDR, 70 GO terms were enriched and are listed in Supplementary Table S4. The top ten listed GO terms include axon guidance and the regulation of transcription from RNA polymerase II promoter, organ morphogenesis, embryonic development, apoptotic processes and cardiac muscle cell proliferation. The *PRDM16* gene with the Bonferroni significant CpG was selected to conduct a pathway analyses with IPA (Ingenuity Pathway Analysis). According to the gene network diagram, downstream related molecules included expression of *CDKN1A* (cyclin-dependent kinase inhibitor 1A) and *SerpinE1,* protein-protein interaction with *CTBP2* (C-terminal-binding protein 2) and *SKI* (Sloan-Kettering Institute proto-oncogene), and DNA-protein interaction with *HGF* (hepatocyte growth factor) (S2 Figure). *PRDM16* also indirectly modifies histone H3.

Mediation analysis

To gain insight in the role of DNA methylation in pVSD pathogenesis, we examined whether the effects of prenatal conditions on pVSD risk are mediated by changes in DNA methylation. Mediation analysis using the methylation first PC suggests that the diseases risks due to the prenatal conditions maternal age at conception, gestational age and birth weight (adjusted for gestational age) are mediated (indirect effects) by DNA methylation (Table 3). The methylation PC was formed based on the eight CpGs identified by the EWAS at 15% FDR to summarise the overall methylation variation and alleviate the multiple testing burden.

Table 3 | Mediation analysis on methylation principal components

Exposure	Effects	Estimate	Standard error	z score	*P* value
Maternal age at conception	indirect	0.067	0.045	1.50	0.134
	total	0.129	0.079	1.63	0.102
Duration of pregnancy (in days)	indirect	-0.093	0.043	-2.17	0.030
	total	-0.128	0.082	-1.55	0.120
Birthweight adjusted for GA	indirect	-0.081	0.042	-1.91	0.056
	total	-0.146	0.084	-1.73	0.083
Birthweight	indirect	-0.119	0.041	-2.87	0.004
	total	-0.191	0.083	-2.32	0.021

GA, gestational age.

Bisulphite pyrosequencing validation

Next, we used bisulphite pyrosequencing in the same samples to validate our top three candidate CpG loci (cases n=31, controls n=61). Within *PRDM16*, mean methylation in both the CpG of interest as well as two surrounding CpGs was significantly different between cases and controls (+3.6% in cases, *P*=0.04; +3.7% in cases, *P*=0.01; +3.4% in cases, *P*=0.04) (Table 4). Within *PPFIA4* and *ESAM*

the CpG of interest did not achieve significance (*P*=0.052 and *P*=0.156 respectively) but the trends were consistent with the 450k array. One CpG surrounding the CpG of interest within *PPFIA4* was significantly differentially methylated between cases and controls (+1.8% in cases, *P*=0.04).

Table 4 | Bisulphite pyrosequencing validation of CpG sites within *PRDM16, ESAM* and *PPFIA4*

Locus	Cases mean (SD)	Controls mean (SD)	*P* value
PRDM16			
#CpG 1	n=31	n=59	
	47.52 (8.81)	44.43 (7.85)	0.092
#CpG 2	n=31	n=59	
	33.68 (7.99)	30.11 (7.49)	0.039
#CpG 3[a]	n=31	n=59	
	18.91 (6.46)	15.24 (5.76)	0.007
#CpG 4	n=31	n=59	
	25.04 (7.75)	21.66 (7.16)	0.042
ESAM			
#CpG 1[a]	n=31	n=61	
	57.80 (3.63)	58.92 (3.49)	0.156
#CpG 2	n=31	n=61	
	10.75 (1.34)	10.66 (1.62)	0.799
#CpG 3	n=30	n=61	
	27.16 (2.66)	27.86 (2.90)	0.270
#CpG 4	n=31	n=61	
	9.11 (1.15)	9.19 (1.45)	0.795
PPFIA4			
#CpG 1[a]	n=31	n=61	
	2.56 (3.67)	1.22 (0.75)	0.053
#CpG 2	n=31	n=61	
	2.67 (4.16)	1.15 (0.81)	0.052
#CpG 3	n=31	n=60	
	2.43 (3.46)	1.21 (0.68)	0.060
#CpG 4	n=31	n=61	
	2.55 (3.09)	1.43 (0.61)	0.055
#CpG 5	n=31	n=61	
	4.67 (4.74)	2.83 (0.90)	0.040

[a] CpG of interest

Discussion

In this study we identified a novel CpG locus (cg17001566) that reaches Bonferroni significance in the *PRDM16* gene associated with pVSD. This CpG was validated by bisulphite pyrosequencing. Previous research on *PRDM16* focused on its well-known function in brown fat determination and adiposity, however, accumulating evidence indicates the novel function of *PRDM16* in cardiac development (89-92). Animal models and studies using human cardiac tissue have shown that disruptions in *PRDM16* function affect cardiac development. Arndt et al. showed in both fetal and adult human hearts the expression of *PRDM16* in cardiomyocytes and interstitial cells (93). Also in mice, *PRDM16* is expressed throughout the ventricular myocardium, including endocardium and epicardium, and in the ventricles of the mouse heart (94). In adult mice *PRDM16* was predominantly restricted to the nuclei of cardiomyocytes. A genome-wide association study has linked a *PRDM16* SNP to QRS duration implying a role in cardiac function (95). This gene has been previously described in relation to clefting disorders, heterochromatin integrity, cardiomyopathy and as a physiologic regulator of hematopoietic stem cells (HSC) (93-97).

PRDM16 functions as a repressor of *TGFβ* signaling (transforming growth-factor beta) which controls tissue morphogenesis and is crucial during embryonic development. *In vitro* experiments show that *TGFβ* signaling is important for the generation and remodeling of cushion mesenchymal cells of the outflow tract, which contributes to the ventricular septum (98). *TGFβ2* knock out mice show severe cardiac malformations including abnormal endocardial outflow tract cushions and pVSDs (99). Moreover, *PRDM16* is a protein that can bind regulating Smads, including *TGFβ*- and BMP (bone morphogenetic proteins)-regulated Smads, and inhibitory Smads (100). *TGFβ* and BMPs are important for cell proliferation, differentiation and apoptosis, and Smad proteins are involved in the mediation of these effects. Of interest is that mice with deficiencies in either Smad6 or Smad7 are associated with multiple cardiovascular defects, including VSDs (101).

PRDM16 downregulates SerpinE1 and the homologue of *PRDM16* (minus a PR domain) *EVI1* (ectropic virus integration site 1) downregulates SerpinB2 (102). *PRDM16* and *EVI1* have many similarities (103). In mice, at 12.5 days post-coitum when the ventricles are partially separated EVI1 is highly expressed in both the proximal (conal) and distal (truncal) outflow tract endocardial cushions and cardiac valves, whereas the surrounding endocardium, myocardium and blood vessels are negative (104).

Looking at downstream related molecules in the pathway analysis, *SKI* negatively regulates *TGFβ* and interacts with Smad2, Smad3 and Smad4. *SKI* zebrafish morphant embryos show severe cardiac anomalies with malformations in the outflow tract and therefore implicate the requirement for *SKI* in early cardiovascular development (105). *CTBP2,* which is downregulated by *PRDM16,* also interacts with *EVI1* (106).

Seven CpG loci mapped to eight genes are identified at a 15% FDR threshold but did not reach Bonferroni statistical significance. They might provide novel insights in pVSD pathophysiology given their biological functions. All genes except one are involved in embryogenesis, cardiogenesis or related to severe CHD (S5 Table).

Multiple GO terms in biological processes seem over-represented within our pVSD cases. The most enriched GO term is related to axon guidance. During heart development axon guidance cues are

critical for the migration of cardiac neural crest cells and generating the endocardial cushions (107). Recently, an abnormal axon guidance protein has been related in a case report to a patient with multiple congenital heart defects including a ventricular septal defect (108). Both positive and negative regulation of transcription from RNA polymerase II promoter seem highly over-represented GO terms in pVSD cases. RNA polymerase II is likely to be essential during embryonic development and gene transcription by RNA polymerase II is affected by DNA methylation (109,110). Also CpGs within genes involved in the regulation of organ morphogenesis, embryonic development, apoptotic processes and cardiac muscle cell proliferation seem to be over-represented in our pVSD cases.

Mediation effects of maternal periconceptional conditions are shown, which suggests a potential role for DNA methylation in the disease mechanism supporting a potential functional link between 1-C metabolism and pVSD risk.

We validated methylation differences in the three top candidate CpG loci identified by the 450k array using bisulphite pyrosequencing. This quantitative real-time sequencing technology allows the measurement of a specific single CpG. Due to financial reasons only a subset was analysed with a 1:2 case:control ratio. Even with a much smaller sample size the CpG locus within *PRDM16* could be validated. Unfortunately it was not possible to find an external replication cohort.

Some limitations of this study have to be addressed. Critical considerations on EWAS is in place, and some recommendations for a standardised EWAS methodology are provided by Michels et al. (111). Firstly, choosing the appropriate tissue is substantial given tissue-specific gene regulation, however, it is not possible to obtain cardiac tissue from especially healthy control children – a disadvantage of human studies. For that reason we used PWBC as easy to access surrogate tissue in young children. Ma et al. have shown that locus-specific methylation differences between tissues are highly consistent across individuals (112). Also, PWBC and cardiac tissue have a shared developmental origin since the lateral plate mesoderm in human embryos give rise to both the heart and blood cells in the circulatory system (113). Thus the PWBC may carry the methylation fingerprint of cardiac tissue. However, it remains to be elucidated whether DNA methylation derived from whole blood can serve as a biomarker for epigenetic regulation of cardiac tissue in clinical practice.

Secondly, the heterogeneity of PWBC as potential confounder in epigenetic studies has to be considered. Differences in PWBC methylation could result from varying cell-type proportions, but is also dependent on the pathways and genes being studied (86,113). Therefore we applied Houseman's method to correct for cell type proportions (114,115). The identified CpG loci in our study could not be explained by differential cell mixtures, since the methylation values are not significantly correlated with the predicted cell proportions. Moreover, the controlled genomic inflation factor (lambda=1.02) indicates that the analyses are adequately adjusted for cell mixtures.

Thirdly, the effect sizes of DNA methylation changes are small in our study. Previous research has shown that small changes in DNA methylation can result in large phenotypic changes (116). The assumption following this reasoning is that (small) changes in DNA methylation can result in aberrant RNA and protein expression. Comparable to genome-wide association studies is that one locus or gene can only explain a small fraction of the disease risk. The pathophysiology of pVSD is very likely a complex process of accumulation and interaction of risk factors and the effect size of

one locus is expectable small. Last of all, the effect size could be attenuated by using PWBC and not the tissue of interest.

In summary, we have identified at Bonferroni epigenome-wide significance level one CpG locus within *PRDM16* that shows differential methylation which is suggested to be associated with pVSD pathogenesis. In addition, seven additional CpG loci were identified at 15% Benjamini-Hochberg FDR. These findings provide novel insights in understanding the pathogenesis of pVSD in human. Epigenetics as underlying mechanism of pVSD in a complex interplay between genetic, environmental and lifestyle factors offers possibilities for future personalised prediction, treatment and prevention. Therefore, future research is warranted to reveal whether our epigenetic findings can be replicated in other studies.

Supplementary material

Table S1 | Primer design

Gene Name	Genomic location[1]	Number of CpG sites analysed	Primer sequence (5'-3')[2]
PRDM16	chr1:2990408-2990754	4 CpG sites	F: AGGAGGTGTTTTGTTTTTTAAGTGTA R: AATCCCCTCCTCCTAACCT S: GATGTAGTAGGGAATTTAGGTA
ESAM	chr11:124628803-124629011	4 CpG sites	F: GGGGAGTATAGTATTTATTGGGTTAGGTT R: CCACTTACTTCCCTAATTTTCCTAAA S: AATTAGAAGGTTTTTTAAATTTTT
PPFIA4	chr1:203044827-203044953	5 CpG sites	F: TTTGGGGTGTTAGGAAGAAG R: ATCCACCCTAAAAACCCTACA S: GGTGTTAGGAAGAAGT

[1] Genome Research Consortium human genome build 37 (GRCh37)/UCSC human genome 19 (hg19)
[2] Forward PCR primer, Reverse PCR primer and Sequencing primer designed using the PyroMark Assay Design 2.0 software. Reverse PCR primer: biotinylated primer.

Table S2 | Differential cell distribution

Cell type	Case	Control	*P* value
Neutrophil	0.269 (0.131)	0.272 (0.119)	0.848
CD4+ T	0.168 (0.081)	0.176 (0.067)	0.434
CD8+ T	0.241 (0.071)	0.259 (0.068)	0.043
B cell	0.150 (0.046)	0.141 (0.038)	0.114
NK cell	0.018 (0.030)	0.006 (0.017)	0.001
Eosinophil	0.030 (0.039)	0.023 (0.031)	0.202
Monocyte	0.094 (0.031)	0.088 (0.027)	0.157

Values are means (SD)

Table S3 | One-sided Kolmogorov-Smirnov test on enrichment of differential CpGs in a particular type of region

Region	*P* value
DHS	0.398
Enhancer	0.899
CDMR	0.553
DMR	0.020
RDMR	0.728
Island	4.55E-31
N Shelf	0.998
S Shelf	0.958
N Shore	0.919
S Shore	0.996
TSS1500	0.175
TSS200	3.89E-25
5'UTR	0.003
1st Exon	9.78E-09
Body	1.000
3'UTR	0.973

Table S4 | Significant GO enrichment results

GO.ID	Term	Annotated	elimKS *P* value	Adjusted *P* value
GO:0007411	axon guidance	340	1.20E-14	5.52E-11
GO:0000122	negative regulation of transcription from RNA polymerase II promoter	476	1.20E-11	2.76E-08
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	672	1.90E-08	2.91E-05
GO:2000027	regulation of organ morphogenesis	135	3.40E-06	0.004
GO:0046777	protein autophosphorylation	172	6.80E-06	0.006
GO:0045995	regulation of embryonic development	81	1.10E-05	0.007
GO:0007268	synaptic transmission	614	1.10E-05	0.007
GO:0001764	neuron migration	92	1.20E-05	0.007
GO:0043066	negative regulation of apoptotic process	538	1.40E-05	0.007
GO:0060045	positive regulation of cardiac muscle cell proliferation	19	3.60E-05	0.015
GO:0060021	palate development	68	3.70E-05	0.015
GO:0009952	anterior/posterior pattern specification	201	3.90E-05	0.015
GO:0003171	atrioventricular valve development	11	4.10E-05	0.015
GO:0045666	positive regulation of neuron differentiation	61	4.90E-05	0.016
GO:0007409	axonogenesis	488	5.80E-05	0.017
GO:0060443	mammary gland morphogenesis	50	6.10E-05	0.017
GO:0007420	brain development	471	6.40E-05	0.017
GO:0050770	regulation of axonogenesis	91	8.00E-05	0.020
GO:0030514	negative regulation of BMP signaling pathway	34	8.90E-05	0.022
GO:0003209	cardiac atrium morphogenesis	25	0.000	0.027
GO:0048706	embryonic skeletal system development	111	0.000	0.027
GO:0002076	osteoblast development	19	0.000	0.027
GO:0060572	morphogenesis of an epithelial bud	16	0.000	0.027
GO:0060348	bone development	95	0.000	0.027
GO:0060070	canonical Wnt receptor signaling pathway	174	0.000	0.027

Table S4 | *(Continued)*

GO.ID	Term	Annotated	elimKS *P* value	Adjusted *P* value
GO:0060412	ventricular septum morphogenesis	28	0.000	0.027
GO:0002064	epithelial cell development	80	0.000	0.027
GO:0045599	negative regulation of fat cell differentiation	28	0.000	0.027
GO:0003161	cardiac conduction system development	11	0.000	0.027
GO:0060379	cardiac muscle cell myoblast differentiation	11	0.000	0.027
GO:0003179	heart valve morphogenesis	23	0.000	0.027
GO:0001759	organ induction	24	0.000	0.029
GO:0045785	positive regulation of cell adhesion	118	0.000	0.031
GO:0021537	telencephalon development	148	0.000	0.034
GO:0042307	positive regulation of protein import into nucleus	59	0.000	0.034
GO:0048010	vascular endothelial growth factor receptor signaling pathway	43	0.000	0.035
GO:0055012	ventricular cardiac muscle cell differentiation	20	0.000	0.035
GO:0007179	transforming growth factor beta receptor signaling pathway	160	0.000	0.035
GO:0051899	membrane depolarization	78	0.000	0.036
GO:0050768	negative regulation of neurogenesis	88	0.000	0.036
GO:0008038	neuron recognition	28	0.000	0.036
GO:0002053	positive regulation of mesenchymal cell proliferation	37	0.000	0.036
GO:0001657	ureteric bud development	97	0.000	0.037
GO:0044087	regulation of cellular component biogenesis	328	0.000	0.038
GO:0061180	mammary gland epithelium development	68	0.000	0.038
GO:0030856	regulation of epithelial cell differentiation	54	0.000	0.038
GO:0007184	SMAD protein import into nucleus	18	0.000	0.038
GO:0061036	positive regulation of cartilage development	10	0.000	0.038
GO:0090263	positive regulation of canonical Wnt receptor signaling pathway	47	0.000	0.040
GO:0043408	regulation of MAPK cascade	428	0.000	0.042

Table S4 | *(Continued)*

GO.ID	Term	Annotated	elimKS *P* value	Adjusted *P* value
GO:0031998	regulation of fatty acid beta-oxidation	14	0.000	0.042
GO:0018107	peptidyl-threonine phosphorylation	50	0.000	0.042
GO:0002062	chondrocyte differentiation	68	0.001	0.043
GO:0051090	regulation of sequence-specific DNA binding transcription factor activity	311	0.001	0.044
GO:0016331	morphogenesis of embryonic epithelium	137	0.001	0.044
GO:0008543	fibroblast growth factor receptor signaling pathway	137	0.001	0.044
GO:0035556	intracellular signal transduction	1742	0.001	0.046
GO:0007163	establishment or maintenance of cell polarity	109	0.001	0.046
GO:1901213	regulation of transcription from RNA polymerase II promoter involved in heart development	13	0.001	0.046
GO:0048538	thymus development	32	0.001	0.047
GO:0007173	epidermal growth factor receptor signaling pathway	150	0.001	0.047
GO:0043507	positive regulation of JUN kinase activity	57	0.001	0.047
GO:0071300	cellular response to retinoic acid	49	0.001	0.047
GO:0008284	positive regulation of cell proliferation	605	0.001	0.047
GO:0060602	branch elongation of an epithelium	17	0.001	0.049
GO:0001934	positive regulation of protein phosphorylation	534	0.001	0.049
GO:0030512	negative regulation of transforming growth factor beta receptor signaling pathway	62	0.001	0.050
GO:0007044	cell-substrate junction assembly	55	0.001	0.050

Table S5 | Overview of the literature on the genes identified at a 15% false discovery rate threshold and the association with embryogenesis, cardiogenesis or phenotype

ESAM

- Expressed in mouse embryos in the outflow tract early in development and mediates cell-cell adhesion in endothelial cells.
- *Hirata K, Ishida T, Penta K, Rezaee M, Yang E, Wohlgemuth J, et al. Cloning of an immunoglobulin family adhesion molecule selectively expressed by endothelial cells. J Biol Chem. 2001;276(19):16223-31.*

PPFIA4

- Expressed in heart tissues and belongs to the liprin-alpha gene family.
- *Serra-Pages C, Medley QG, Tang M, Hart A, Streuli M. Liprins, a family of LAR transmembrane protein-tyrosine phosphatase-interacting proteins. J Biol Chem. 1998;273(25):15611-20.*

NFIA

- Regulates tissue-specific gene expression during mammalian embryogenesis.
- *Chaudhry AZ, Lyons GE, Gronostajski RM. Expression patterns of the four nuclear factor I genes during mouse embryogenesis indicate a potential role in development. Dev Dyn. 1997;208(3):313-25.*

HNRNPU

- Mediates the long-range regulation of *Shh* expression during limb development. It is proposed that distant cis-elements may modulate gene expression by altering the affinity of *HNRNPU* for certain mediator proteins and nuclear relocation. Children with Holt-Oram syndrome are affected with upper limb malformations and cardiac septation defects caused by a mutation within *HNRNP.*
- *Zhao J, Ding J, Li Y, Ren K, Sha J, Zhu M, et al. HnRNP U mediates the long-range regulation of Shh expression during limb development. Hum Mol Genet. 2009;18(16):3090-7.*
- *Basson CT, Bachinsky DR, Lin RC, Levi T, Elkins JA, Soults J, et al. Mutations in human TBX5 cause limb and cardiac malformation in Holt-Oram syndrome. Nat Genet. 1997;15(1):30-5.*

HIF3A

- The splicing variant of *HIF3A* is *NEPAS* which is pertinent to heart development during the embryonic stages. Recent studies including two EWAS have described *HIF3A* in relation to body-mass index and (childhood) obesity.
- *Yamashita T, Ohneda O, Nagano M, Iemitsu M, Makino Y, Tanaka H, et al. Abnormal heart development and lung remodeling in mice lacking the hypoxia-inducible factor-related basic helix-loop-helix PAS protein NEPAS. Mol Cell Biol. 2008;28(4):1285-97.*
- *Demerath EW, Guan W, Grove ML, Aslibekyan S, Mendelson M, Zhou YH, et al. Epigenome-wide association study (EWAS) of BMI, BMI change and waist circumference in African American adults identifies multiple replicated loci. Hum Mol Genet. 2015;24(15):4464-79.*
- *Dick KJ, Nelson CP, Tsaprouni L, Sandling JK, Aissi D, Wahl S, et al. DNA methylation and body-mass index: a genome-wide analysis. Lancet. 2014;383(9933):1990-8.*
- *Wang S, Song J, Yang Y, Zhang Y, Wang H, Ma J. HIF3A DNA Methylation Is Associated with Childhood Obesity and ALT. PLoS One. 2015;10(12):e0145944.*

CDK4

- Severe heart defects are reported in *CDK4* double knockout mice.
- *Berthet C, Klarmann KD, Hilton MB, Suh HC, Keller JR, Kiyokawa H, et al. Combined loss of Cdk2 and Cdk4 results in embryonic lethality and Rb hypophosphorylation. Dev Cell. 2006;10(5):563-73.*

MOAP1

- Induces apoptosis.
- *Tan KO, Tan KM, Chan SL, Yee KS, Bevort M, Ang KC, et al. MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains. J Biol Chem. 2001;276(4):2802-7.*

C14orf109

- Has to date not been described in relation to embryogenesis or phenotypes.

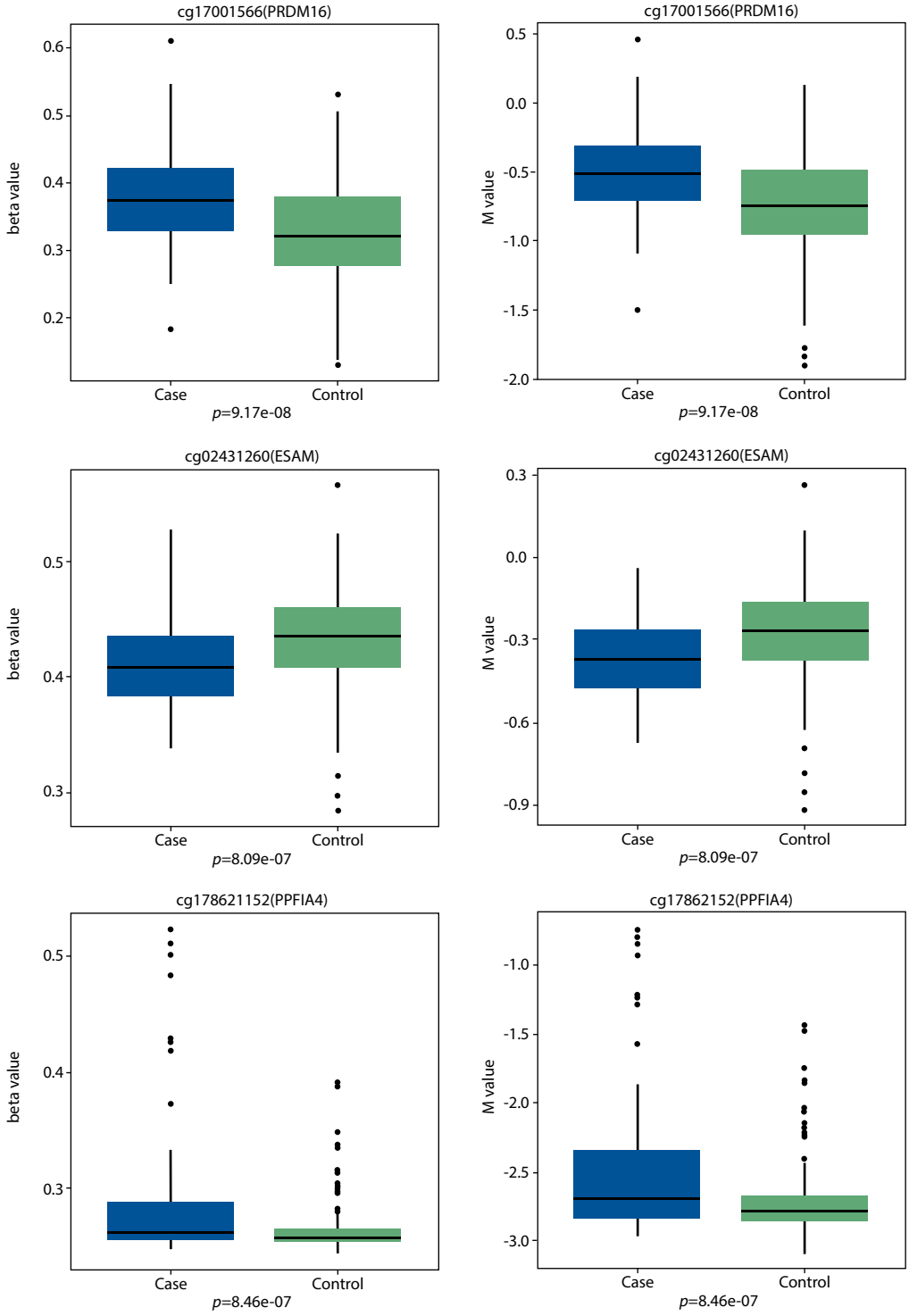
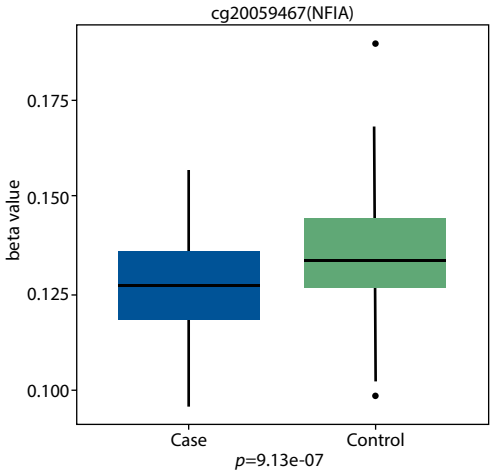


Figure S1 | Boxplots of significant CpGs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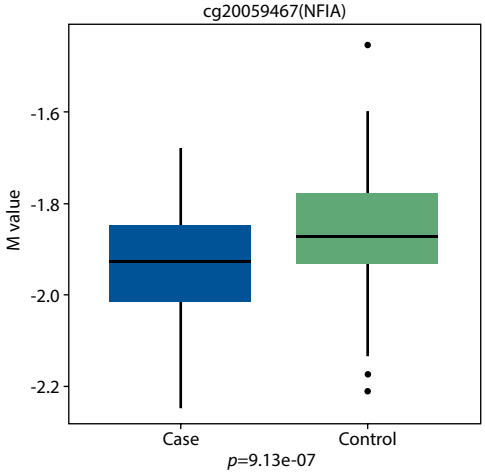
0.150
0.125
0.100
Case
Control
p=9.13e-07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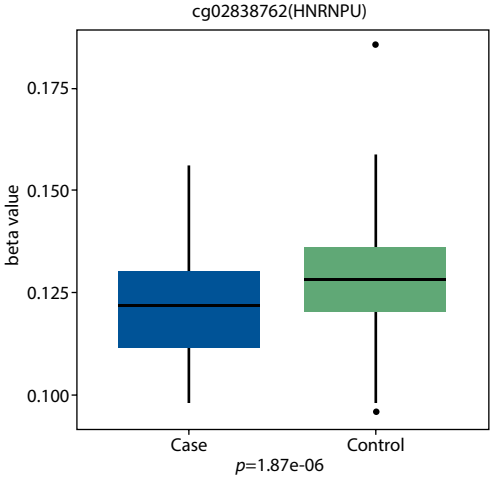
-2.0
-2.2
Case
Control
p=9.13e-07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beta value
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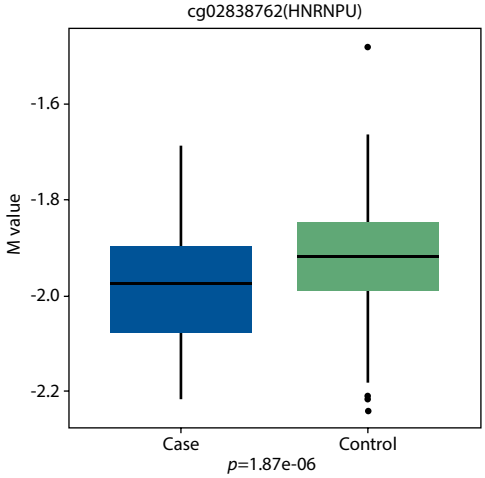
0.150
0.125
0.100
Case
Control
p=1.87e-06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M value
-1.6
-1.8
-2.0
-2.2
Case
Control
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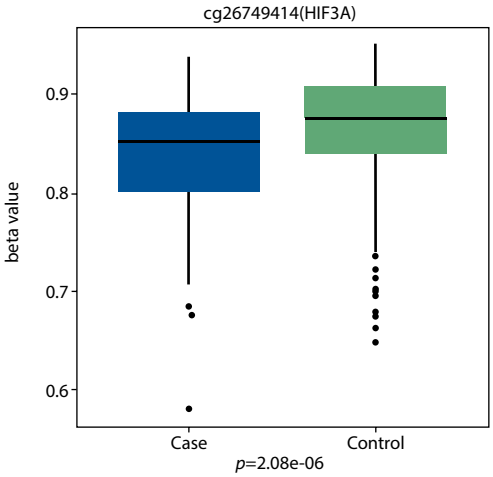
cg26749414(HIF3A)
beta value
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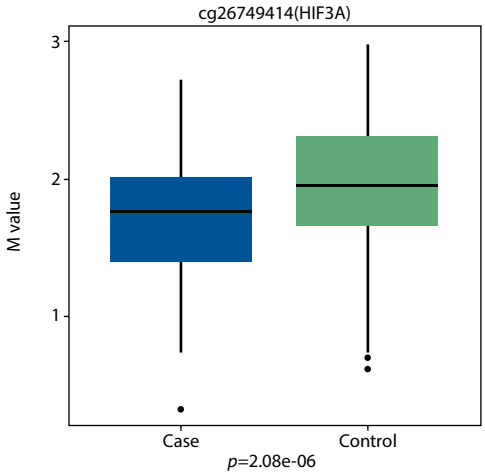
0.8
0.7
0.6
Case
Control
p=2.08e-06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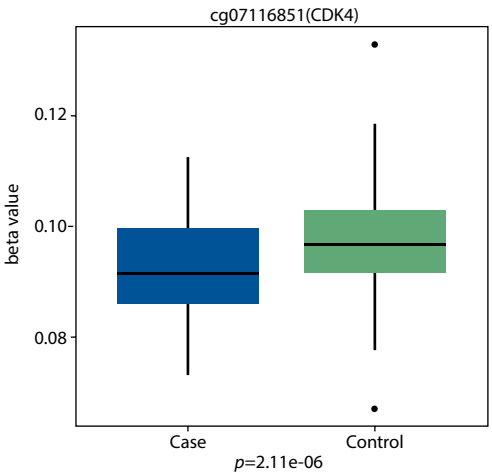
M value
3
2
1
Case
Control
p=2.08e-06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0.10
0.08
Case
Control
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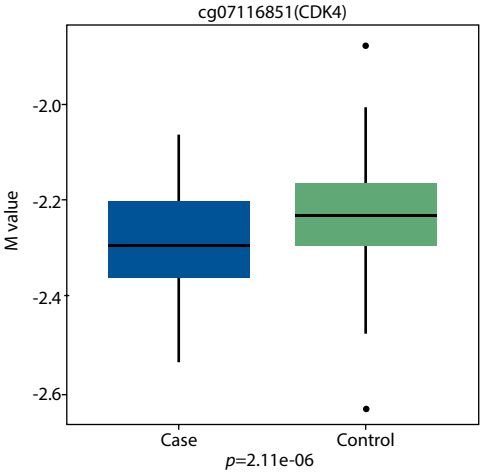
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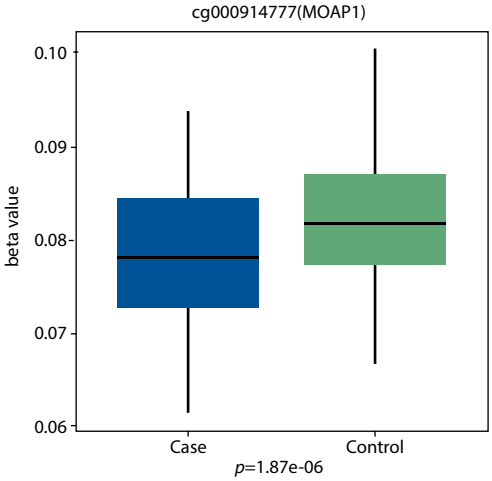
-2.2
-2.4
-2.6
Case
Control
p=2.11e-06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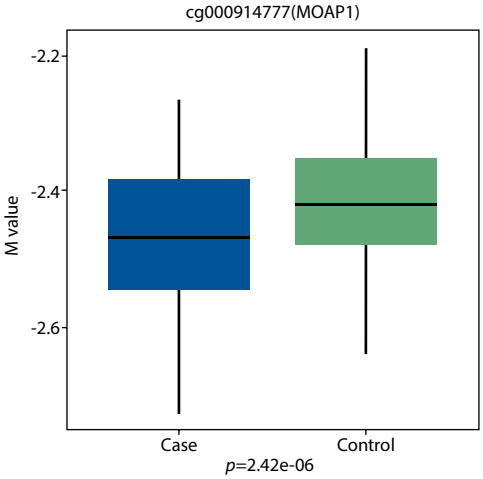
beta value
0.10
0.09
0.08
0.07
0.06
Case
Control
p=1.87e-06


cg000914777(MOAP1)
M value
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-2.4
-2.6
Case
Control
p=2.42e-06

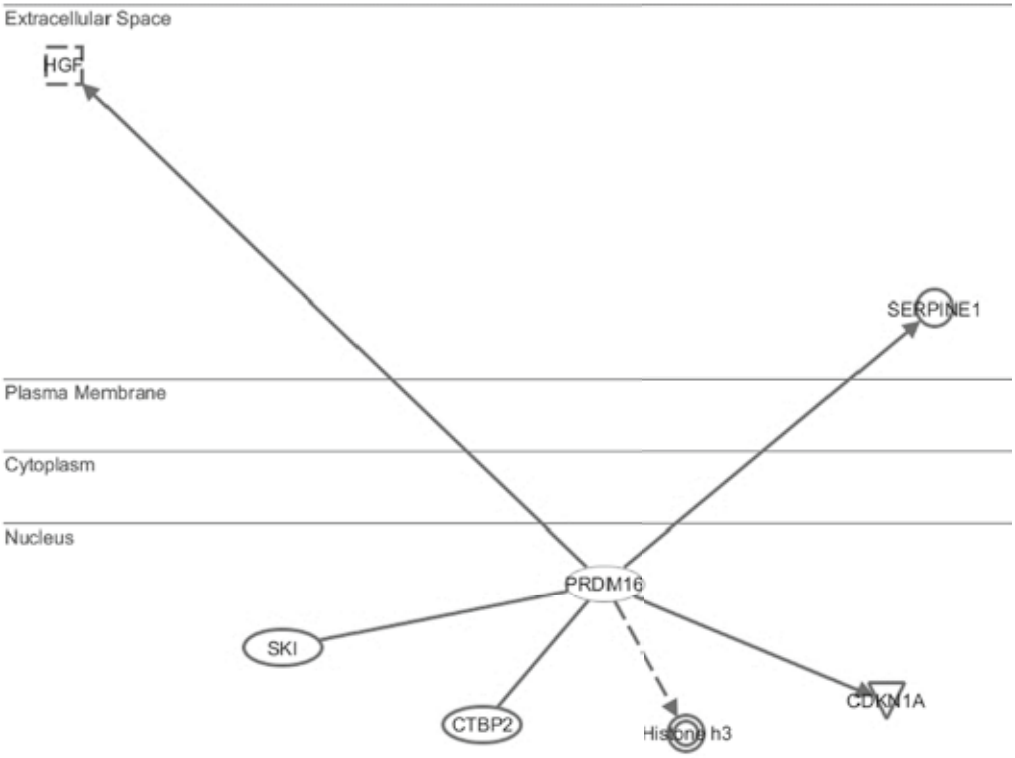

Figure S2 | Gene network diagram

Chapter 5

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Genome-wide methylation analysis in perimembranous ventricular septal defects and Down syndrome

Abstract

Background Approximately 50% of children born with Down syndrome (DS) have a congenital heart disease (CHD). Presumably trisomy 21 alone is not enough to cause CHD. The complexity of CHD in DS has been studied before in which gene-environment interactions seem to contribute to CHD risk. We investigated epigenome-wide DNA methylation at 5'-cytosine-phosphate-guanine (CpG) loci in blood of children with both DS and a perimembranous ventricular septal defect (pVSD) and in control children of the same age.

Methods At 17 months of age, 10 case children with both DS and pVSD and 196 healthy control children were included for analysis. We performed an epigenome-wide association study (EWAS) in DNA extracted from peripheral white blood cells (PWBC) to investigate 455,741 CpG loci using the Illumina Infinium® HumanMethylation450 BeadChip. The differentially methylated CpG loci were used to test the enrichment of pVSD-associated CpG loci from a previous EWAS on pVSD in non-DS children.

Results pVSD-DS cases show a distinctive methylation profile of global hypermethylation in all autosomes except chromosome 21. Using Bonferroni correction, we identified 3,889 differentially methylated CpGs within 1,625 genes. Among these, 2,096 CpGs within 869 genes showed a mean methylation difference of at least 10%. 1,593 of these CpGs were hypermethylated in case children and 503 in control children. Differentially methylated CpGs were enriched for pVSD-associated CpGs.

Conclusion Children at 17 months of age with both DS and pVSD show global hypermethylation of PWBC DNA. Our data suggest that CpGs associated with pVSD in non-DS may also be involved in the pathogenesis of pVSD in DS.

Introduction

Congenital heart diseases (CHDs) are the most common congenital malformations in humans worldwide with a birth prevalence rate of 0.6 to 1.9 in 100 newborns per year (57). The prevalence is strongly increased in individuals with Down syndrome (DS) as approximately 50% of children born with DS have a CHD (117). The most common forms of CHD in DS are the atrioventricular septal defect (AVSD) and ventricular septal defect (VSD) occurring in 90% of cases.

Since 50% of children with DS have a nonmalformed heart it can be concluded that DS alone is not enough to cause CHD. The complexity of CHD in DS has been investigated before in which a combination of both genetic variations and environmental factors contribute to CHD risk (118). One of the proposed mechanisms of gene-environment interactions is epigenetics in which DNA methylation at 5'-cytosine-phosphate-guanine (CpG) loci is the best-studied epigenetic modification.

In DS it has been shown that there are large gene-specific CpG methylation alterations (119). Aberrant DNA methylation has been found in DS chorionic villi samples, which supports derangements in epigenetic programming of the DS phenotype during the earliest weeks of development (120). Epigenetic changes of global DNA hypermethylation in DS are also found in DS placental tissue in early gestation (121).

In DNA methylation, folate-mediated one-carbon metabolism is essential. The role of maternal folate is supported by data suggesting that a lack of maternal folic acid supplementation is associated with septal defects in children with DS in comparison to children with DS and no CHD (14,122). Previous studies indicate that abnormal folate metabolism and a single nucleotide polymorphism in the methylenetetrahydrofolate reductase (*MTHFR*) gene are maternal risk factors for DS (123-125). A meta-analysis of 34 studies also shows that the maternal *MTHFR* 677T polymorphism is a risk factor for DS (126). In children with DS it has been shown that plasma levels of homocysteine, methionine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) are significantly decreased and their lymphocyte DNA is hypermethylated relative to that of normal siblings (127). Complex CHD is also associated with higher concentrations of the methylation biomarkers SAM, SAH and folate in blood. Seemingly contradictory, we have previously published that a child with DS and CHD seems to be associated with a global hypomethylation status (81). This is in line with the decreased SAM/SAH ratio as a marker of maternal hypomethylation observed in the mothers of these DS children (60). This hypomethylation was not found in mothers with nonsyndromic CHD offspring, suggesting perhaps a different underlying mechanism for CHD. However, these studies use a proxy measure for hypomethylation and do not take into account the global hypermethylation status in DS outnumbering hypomethylation in single CpG loci.

Recently, we performed an epigenome-wide association study (EWAS) on pVSD (perimembranous ventricular septal defects) in nonsyndromic children [Wijnands et al. unpublished]. Since CHD is not unique to people with DS we hypothesise that the same CpG risk loci could be identified in both DS and non-DS children with CHD.

In the present study, we used the Illumina Infinium® HumanMethylation450 BeadChip (Illumina, Inc., San Diego, CA, USA) to perform an EWAS in DNA extracted from peripheral white blood cells (PWBC)

of very young children with pVSD and DS (pVSD-DS) and healthy controls of the same age to identify any loci associated with pVSD in children with DS, and compare these data with loci found in non-DS children with pVSD.

Methods

Study population

Sampling of the participants for this case-control study has been previously described in detail (35). Cases were diagnosed with both DS and pVSD. In all cases (n=10), pVSD was diagnosed by two paediatric cardiologists using echocardiography and/or cardiac catheterisation and/or surgery data. Control children (n=195) had no chromosomal abnormalities or major congenital malformations according to the medical records and regular health checks by physicians of the child health care centres.

DNA isolation and methylation protocol

Genomic DNA was isolated from 0.2 mL EDTA-whole blood using the Total Nucleic Acid Extraction kit on a MagNA PureLC (Roche Molecular Biochemicals, Mannheim, Germany). Isolated genomic DNA (500 ng of input per sample) was sodium bisulphite treated using the EZ-96 DNA methylation kit (Shallow; Zymo Research, Irvine, CA, USA) according to Illumina's protocol. The Illumina Infinium® HumanMethylation450 BeadChips were processed according to the manufacturer's protocol.

Preprocessing of data

A quality control protocol was performed including a sample call rate check, colour balance check and control dashboard check. DNA methylation levels of probes on the sex chromosomes were used to match the gender of the child in order to identify sample mix-ups. Beta Mixture Quantile dilation (BMIQ) was used as intra-array normalisation (128). Probes with detection *P* values>0.01 in more than 5% of samples (n=1,721), probes with single-nucleotide polymorphisms (MAF>0.05, European population, 1000 Genomes Project) (n=17,196), and probes on the sex chromosomes (n=11,648) were excluded from analysis.

Statistical analysis

To adjust for potential confounding due to cell mixtures, Houseman's method was applied to predict the proportions of leukocyte subtypes with the model trained on the validation data set from Reinius et al. (86,87). Top 500 cell type-informative CpGs were used for prediction. We included the proportions of the subtypes that showed differentiation between pVSD-DS and control samples as covariates in the regression model.

Permutational multivariate analysis of variance (PERMANOVA) was used to test for the overall difference between pVSD-DS and controls based on the Euclidean distances between sample methylation profiles adjusting for children's gender and age, maternal smoking status, bisulphite plates and blood cell mixtures (R package 'vegan') (129). The first two principal coordinates are

plotted with the percentage of explained variability indicated. Principal component analysis (PCA) is performed on the methylation residuals after adjusting for cell mixtures using linear regression.
Methylation β-values were converted to M-values using: M-value=$\log_2$ (β-value/(1-β-value)) (84). To test for the association between case-control status and individual CpG methylation, a multiple linear regression model was fitted using the methylation M-value as the outcome and case-control status as the primary covariate, adjusting for the independent methylation predictors child's gender and age and maternal smoking status, and the potential confounders bisulphite plate and blood cell mixtures (R package 'CpGassoc') (85). Bonferroni correction for 455,741 tests was used to adjust the level of significance and associations were considered epigenome-wide significant where *P* values were less than 1.1e-7.
The one-sided Kolmogorov-Smirnov (KS) test was used to test for enrichment of differential CpGs in a particular type of region based on the distribution of regional *P* values in comparison to the overall *P* value distribution.
Gene ontology (GO) term enrichment analysis was performed using Fisher's exact tests. We focused on GO terms of biological processes only. GO terms with less than 10 genes were excluded for testing. A gene was considered to be differentially methylated when it contained one CpG that reached Bonferroni significance.
Previously, we conducted an EWAS on pVSD in non-DS children, and identified one significant CpG in the *PRDM16* gene after Bonferroni correction. To conduct enrichment analysis of potential pVSD-associated CpGs in the pVSD-DS-associated CpGs identified herein, we relaxed the significance level to expand the list of differential CpG loci. At different significance levels (raw *P* cut-off: 0.01-0.00001), we used Fisher's exact test to test for enrichment of these potential pVSD-associated CpGs in the differentially methylated pVSD-DS CpG loci. Lastly, a gene-level analysis of the *PRDM16* gene was performed.
Statistical analyses were performed in the R environment for statistical computing (R 3.0.1).

Results

Characteristics of the children

In total, 10 case children and 196 control children were included for analysis. Characteristics of the children and their mothers are shown in Table 1. Mothers of case children used significantly less often folic acid supplements in the periconception period compared to mothers of control children, 20% and 56% respectively (*P*=0.047). Maternal age at conception was not significantly different between cases and controls, 32.2 and 30.3 years of age respectively (*P*=0.096).

Predicting leukocyte proportions based on methylation data

Leukocyte proportions predicted by Houseman's method showed differential cell populations of CD4+ T-cells, B-cells, neutrophils and eosinophils between case and control samples. Therefore, these cell proportions were used to adjust for confounding due to cell mixtures (Supplementary Table S1*)*.

Table 1 | Characteristics of children and their mothers

	Cases DS-pVSD (n=10)	Controls (n=196)	*P* value
Child			
Age at study moment (in months)	16.3	16.4	0.927
Gender (female) n(%)	5 (50)	108 (55)	0.757
Mother			
Age at conception (in years)	32.2	30.3	0.096
Periconceptional folic acid use n (%)	2 (20)	109 (56)	0.047
Body mass index (kg/m^2)	25.3	24.7	0.771

Global analysis of methylation profiles

PCA revealed that pVSD-DS leukocytes have a distinctive methylation profile (Figure 1A). pVSD-DS leukocytes show global hypermethylation in all autosomes except chromosome 21 (Figure 1B). Chromosome 21 is the only autosome that is lower methylated in pVSD-DS children in comparison with healthy control children.

Epigenome-wide association analysis

Epigenome-wide association analysis was performed to identify differentially methylated CpG loci. We observed widespread differentially methylated CpGs indicated as strong deviation of the distribution of association *P* values from the null distribution (Figure 2A). The association *P* values were significantly lower in the DHS, enhancer, CDMR, DMR, RDMR, CpG shore and TSS1500 regions based on the KS test (Supplementary Table S2). Using Bonferroni correction for 455,741 tests, we identified 3,889 differentially methylated CpGs within 1,625 genes. Among these, 2,096 CpGs within 869 genes showed a mean methylation difference of at least 10%. 1,593 of these CpGs were hypermethylated in case samples and 503 in control samples. These CpGs were mostly enriched in chromosome 5, 8, 19, 21 and 22 (Fisher's test, $P<0.05$, Figure 2B).

Functional analysis

Using GO term enrichment analysis, a total of 4,084 GO terms were scored. Bonferroni significant GO terms are shown in Table 2. The top five enriched GO terms include "anatomical structure development", "single-multicellular organism process", "multicellular organismal process", "single-organism developmental process" and "developmental process".

Enrichment analysis

Enrichment analysis showed that candidate pVSD-associated CpG loci from our previous study on pVSD in non-DS children were enriched in the significant pVSD-Down loci (Table 3), and the enrichment was very robust to the *P* value threshold used.

Gene-level analysis of *PRDM16* shows that several CpGs in *PRDM16* are significantly differently methylated between pVSD-DS and control children (Table 4) .

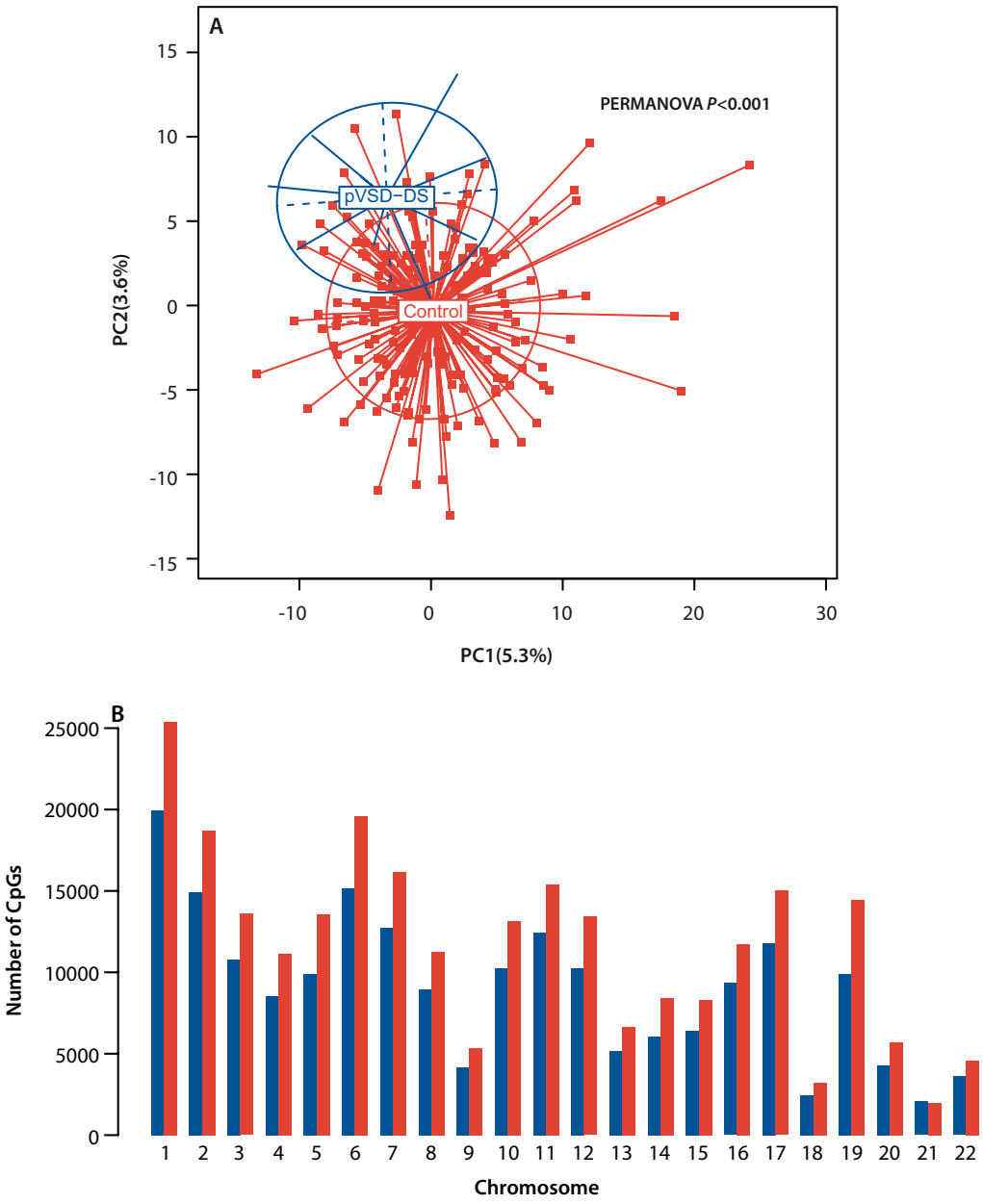

Figure 1 | Global methylation difference between pVSD-DS and healthy children

(A) CMD (colour magnitude diagram) plot of methylation profiles in pVSD-DS and control children

(B) Histogram of methylation profiles for each chromosome in pVSD-DS and control children.

Red: mean methylation pVSD-Down > control; Blue: mean methylation pVSD-Down < control.

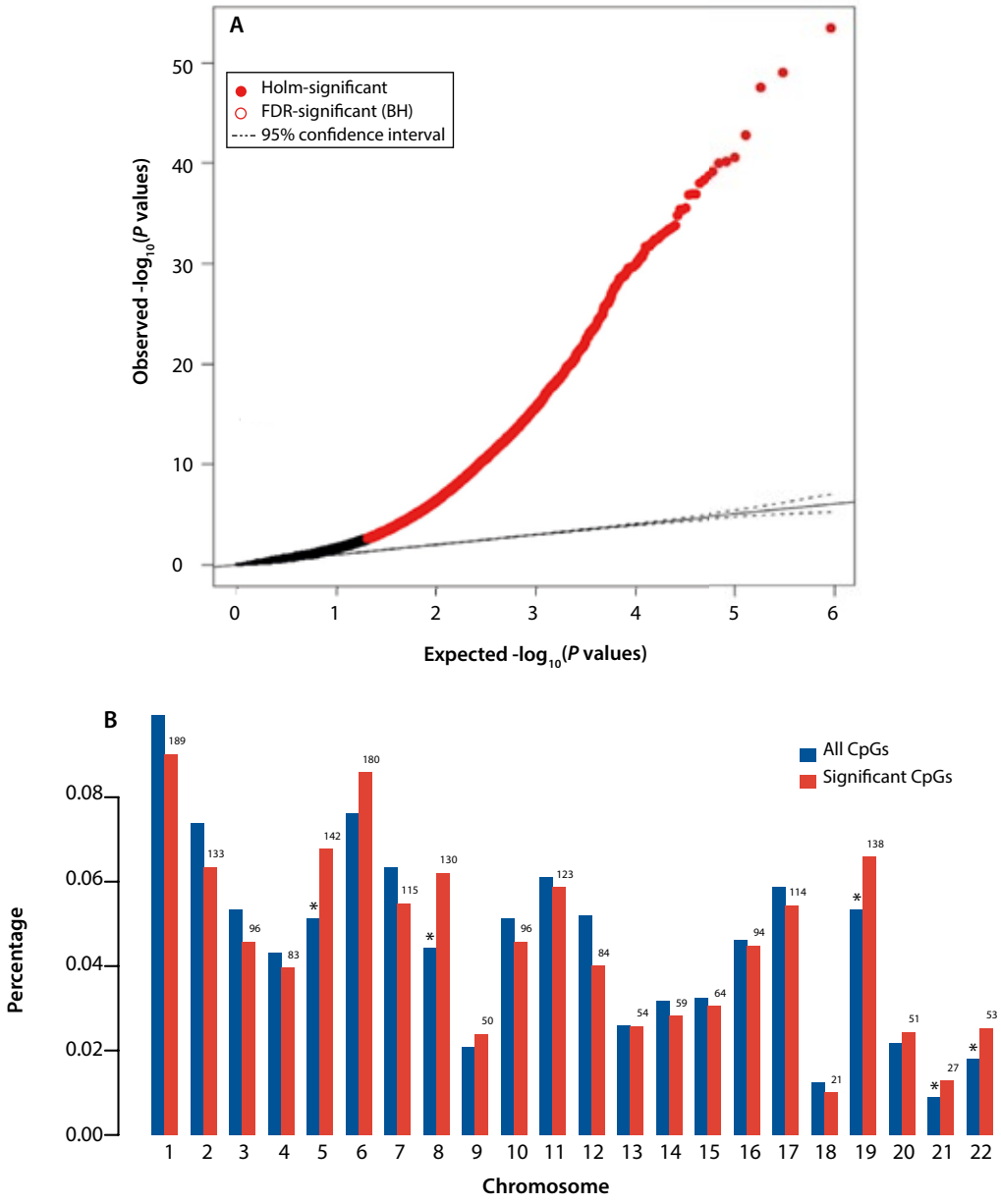

Figure 2 | Widespread differential CpG methylation between pVSD-DS and control

(A) Quantile-quantile plot of linear regression test for case-control status

(B) Distribution of Bonferroni significant CpGs on the chromosomes

* Enrichment $P<0.05$

Table 2 | GO Term enrichment analysis

GO.ID	Term	Annotated	Significant	Expected	Fisher
GO:0048856	anatomical structure development	3590	449	313.92	5.6e-20
GO:0044707	single-multicellular organism process	4902	567	428.65	1.1e-18
GO:0032501	multicellular organismal process	5065	578	442.9	9.0e-18
GO:0044767	single-organism developmental process	3178	400	277.9	1.0e-17
GO:0032502	developmental process	4069	485	355.81	1.6e-17
GO:0007275	multicellular organismal development	3613	438	315.93	1.1e-16
GO:0048731	system development	3113	389	272.21	1.4e-16
GO:0007154	cell communication	4291	498	375.22	1.0e-15
GO:0023052	signaling	4190	485	366.39	6.2e-15
GO:0044700	single organism signaling	4190	485	366.39	6.2e-15
GO:0007399	nervous system development	1597	225	139.65	2.3e-14
GO:0009653	anatomical structure morphogenesis	1928	257	168.59	1.5e-13
GO:0048646	anatomical structure formation involved in morphogenesis	1513	212	132.3	2.9e-13
GO:0040011	locomotion	1170	174	102.31	3.7e-13
GO:0048869	cellular developmental process	2643	328	231.11	3.9e-13
GO:0044699	single-organism process	9634	936	842.43	1.6e-12
GO:0030154	cell differentiation	2482	309	217.04	1.8e-12
GO:0044763	single-organism cellular process	8734	860	763.73	2.6e-11
GO:0007165	signal transduction	3706	422	324.07	2.6e-11
GO:0030030	cell projection organization	883	135	77.21	3.4e-11
GO:0007166	cell surface receptor signaling pathway	2124	264	185.73	1.8e-10
GO:0007155	cell adhesion	813	124	71.09	2.8e-10
GO:0022610	biological adhesion	815	124	71.27	3.3e-10
GO:0048468	cell development	1403	188	122.68	4.4e-10

Table 2 | *(Continued)*

GO.ID	Term	Annotated	Significant	Expected	Fisher
GO:0048699	generation of neurons	1008	144	88.14	9.9e-10
GO:0022008	neurogenesis	1075	150	94	2.2e-09
GO:0000902	cell morphogenesis	894	130	78.17	2.3e-09
GO:0006928	cellular component movement	1126	155	98.46	3.1e-09
GO:0050896	response to stimulus	5896	607	515.57	6.5e-09
GO:0048513	organ development	2224	266	194.47	8.0e-09
GO:0032989	cellular component morphogenesis	950	134	83.07	9.2e-09
GO:0048812	neuron projection morphogenesis	539	87	47.13	1.1e-08
GO:0000904	cell morphogenesis involved in differentiation	661	101	57.8	1.3e-08
GO:0007167	enzyme linked receptor protein signaling pathway	822	119	71.88	1.5e-08
GO:0007409	axonogenesis	488	80	42.67	2.2e-08
GO:0048667	cell morphogenesis involved in neuron differentiation	534	85	46.69	3.1e-08
GO:0031175	neuron projection development	656	99	57.36	3.5e-08
GO:0048858	cell projection morphogenesis	639	97	55.88	3.6e-08
GO:0048870	cell motility	853	121	74.59	3.8e-08
GO:0051674	localization of cell	853	121	74.59	3.8e-08
GO:0030182	neuron differentiation	928	128	81.15	7.8e-08
GO:0048666	neuron development	756	109	66.11	8.2e-08
GO:0032990	cell part morphogenesis	650	97	56.84	8.5e-08
GO:0007411	axon guidance	340	60	29.73	9.7e-08
GO:0016477	cell migration	787	112	68.82	1.1e-07
GO:0031589	cell-substrate adhesion	198	41	17.31	1.4e-07
GO:0009966	regulation of signal transduction	1655	201	144.72	3.0e-07
GO:0065007	biological regulation	7758	755	678.39	4.6e-07

Table 2 | *(Continued)*

GO.ID	Term	Annotated	Significant	Expected	Fisher
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	578	86	50.54	5.4e-07
GO:0051716	cellular response to stimulus	4564	475	399.09	5.6e-07
GO:0048583	regulation of response to stimulus	2145	248	187.57	5.9e-07
GO:0023051	regulation of signaling	1888	221	165.09	1.2e-06
GO:0006935	chemotaxis	556	82	48.62	1.4e-06
GO:0042330	taxis	556	82	48.62	1.4e-06
GO:0010646	regulation of cell communication	1895	221	165.71	1.6e-06
GO:0007160	cell-matrix adhesion	129	29	11.28	1.6e-06
GO:0050793	regulation of developmental process	1347	165	117.79	2.6e-06
GO:0050794	regulation of cellular process	6928	678	605.81	3.3e-06
GO:0042060	wound healing	562	81	49.14	4.2e-06
GO:0048514	blood vessel morphogenesis	399	62	34.89	5.3e-06
GO:0001525	angiogenesis	324	53	28.33	5.7e-06
GO:0031295	T cell costimulation	64	18	5.6	5.8e-06
GO:0072358	cardiovascular system development	713	97	62.35	6.0e-06
GO:0072359	circulatory system development	713	97	62.35	6.0e-06
GO:0031294	lymphocyte costimulation	65	18	5.68	7.3e-06
GO:0007599	hemostasis	459	68	40.14	9.9e-06
GO:2000026	regulation of multicellular organismal development	1035	130	90.5	1.0e-05
GO:0002009	morphogenesis of an epithelium	373	58	32.62	1.0e-05
GO:0050870	positive regulation of T cell activation	156	31	13.64	1.1e-05
GO:0007044	cell-substrate junction assembly	55	16	4.81	1.2e-05

Table 3 | Enrichment of pVSD-associated CpGs in an earlier EWAS study on perimembranous ventricular septal defects at different significance levels

P value cutoff threshold	0.1	0.05	0.01	0.005	0.001	5.00E-04	1.00E-05
Number of significant CpGs from previous study	45844	23081	4732	2429	545	277	14
Odds ratio	1.54	1.71	2.23	2.01	3.3	4.82	8.94
Lower CI	1.4	1.52	1.78	1.43	1.83	2.37	0.21
Upper CI	1.68	1.92	2.76	2.73	5.5	8.77	59.5
P value	2.24E-19	9.80E-18	5.20E-11	5.63E-05	9.45E-05	3.29E-05	0.113

Discussion

In this study, we examined epigenome-wide DNA methylation in children with both DS and pVSD. Children with both DS and pVSD show global hypermethylation of PWBC DNA in all autosomes except chromosome 21. Significantly differently methylated CpGs from our previous study on pVSD in nonsyndromic children are enriched in children with pVSD and DS [Wijnands et al. unpublished]. This might indicate that these CpGs may also be involved in the pathogenesis of pVSD in children with DS. Also *PRDM16*, a candidate-gene previously found in our EWAS on nonsyndromic pVSD and known as a repressor of *TGFβ* signaling (transforming growth-factor beta), seems differently methylated between pVSD-DS and controls.

We observed differential cell populations of CD4+ T-cells, B-cells, neutrophils and eosinophils between pVSD-DS cases and healthy controls. It has been shown before that DS is characterised by defects in the immune system including mild to moderate T and B cell lymphopenia (130). This is supported by the fact that many differentially methylated genes are involved in development and functioning of white blood cells (75). Based on our data, we conclude that blood leukocyte mixture is a potential confounder for this association study, therefore we applied Houseman's method to correct for cell type proportions (87).

Looking at our data, chromosome 21 is the only autosome not hypermethylated in case children. Previous research showed hypermethylation over hypomethylation in DS placenta villi in all autosomes including chromosome 21 (121). It remains to be seen whether these inconsistent findings could perhaps be explained by differences in tissue, intraindividual changes over time during different stages in development or differences in the genetic defect.

We measured DNA extracted from PWBC at a mean age of 17 months. Choosing the appropriate tissue is key, however, in clinical practice it is difficult if not impossible in many cases to obtain heart tissue, especially from healthy control children. Interestingly, the results of our functional analyses are comparable with the results of a recent study on heart tissue. Serra-Juhé et al. showed in fetal heart tissue the five top enriched gene-ontology based sets which comprised similar to our top five enriched GO terms the sets "anatomical structure development", "single-multicellular organism process" and "single-organism developmental process" (131). This is a distinct observation since our

Table 4 | Gene-level analysis of *PRDM16*

Probe ID	Chromosome	Mapinfo (bp)	UCSC RefGene name	UCSC RefGene Group	Control methylation	Case methylation	Mean absolute difference	Mean relative difference	*P* value	FDR-adjusted *P* value
cg25618424	1	2989307	PRDM16	Body	57.38	69.12	+11.74	+20.46	6.13E-11	1.71E-08
cg22510139	1	3058822	PRDM16	Body	43.60	59.82	+16.23	+37.22	2.76E-13	1.38E-10
cg24460544	1	3085446	PRDM16	Body	25.50	36.07	+10.57	+41.44	6.54E-08	8.19E-06
cg21475097	1	3164642	PRDM16	Body	21.81	10.68	-11.13	-51.05	2.42E-11	7.59E-09
cg04873098	1	3165191	PRDM16	Body	64.48	42.96	-21.53	-33.38	2.09E-16	2.07E-13
cg24807850	1	3165391	PRDM16	Body	83.45	68.34	-15.11	-18.11	7.93E-15	5.58E-12
cg27171194	1	3229579	PRDM16	Body	47.36	60.87	+13.50	+28.51	1.51E-14	1.02E-11
cg10893986	1	3229739	PRDM16	Body	10.67	21.21	+10.54	+98.86	1.43E-10	3.66E-08
cg09845604	1	3229921	PRDM16	Body	24.01	38.35	+14.34	+59.74	2.07E-09	3.98E-07
cg16394551	1	3230357	PRDM16	Body	43.20	60.85	+17.65	+40.87	8.83E-16	7.62E-13
cg15491247	1	3230424	PRDM16	Body	50.19	63.22	+13.03	+25.95	1.05E-12	4.53E-10

data is based on methylation in DNA leukocytes, while Serra-Juhé et al. did not find these sets to be enriched in blood and point out the importance of analysing the affected tissue.

DS is associated with a broad spectrum of phenotypes and the clinical presentation is complex and variable (25). The DS phenotype can be caused by three genetic defects which are non-disjunction resulting in trisomy of chromosome 21 (95%), unbalanced translocation of chromosome 21 (3-4%) or mosaicism of chromosome 21 (1-2%) (26). Epigenetics has been proposed as a causative mechanism of maternal meiotic non-disjunction resulting in trisomy 21. Unfortunately the genetic defect in our study group is unknown.

Strength of our study population is that DS cases have a homogenous CHD phenotype. Sailani et al. recently showed in DS monozygotic twins discordant for CHD that the direction of DNA methylation differs between VSD and AVSD (132). These differences in DNA methylation (either up- or downregulation) between different heart phenotypes highlight the importance of a homogenous case sampling.

Some limitations of this study have to be addressed. Firstly, some critical remarks on the Illumina Infinium® HumanMethylation450 BeadChip are in place. It has been shown that it is critical to correct for the two different probe design types (133). We did not perform a between-array normalisation because the case samples have a trisomy of chromosome 21 resulting in (epi)genomic imbalance. Therefore we used BMIQ normalisation, after which the peaks of the type I probes and type II probes matched very well (Supplementary Figure S3). Though no between-array normalisation was employed, the overall distributions over all chromosomes or separate chromosomes were very similar. There was no obvious deviation of the methylation distribution of pVSD-DS samples from the control samples.

Secondly, the differential methylation we have found could be considered the outcome of an interaction between both pVSD and DS. Unfortunately, we do not have data on DS children without a heart defect. Therefore, it is not possible to differentiate between differential methylation involved in DS and differential methylation involved in pathophysiology of pVSD. Global DNA hypermethylation has been reported in several studies, however, it is not consistently reported which percentage of these DS participants also has a CHD (119-121). In our data, enrichment analysis suggests that methylation changes previously found in pVSD children might also contribute to pVSD in DS. The results are very robust to the threshold used to pick up the a priori defined CpG loci.

Several EWAS on DS have been performed showing a state of global hypermethylation, however, epigenetic data focussing on CHD in DS is limited (119-121). This study implicates that perturbations in DNA methylation might play a role in the pathogenesis of pVSD in DS. Children at 17 months of age with both DS and pVSD show global hypermethylation of PWBC DNA. Our data suggest that CpGs associated with pVSD in nonsyndromic children may also be involved in the pathogenesis of pVSD in children with DS. Future research will have to emphasise the role of aberrant DNA methylation in the pathophysiology of CHD in DS.

Supplementary material

Table S1 | Differential cell distribution

Cell type	Case	Control	*P* value
Neutrophil	0.272	0.388	0.00104
CD4+ T	0.176	0.122	0.00853
CD8+ T	0.259	0.264	0.861
B cell	0.141	0.0821	1.89e-06
Eosinophil	0.0234	0.00523	0.00742
Monocyte	0.0883	0.0951	0.548
NK cell	0.00631	0.0111	0.466

Table S2 | One-sided Kolmogrov-Smirnov test on enrichment of differential CpGs in a particular type of region

Region	*P* value	Region	*P* value
DHS	1.29e-92	N Shore	4.9e-101
Enhancer	4.98e-64	S Shore	4.21e-101
CDMR	1.1e-23	TSS1500	9.84e-21
DMR	7.4e-08	TSS200	0.988
RDMR	2.78e-128	UTR3	0.813
Island	0.998	UTR5	0.997
N Shelf	0.999	1st Exon	0.985
S Shelf	0.992	Body	0.747

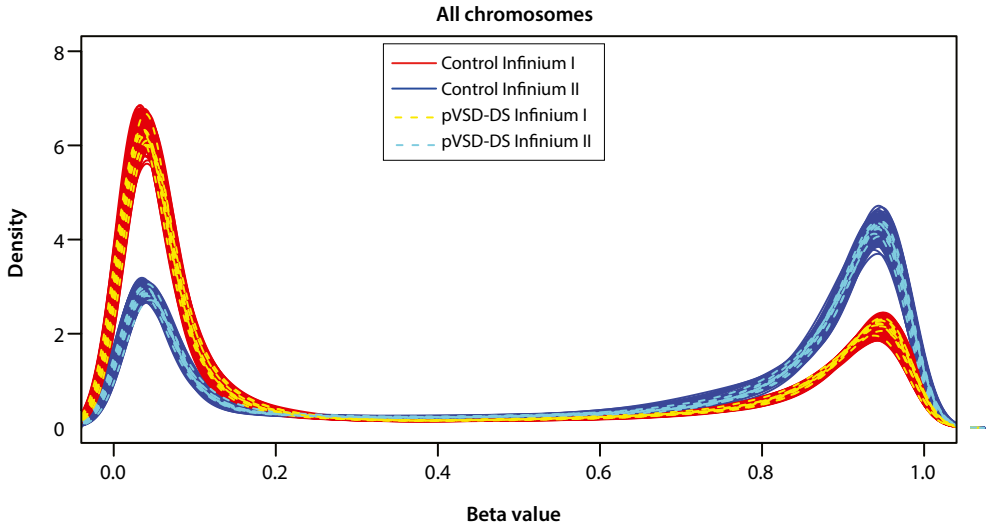

Figure 3 | Density plot of the beta values for the two probe design types (Infinium I and Infinium II) after BMIQ normalisation

pVSD, Perimembranous ventricular septal defect. DS, Down syndrome.

Part II

Cardiovascular risk factors

Chapter 6

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The periconception maternal cardiovascular risk profile influences human embryonic growth trajectories in IVF/ICSI pregnancies

Abstract

Background Maternal high blood pressure and smoking affect placental function, accompanied by increased risk of fetal growth restriction and low birthweight. Mothers who experience pregnancies complicated by fetal growth restriction are at increased risk of CV disease in later life. We aimed to study if the maternal cardiovascular (CV) risk profile is associated with human embryonic growth trajectories and whether the mode of conception affects this association.

Methods In a prospective periconception birth cohort conducted in a tertiary hospital, 111 singleton ongoing pregnancies without pre-existing maternal disease, reliable pregnancy dating and nonmalformed live borns were investigated. Spontaneously conceived pregnancies with a reliable first day of the last menstrual period and a regular menstrual cycle of 25 to 31 days only (n=66) and *in vitro* fertilisation (IVF)/intra-cytoplasmic sperm injection (ICSI) pregnancies (n=45) were included. Women underwent weekly three-dimensional ultrasound scans (3D US) from 6 up to 13 weeks gestational age. To estimate embryonic growth, serial crown-rump length (CRL) measurements were performed using V-Scope software in a BARCO I-Space. Maternal characteristics and CV risk factors were collected by self-administered questionnaires. The CV risk profile was created based on a score of risk factors, including maternal age, body-mass index, CV disease in the family, diet and smoking. Quartiles of the CV risk score were calculated. Associations between the CV risk score and embryonic growth were assessed using square root transformed CRL in multivariable linear mixed model analyses.

Results From the 111 included pregnancies 696 3D US datasets were obtained of which 637 (91.5%) CRLs could be measured. In the total group, CV risk score was inversely but not significantly associated with embryonic growth (-0.03√mm; *P*=0.291). Stratified by mode of conception, the CV risk score was inversely and significantly associated with embryonic growth (β=-0.04√mm; *P*=0.025, adjusted for possible confounders) in the IVF/ICSI group. Compared with the first quartile, embryos in the upper quartile were 10.4% smaller at 6^{+0} weeks (4.4mm vs. 4.9mm) and 3.1% smaller at 12^{+0} weeks (56.5mm vs. 58.4mm) of gestation. Although the CV risk score was slightly, but significantly, higher in women conceiving spontaneously compared to those undergoing IVF/ICSI treatment (CV risk score=2.06 (SD 1.23) and 1.60 (SD 1.15) respectively), no association was established with embryonic growth in that particular group.

Conclusions This small study suggests that the maternal CV risk profile is inversely associated with first trimester embryonic growth trajectories in IVF/ICSI pregnancies, but not in spontaneously conceived pregnancies. Differences in embryonic growth between pregnancies conceived spontaneously and after IVF/ICSI treatment in relation with CV risk factors substantiate the importance of more investigation into differences in sensitivity of endometrial, endothelial, placental and embryonic tissues.

Introduction

The periconception period is critical for gametogenesis, embryogenesis and placentation (14). Events during this time window can result in subfertility and vascular-related pregnancy complications, such as fetal growth restriction and pre-eclampsia that mainly originates in the placenta and largely present in the second half of pregnancy (134,135). Unhealthy lifestyle such as an insufficient diet, smoking and (social) alcohol use, occur in more than 80% of pregnancies and detrimentally affect reproduction. These poor behaviours partially act through interactions with vascular-related pathways such as one-carbon (1-C) metabolism, which has been implicated in growth and development of the gametes, embryo, placenta and fetus (14,136-138).

Previous studies showed that women with CV disease or risk equivalents are more likely to experience a pregnancy complicated by fetal growth restriction and low birthweight (139). Furthermore, mothers with pregnancies complicated by fetal growth restriction are at an increased risk of CV disease in later life (140-142).

We hypothesise that the maternal cardiovascular risk profile is associated with human embryonic growth trajectories *(*Figure 1). The suggested mechanism behind this hypothesis is that increased maternal CV disease risk will result in endothelial dysfunction and reduced endometrium receptivity which will result in impaired placental function and embryonic growth. However, this might be different in pregnancies conceived after *in vitro* fertilisation (IVF) with or without intracytoplasmic sperm injection (ICSI) since the endometrial receptivity and endocrine environment are altered. This is important since the human endometrium decidualises in response to endocrine rather than embryonic cues (Macklon and Brosens, 2014). Studies on endometrial histology show a wide range of abnormalities during various stimulation protocols (Zandstra, et al., 2015). To consider the possible indirect effects of IVF/ICSI treatment through influences on placentation and endometrium receptivity we also presented the stratified analysis.

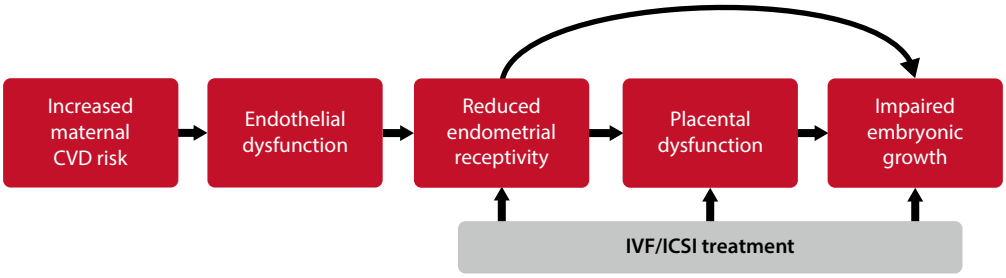

Figure 1 | Hypothesis

Methods

This study was embedded in the Predict Study, a prospective periconception hospital-based birth cohort, conducted at the department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands (27). All women of at least 18 years of age with intrauterine singleton pregnancies of 6-8 weeks of gestation at enrolment were eligible for participation during 2009 and 2010.

Ultrasound scans

Women received weekly transvaginal three-dimensional ultrasound scans (3D US) from enrolment up to 13 weeks gestational age (GA). Ultrasound scans were performed with a 6-12 MHz transvaginal probe using GE Voluson E8 equipment and 4D View software (General Electrics Medical Systems, Zipf, Australia). The obtained 3D-data sets were transformed to Cartesian (rectangular) volumes and transferred to the BARCO I-Space (Barco N.V., Kortrijk, Belgium) at the department of Bioinformatics, Erasmus MC, University Medical Centre. This is a four-walled CAVE™-like (Cave Automatic Virtual Environment) virtual reality system, allowing depth perception and interaction with the projected images (143). CRL measurements were performed offline in the I-Space using the V-Scope software by placing the callipers at the outer side of crown and rump in the mid-sagittal plane (144). The BARCO I-Space and V-scope software use the benefit of the third dimension which enables the performance of highly precise and reliable first trimester embryonic measurements *in vivo* (27,145,146). All CRL measurements were performed three times by the same researcher and the mean of these three measurements was used in the analyses.

Questionnaires

At enrolment participants completed a self-administered general questionnaire containing items on maternal age, anthropometrics, ethnicity, education, family history, obstetrical history, and periconception exposures such as folic acid supplement use and diet. Data on fetal gender and pregnancy outcome were obtained after birth from medical records. Ethnicity and educational level were classified according to the definitions of Statistics Netherlands (67). Smoking was defined as smoking any number of cigarettes until the moment of pregnancy recognition. Folic acid supplement use was defined as the daily intake of a folic acid supplement of 0.4-0.5 mg. Diet was determined using six questions on the adequacy of the intake based on Dutch guidelines of the main food groups: whole wheat, vegetables, fruit, unsaturated oils, meat and fish. Based on the answers to these questions the validated Preconception Dietary Risk (PDR) score was calculated, which ranges from zero to six, where a score of six points represents a highly inadequate food group intake (147).

Cardiovascular risk score

We constructed the CV risk score based on risk assessment tools from the Framingham Heart Study and the American Heart Association comprised of maternal age, body-mass index (BMI), CV disease in the family, diet and smoking (148,149). The CV risk score ranged from zero to eight, where a score of eight indicates the highest number of risk factors. CV risk factors were weighed according to their increased risk for CV disease and known effect on fetal growth.

Pregnancy dating

Data on the first day of the last menstrual period (LMP) and data of regularity and duration of the menstrual cycle were obtained in a personal interview by the researcher performing the ultrasound at the first visit. GA was calculated from the LMP in spontaneously conceived pregnancies, from the date of oocyte retrieval plus 14 days in pregnancies conceived through IVF/ ICSI procedures, from the LMP or insemination date plus 14 days in pregnancies conceived through intra-uterine insemination, and from the day of embryo transfer plus 17 or 18 days in pregnancies originating from the transfer of cryopreserved embryos, depending on the number of days between the oocyte retrieval and cryopreservation of the embryo. When the menstrual cycle was regular, but >3 days different from 28 days, the GA was adjusted for the duration of the menstrual cycle.

If the first day of the LMP was unknown in spontaneously conceived pregnancies, or if the observed CRL differed by >6 days from the expected CRL according to the Robinson curve, pregnancies were excluded from the analysis (150). We included spontaneously conceived pregnancies and pregnancies conceived through IVF/ICSI using biological oocytes from the participating mother-to-be only. Ectopic pregnancies and pregnancies that ended in a miscarriage before 16 weeks GA and congenital malformations were excluded.

Statistical analysis

To assess associations between the CV risk score and serial CRL measurements we performed a linear mixed model analysis. CRL measurements were entered as response and CV risk score as a function of GA as predictor. Square root transformation of the serial CRL measurements was performed to achieve linearity with GA and a constant variance. To model the within subject correlation we used random intercepts and GA.

Firstly, we presented a multivariable analysis. Secondly, we presented a stratified analysis. For both analyses, we started with a univariable model adjusted for GA only. In the second or fully adjusted model we adjusted for all potential confounding variables. As potential confounders we considered the moment of initiation of folic acid use, parity, ethnicity, educational level, fetal gender and interaction terms between all aforementioned covariates and GA. The third and final model was derived from the fully adjusted model after hierarchical backward elimination of all covariates with $P<0.20$ (151). In all analyses, $P<0.05$ was considered statistically significant.

Data analysis was performed using IBM SPSS Statistics for Windows software (version 20.0; IBM, Armonk, NY, USA) and SAS for Windows software (version 9.2; SAS Institute Inc., Cary, NC, USA).

Ethical approval

This study has been approved by the Central Committee on Research in The Hague and the local Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Centre. At enrolment, all participants signed a written informed consent form.

Results

Two hundred and fifty-nine singleton pregnancies were enrolled (Figure 2). We excluded 69 pregnancies in total, because they were conceived by oocyte donation (n=2) or ended in a miscarriage (n=43). Pregnancies were also excluded in case of an ectopic pregnancy (n=1), unknown first day of the LMP (n=12), missing questionnaires (n=3) or if they resulted in fetal/neonatal death (n=5) or in case of fetuses with a congenital malformation (n=3). Excluded pregnancies were comparable in baseline characteristics (Supplementary Table S1). In addition, 52 women with one or more pre-existing diseases, past medical history or malformations were excluded (diabetes mellitus (n=2), thyroid disease (n=9), asthma (n=9), hypertension (n=6), epilepsy (n=3), thrombosis (n=5), rheumatic disorder (n=3), heart disease (n=3), cancer (n=3), uterine anomaly (n=2), Ehlers-Danlos syndrome (n=1), hematologic disease (n=2), psychiatric disorder (n=4), gastrointestinal disease (n=3), metabolic disorder (n=1) and previous gynaecological surgery (n=3)). An additional number of 27 spontaneously conceived pregnancies were excluded because GA was not based on a strictly regular menstrual period of 28 ± 3 days. In total, 111 pregnancies were available for analysis, including 67% derived from the Obstetrics and Gynaecology outpatient clinic at the Erasmus MC, University Medical Centre, and 33% from outside the hospital. Of these 111 pregnancies 45 were conceived through IVF/ICSI treatment.

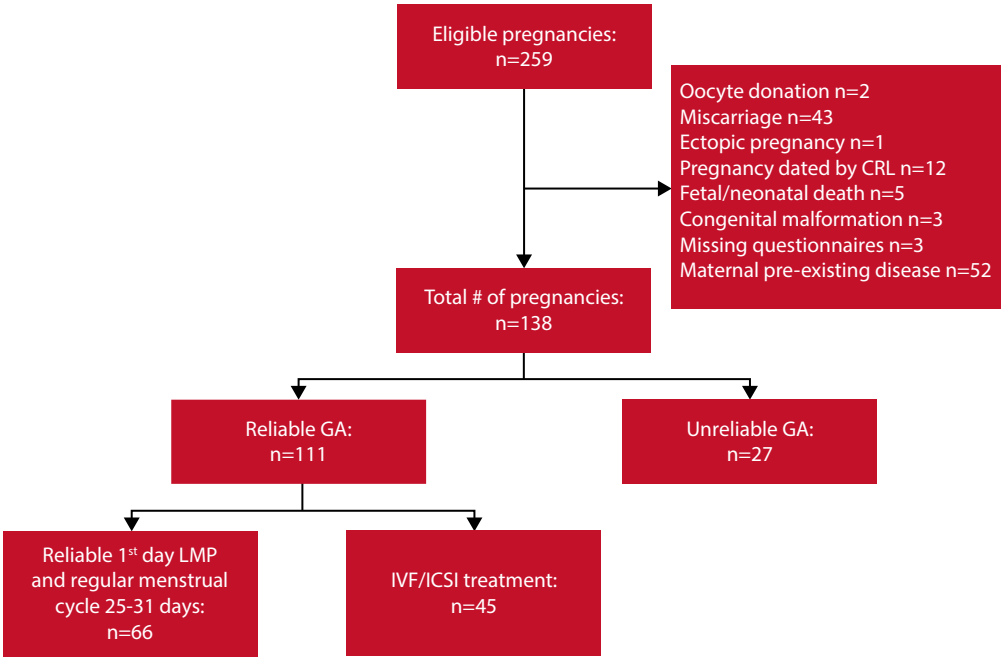

Figure 2 | Flowchart of the study population

CRL, crown-rump length; GA, gestation age; LMP, last menstrual period.

Median GA at enrolment was 6^{+6} (range 6^{+0}-8^{+6}) weeks and the median number of ultrasound visits per pregnancy was 6 (range 4-8). In total 696 3D US were performed. A number of 637 (91.5%) 3D US scans were of sufficient quality to measure CRL. Information from medical records was obtained for 111 pregnancies (100%).

Maternal and pregnancy characteristics are shown in Table 1. Women in the spontaneously conceived and in the IVF/ICSI group were on average 32.1 (SD 4.7) and 32.9 (SD 4.3) years of age respectively. Women in the two groups were both highly educated (68% and 53% respectively) and the majority of Dutch origin (74% and 84% respectively). These characteristics were not significantly different between spontaneously conceived and IVF/ICSI pregnancies. In the IVF/ICSI group, all women (100%) initiated folic acid supplement use preconceptional compared to 72% of women with spontaneously conceived pregnancies. In the IVF/ICSI group women were less likely to smoke compared to the women in the spontaneously conceived pregnancy group (9% vs. 24% respectively, *P*=0.039). Both groups showed a comparable GA at delivery and birth weight. Fetal pregnancy complications occurred in 15% of spontaneously conceived pregnancies and 7% of IVF/ICSI pregnancies (*P*=0.172). The mean CV risk scores were for the spontaneous and IVF/ICSI group 2.06 (range 0.17-6.17) and 1.60 (range 0.17-4.33) (*P*=0.049), respectively (Table 2). From all CV risk factors, only smoking was significantly different between both groups

Table 3 and Table 4 show the results of the linear mixed model analyses. In the total group, CV risk score was inversely but not significantly associated with embryonic growth (-0.03√mm; *P*=0.291) (Table 3). Stratified by mode of conception, the univariable model of CRL as a function of GA adjusted CV risk score showed no association between CV risk score and CRL (β=-0.01√mm; *P*=0.75) in spontaneous pregnancies (Table 4). In the IVF/ICSI pregnancies, the association between CV risk score and CRL was marginally statistically significant (β=-0.03√mm; *P*=0.08).

In spontaneous pregnancies, CV risk score was not significantly associated with embryonic growth trajectories in neither the saturated (β=-0.00√mm; *P*=0.93) nor the final model after backward elimination (β=-0.01√mm; *P*=0.72). In IVF/ICSI pregnancies on the other hand, the association between CV risk score and CRL was significantly associated with embryonic growth trajectories in both the saturated (β=-0.04√mm; *P*=0.03) and the final model (β=-0.04√mm; *P*=0.03). The estimated multivariable regression lines for the final models are shown in Figure 3 for the lowest CV risk (first quartile, mean 0.54 (SD 0.28)) and the highest CV risk (fourth quartile, mean 3.15 (SD 0.58)) in spontaneously conceived pregnancies, and for the lowest CV risk (first quartile, mean 0.54 (SD 0.28)) and the highest CV risk (fourth quartile, mean 3.65 (SD 0.93)) in IVF/ICSI pregnancies. Compared with the first CV risk quartile, embryos in the upper quartile were 10.4% smaller at 6^{+0} weeks (4.4mm vs. 4.9mm) and 3.1% smaller at 12^{+0} weeks (56.5mm vs. 58.4mm) of gestation in the IVF/ICSI group.

Table 1 | General maternal characteristics

Variable	Spontaneous pregnancies n=66 (59%)	IVF/ICSI pregnancies, n=45 (41%)	*P* value
Maternal			
Age in years (mean ± SD)	32.1 (4.7)	32.9 (4.3)	0.397
Body mass index (kg/m^2)	24.6 (3.8)	24.0 (3.4)	0.348
Ethnicity			0.149
Dutch	49 (74)	38 (84)	
Western-other	4 (6)	4 (9)	
Non-Western	13 (20)	3 (7)	
Educational level			0.277
Low	4 (6)	5 (11)	
Intermediate	16 (25)	16 (36)	
High	43 (68)	24 (53)	
Folic acid supplement use			0.407
No	1 (2)	0 (0)	
Yes	65 (99)	45 (100)	
Preconception initiation	46 (72)	45 (100)	0.000
Periconception			
Smoking	16 (24)	4 (9)	0.039
Alcohol	35 (53)	13 (29)	0.064
Nulliparous	38 (58)	37 (82)	0.018
Primigravida	20 (30)	27 (60)	0.002
Conception			NA
Spontaneous	63 (96)	0 (0)	
Assisted reproductive technology	3 (5)	45 (100)	
Newborn			
Birth weight in grams (mean ± SD)	3347 (621)	3341 (543)	0.606
Gestational age at delivery, weeks $^{+\text{ days}}$	39^{+1} (26^{+5}-42^{+0})	39^{+1}(31^{+3}-41^{+3})	0.926
Male gender	32 (48)	16 (36)	0.177
Fetal complication	10 (15)	3 (7)	0.172
Low birthweight (<2500g)	5 (8)	2 (4)	0.505
Premature delivery (<37 weeks)	4 (6)	2 (4)	0.712
SGA (<10th customised centile)	7 (11)	1 (2)	0.086

Data are presented as number of observations (%) unless otherwise specified.
SD, standard deviation. SGA; small for gestational age.

Table 2 | Cardiovascular risk score

Cardiovascular risk factor	Item score	Spontaneous pregnancies n=66	IVF/ICSI pregnancies n=45	*P* value
Positive family history of mother with CV disease	1	15 (23)	6 (13)	0.215
Positive family history of father with CV disease	1	12 (18)	9 (20)	0.810
Smoking[a]	2	16 (24)	4 (9)	0.039
Overweight (BMI 25-30)	1	22 (34)	10 (22)	0.399
Obesity (BMI ≥30)	2	4 (6)	4 (9)	0.399
Age ≥36 years	1	15 (23)	10 (23)	1.000
PDR score				
0	0	2 (3)	0 (0)	
1	1/6	9 (14)	7 (16)	
2	2/6	12 (18)	11 (24)	
3	3/6	17 (26)	16 (36)	
4	4/6	22 (33)	6 (13)	
5	5/6	3 (5)	5 (11)	
6	1	1 (2)	0 (0)	
Mean CV risk score (SD) range	**0-8**	**2.06 (1.23)**	**1.60 (1.15)**	0.049

BMI, body-mass index; PDR, preconception dietary risk; CV, cardiovascular; SD, standard deviation.
[a] Smoking was defined as smoking any number of cigarettes until the moment of pregnancy recognition.

Table 3 | Effect estimates of cardiovascular risk score for embryonic crown-rump length (CRL) derived from linear regression models

	CRL (√mm) n=111 (696 3D US)	
Model	**β CV risk score (SE)**	***P* value**
1. Unadjusted	-0.02532 (0.02368)	0.2856
2. Fully adjusted	-0.03171 (0.02514)	0.2079
3. Adjusted	-0.02516 (0.02381)	0.2912

3D US, 3D ultrasound scans; CV, cardiovascular.
Model 1: CRL adjusted for gestational age (GA) and mode of conception.
Model 2: Analysis adjusted for GA, mode of conception, moment of folic acid use, parity, ethnicity, educational level, fetal gender and interaction terms between all aforementioned covariates and GA.
Model 3: Analysis adjusted for GA, mode of conception, parity, gender, and interaction terms between GA and mode of conception, GA and parity , and GA and gender.

Table 4 | Effect estimates of cardiovascular risk score for embryonic crown-rump length (CRL) derived from linear regression models stratified for mode of conception

Model	Spontaneous pregnancies CRL (√mm) n=66 (406 3D US)		IVF/ICSI pregnancies CRL (√mm) n=45 (290 3D US)	
	β CV risk score (SE)	*P* value	β CV risk score (SE)	*P* value
1. Unadjusted	-0.01185 (0.03658)	0.7462	-0.03323 (0.01898)	0.0817
2. Fully adjusted	-0.00400 (0.04420)	0.9280	-0.04378 (0.01941)	0.0254
3. Adjusted	-0.01472 (0.04063)	0.7175	-0.04269 (0.01889)	0.0251

Model 1: CRL adjusted for gestational age (GA).
Model 2: Analyses of both groups adjusted for GA, parity, ethnicity, educational level, fetal gender and interaction terms between all aforementioned covariates and GA. Analysis of the spontaneously conceived pregnancy group is additionally adjusted for moment of folic acid use.
Model 3: Analysis of the spontaneously conceived pregnancy group with reliable GA is adjusted for ethnicity and educational level. Analysis of the IVF/ICSI group is adjusted for GA, ethnicity, parity, gender, and interaction terms between GA and ethnicity, GA and parity, and GA and gender.

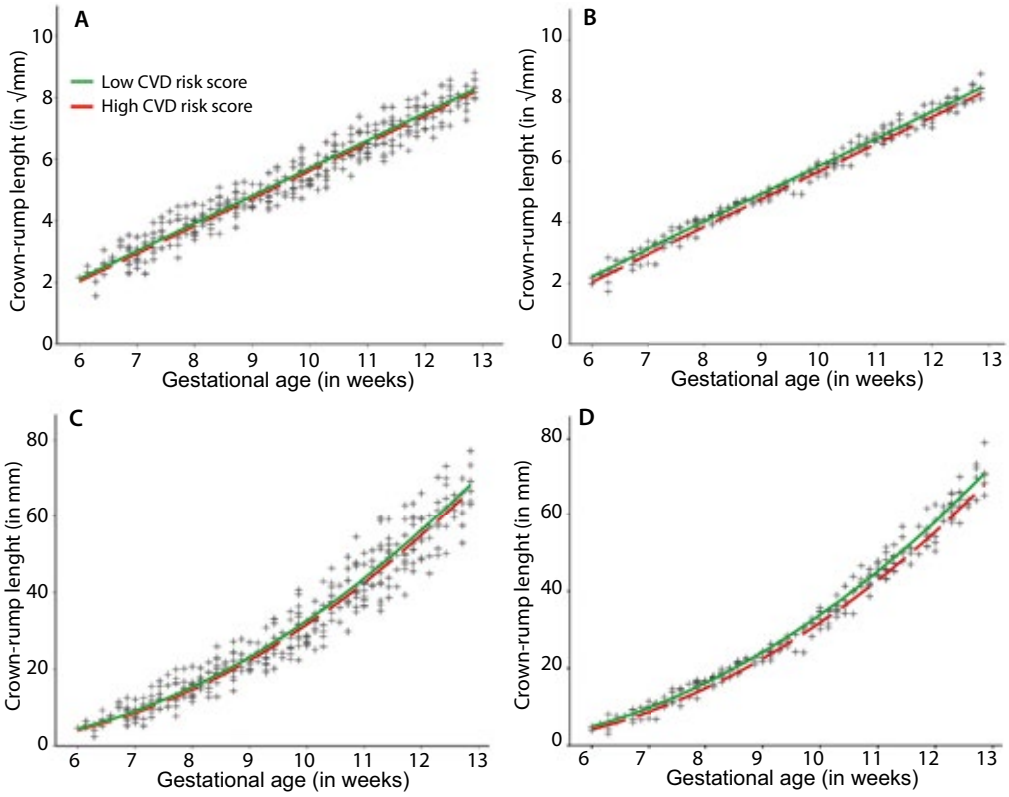

Figure 3 | Regression lines crown-rump length versus gestational age

Estimated multivariable regression lines for high and low CV risk score in spontaneously conceived pregnancies (A) and IVF/ICSI pregnancies (B) and after transformation to the original scale for spontaneously conceived pregnancies (C) and IVF/ICSI pregnancies (D).

Discussion

In a prospective periconception hospital-based birth cohort we observed that the maternal CV risk profile is inversely associated with embryonic growth trajectories in IVF/ICSI pregnancies, but not in spontaneously conceived pregnancies.

So far, only a few longitudinal ultrasound studies on human embryonic growth trajectories have been conducted (152-154). Strengths of our study are that serial CRL measurements were measured both very accurately in a virtual reality system as well as in triplicate by the same researcher. The mean measurement was used in the analyses providing greater precision. Furthermore, we used strict criteria to estimate GA because GA is the main determinant of CRL. We excluded women with an unknown first day of the LMP or with a discrepancy between LMP and CRL dates of more than 6 days, and adjusted GA for the duration of the menstrual cycle when regular but more than three days different from 28 days. However, misclassification of GA may have occurred due to recall bias of the LMP and physiological variations in the timing of ovulation and implantation.

In the present study the CV risk score was significantly associated with embryonic growth trajectories in pregnancies conceived with IVF/ICSI treatment only, which seems surprising since CV score was significantly lower in women undergoing IVF/ICSI treatment. This result can be explained by either information bias of smoking or a real higher sensitivity of these embryos to environmental maternal conditions and exposures due to the cause of subfertility or the IVF/ICSI treatment. Also differences in pregnancy related factors such as uterine fluid or endometrium receptivity could be of importance (155,156). Endometrial histology observations show a wide range of abnormalities during various stimulation protocols (157). Furthermore, studies suggest that the culture medium used for *in vitro* fertilisation affects fetal growth and birth weight (157-159).

This is the first study investigating the association between a maternal CV risk profile and human embryonic growth trajectories. In the future, the extension of the CV risk score with biomarkers such as total cholesterol, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol is challenging to substantiate these first findings. We restricted our analysis to asymptomatic women without any disease and therefore diabetes mellitus and CV disease were not added to the risk score, which may have underestimated the effect sizes. It should be noted that by using CV family history as a risk factor a genetic contribution of derangements in CV pathways cannot be excluded.

There is some evidence that a number of CV risk factors, such as high blood pressure, smoking or increased maternal age, influence embryonic growth (74,135,160). Whether this is a direct or indirect effect needs to be clarified. The 1-C metabolism pathway as an intermediate could be a possible explanation. The relation between 1-C metabolism and poor dietary intake, smoking and age is evident, as well as hyperhomocysteinaemia as a risk factor for CV disease (161). Since embryogenesis is vulnerable to maternal nutrient deficiencies such as insufficient folate and methionine, it is possible that alterations in 1-C metabolism affect critical processes such as DNA synthesis, S-adenosylmethionine mediated DNA and histone methylation, polyamine and phospholipid biosynthesis, and protein, lipid and chromatin methylation (14,162). This might affect embryonic and fetal growth or could even result in congenital malformations.

It is possible that the difference in CRL can only be detected or is only present in the first 8-9 weeks after conception. An explanation is that due to the gradual dissolving trophoblastic plugs of the spiral arteries the nutritional environment of the embryonic tissues is massively changing through which the reflection of epigenetic embryonic effects are diluted and cannot be detected anymore. We have observed the same effect in our other studies as well (163,164). Nevertheless, the early effects on the embryo may still have long term consequences taking into account that the embryonic genome is epigenetically programmed (14,50,165). It is known that the periconception period is highly important and sensitive with respect to epigenetic programming of the gametes, embryo and placenta by DNA methylation of genes implicated in growth and development (14). Therefore, differences in this early time window can have long term health and disease consequences which is in line with the developmental origins of health and disease hypothesis. We have previously shown that embryonic growth is associated with subsequent fetal growth and birth weight (166). Mook-Kanamori et al. shows that first-trimester growth restriction is associated with increased risks of preterm birth, low birth weight, small size for gestational age at birth and postnatal growth acceleration until the age of 2 years (135). Furthermore, it has been reported that impaired first trimester fetal growth is associated with an adverse cardiovascular risk profile in school age children, which suggests that early fetal life might be a critical period for cardiovascular health in later life (167). The numbers in the current study were too small to address the association between embryonic growth and aforementioned adverse pregnancy outcomes.

The current study is based on self-reported periconception exposures. As a result, recall bias cannot be excluded. Since subfertile women have more difficulties to become pregnant and are possibly more aware of any adverse exposures, they may recall their exposures to potential risk factors better. On the other hand, social desired answers cannot be excluded. Recall bias is minimised by blinding the study participants to the hypothesis, however, it cannot be excluded. The generalisability of this study is restricted to pregnancies ending in live borns only. CRL data were not available for most miscarriages since there was no fetal heart action during the first ultrasound visit. The very few ultrasounds scans available were of poor quality whereby a precise measure of CRL could not be provided.

In conclusion, this prospective periconception birth cohort study shows that a maternal CV risk score is inversely associated with human embryonic growth in IVF/ICSI pregnancies. Using a CV risk assessment tool in preconception care could possibly identify mothers-to-be with an increased risk of impaired fetal growth (24). A population based study should be performed to investigate the external validity of our results as well as the association between maternal CV score and adverse pregnancy outcomes.

Supplementary material

Table S1 | Excluded pregnancies

Variable	Exclusions group 1 (n=69)	Exclusions group 2 (n=52)	Inclusions (n=138)
Maternal			
Age in years (mean ± SD)	33.2 (6.2)^{NS}	32.7 (4.9)^{NS}	31.8 (4.8)
Body mass index (kg/m^2)	24.6 (4.9)^{NS}	25.5 (5.0)^{NS}	24.3 (3.6)
Ethnicity			
Dutch	25 (36)^{NS}	39 (75)^{NS}	105 (76)
Western-other	3 (4)^{NS}	3 (6)^{NS}	13 (9)
Non-Western	11 (16)^{NS}	9 (17)^{NS}	19 (14)
Educational level			
Low	6 (9)^{NS}	6 (12)^{NS}	11 (8)
Intermediate	10 (14)^{NS}	16 (31)^{NS}	40 (29)
High	20 (29)^{NS}	26 (50)^{NS}	82 (59)
Folic acid supplement use			
No	0 (0)^{NS}	1 (2)^{NS}	1 (1)
Yes	39 (56)^{NS}	51 (98)^{NS}	137 (99)
Preconception initiation	32(46)^{NS}	41 (79)^{NS}	109 (79)
Periconception			
Smoking	5 (7)^{NS}	7 (13)^{NS}	24 (17)
Alcohol	17 (25)^{NS}	21 (40)^{NS}	64 (46)
Nulliparous	24 (35)^{NS}	28 (54)^{NS}	91 (66)
Primigravida	9 (13)^{NS}	14 (27)^{NS}	55 (40)
Conception			
Spontaneous	19 (27)$^{\#}$	39 (75)^{NS}	66 (48)
Assisted reproductive technology	5 (7)$^{\#}$	13 (25)^{NS}	45 (33)
Newborn			
Birth weight in grams (mean ± SD)	3095 (988)^{NS}	3330 (471)^{NS}	3305 (595)
Gestational age at delivery, weeks $^{+ \text{days}}$	37^{+2} (14^{+3}-41^{+1})$^{\#}$	39^{+0} (35^{+4}-41^{+3}) ^{NS}	39^{+1} (26^{+5}-42^{+0})
Male gender	9 (13)^{NS}	28 (54)^{NS}	63 (46)
Fetal complication			
Low birthweight (<2500g)	2 (3)^{NS}	2 (4)^{NS}	11 (8)
Premature delivery (<37 weeks)	4 (6)^{NS}	3 (6)^{NS}	10 (7)
SGA (<10th customised centile)	0 (0)^{NS}	1(2)^{NS}	11 (8)

Numbers may not add up to the total sample size due to missing values
Exclusions group 1: oocyte donation, miscarriage, ectopic pregnancy, unknown first day of the last menstrual period, missing questionnaires, fetal/neonatal death or congenital malformation
Exclusions group 2: maternal pre-existing diseases, past medical history or malformations
^{NS} No statistically significant difference between this excluded group and the included group
$^{\#}$ Statistically significant difference (*P* value <0.05) between this excluded group and the included group

Chapter 7

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Early life lipid profile and metabolic programming in very young children

Abstract

Background Lipid derangements during early postnatal life may induce stable epigenetic changes and alter metabolic programming. We investigated associations between serum lipid profiles in very young children and DNA methylation of tumor necrosis factor-alpha (*TNFα*) and leptin (*LEP*). Secondly, we explored if the maternal serum lipid profile modifies DNA methylation in the child.

Methods In 120 healthy children at 17 months of age, DNA methylation of *TNFα* and *LEP* was measured in DNA derived from whole blood. Linear mixed models were used to calculate exposure-specific differences and associations.

Results Total cholesterol in children was associated with decreased methylation of *TNF*α (-5.8%, *P*=0.036), and HDL-cholesterol was associated with decreased methylation of both *TNF*α (-6.9%, *P*=0.013) and *LEP* (-3.4%, *P*=0.021). Additional adjustment for gestational age at birth, birth weight, sex, breastfeeding and educational level attenuated the effects, *TNF*α (-6.1%, *P*=0.058) and *LEP* (-3.1%, *P*=0.041). In mothers, HDL-cholesterol only was associated with decreased methylation of *TNF*α in the child (-8.7%, *P*=0.001).

Conclusion Our data support the developmental origins of health and disease hypothesis by showing that total cholesterol and HDL-cholesterol levels in very young children are associated with epigenetic metabolic programming, which may affect their vulnerability for developing cardiovascular diseases in later life.

Introduction

Periconception, prenatal and early postnatal life conditions seem to contribute to the risk of developing late-onset diseases, including cardiovascular and metabolic diseases (168-171). Dyslipidaemia in childhood is becoming increasingly common and depending on the cut-off points used up to 20 percent has at least one abnormal lipid level (172). Poor lifestyles and nutrition are partly responsible for this problem. Cardiovascular risk factors, including dyslipidaemia in childhood, seem to contribute to the risk of developing cardiovascular disease (CVD) in adulthood (173). Not only the formation of lifestyle and diet habits but also developmental programming could be considered a potential mechanism (169,170,173).

The link between early life events and disease risk in later life is often described in relation to DNA methylation, an epigenetic mechanism important for development. Early childhood has been described as a critical developmental window during which perturbations are associated with intergenerational metabolic programming (174). Persons at age 50 exposed to the Dutch famine in early gestation have a more atherogenic lipid profile than those who are not exposed to famine (42). A number of genes are known to influence metabolic pathways, including the non-imprinted genes tumor necrosis factor-alpha (*TNFα*) and leptin (*LEP*). *TNFα* is a proinflammatory cytokine which has been associated with cardiovascular risk, lipid metabolism and insulin resistance (175). Leptin levels have been associated with obesity, hypertension, insulin resistance and type 2 diabetes mellitus. We have previously shown that *LEP* methylation in very young children is associated with the duration of breastfeeding and sex (176).

Whether derangements in serum lipid levels during early postnatal life may influence epigenetic regulation of gene expression and alter metabolic programming is currently unknown. We hypothesise that a derangement in biomarkers of the lipid profile during early postnatal life is associated with epigenetic changes and altered metabolic programming. Therefore, we studied in a cross-sectional study associations between serum lipid levels related to increased risks of CVD (total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol concentrations) and DNA methylation of *LEP* and *TNFα* in healthy young children at the age of 17 months. In addition, effect modification of maternal lipids was studied on the observed associations.

Methods

Study population

In a cross-sectional study, we investigated 120 healthy children (boys n=70, girls n=50) at a mean age of 17 months (SD 2.5). These subjects were previously described and served as controls in a case-control study on congenital heart disease (116). All children were recruited from the public child health care centres of 'Careyn' in the Rotterdam area in the Netherlands. Children were eligible as controls when they did not have a major congenital malformation or chromosomal abnormality according to the regular check up at the child health care centre up to the study moment.

Data collection

At the standardised study moment of 17 months, we collected data about exposures and conditions in the periconception, prenatal and postnatal period. Self-administered questionnaires were send before the hospital visit, which were checked by the researcher for completeness and consistency at the hospital. Extracted data were maternal folic acid supplement use, age and educational level, and child's gestational age at birth, age at study moment, gender, birth weight, duration of breastfeeding and ethnicity. During the hospital visit fasting venous blood samples were drawn from all mothers and nonfasting venous blood samples from all children. 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 4000 x *g* for 10 minutes at 4°C and separated within 1h. All samples were stored at -80°C and analysed anonymously in batches within five months after collection. Serum concentrations of total cholesterol, HDL-cholesterol and triglycerides were analysed using commercially available assays (Wako Diagnostics, Japan). HDL-cholesterol was analysed using the direct HDL assay from Wako Diagnostics. LDL-cholesterol values were estimated using the Friedewald formula (177).

DNA methylation measurements

DNA methylation of 2 genes associated with growth and metabolism was measured in 120 children. Genomic DNA was isolated from whole blood using the salting out method. One-half microgram of genomic DNA was bisulphite-treated using the EZ 96-DNA methylation kit (Zymo Research) on a total of two 96-well plates.

In the *LEP* gene, DNA methylation of seven CpG sites was measured in triplicate using a mass spectrometry-based method (EpiTYPER, Sequenom) (Supplementary Table S2). Methylation per CpG was used to estimate the mean methylation. In the *TNFa* gene, five CpG sites were measured in triplicate and used to estimate mean methylation. These measurements were part of a study on cardiometabolic loci.

Statistical analysis

We applied linear mixed models on the raw data without imputation of missing values to calculate exposure-specific differences and associations. All the analyses accounted for bisulphite plate and correlation between the CpG dinucleotides within a locus. CpG dinucleotides were entered simultaneously with the variable under study as fixed effect with overall DNA methylation as outcome. Serum lipid concentration levels were treated as continuous variables. Absolute changes in DNA methylation are presented as regression coefficient ß with standard error (SE). Z-scores have been calculated so that the resulting estimated effect sizes indicate the methylation change per standard deviation change. Relative changes in percentage of DNA methylation are calculated by dividing the mean methylation with the exposure (i.e. serum lipid levels) by mean methylation without the exposure. Additional analyses were performed adjusting for gestational age at birth, birth weight, sex, breastfeeding and educational level. Blood biomarker levels of children were additionally adjusted for maternal blood biomarker levels. Since multiple comparisons are made we applied Bonferroni correction. For power calculations we used a simplified model assuming a bivariate linear regression analysis with mean methylation as response and the blood biomarkers as

predictor, ignoring bisulphite batches. Since we performed a Bonferroni correction we used an alpha of 0.00625. We used a SD of 4% in mean methylation of *TNFα* and a SD of 3% in mean methylation of *LEP*. In *TNFα*, the power to detect 1% and 1.5% absolute methylation difference was 50% and 95% respectively. In *LEP*, the power to detect 1% and 1.5% absolute methylation difference was 85% and 99% respectively. All statistical analyses were done using IBM SPSS Statistics 20.0 for Windows software (IBM, Armonk, NY, USA).

Ethics committee approval

The study protocol was approved by the Central Committee for Human Research (CCMO) in The Hague, the Netherlands, and the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands. All mothers gave their written informed consent and mothers and their partner gave written informed consent on behalf of their participating child.

Results

Characteristics and methylation

The characteristics of the 120 mothers and children are shown in Table 1. Boys had a significantly higher birth weight, adjusted for gestational age, than girls.

Average DNA methylation in the child for *TNFα* and *LEP* was 17.3% and 23.6%, respectively. Boys had a significantly lower mean methylation of *LEP* than girls, 22.8% and 24.7%, respectively (*P*=0.010). Methylation of the separate CpG sites is shown in Supplementary Table 1. DNA methylation of *TNFα* and *LEP* was significantly correlated (Pearson's *r*=0.327, *P*=<0.001). The serum lipid profiles correlated between mothers and children significantly for total cholesterol (Pearson's *r*=0.311, *P*=0.001), HDL-cholesterol (Pearson's *r*=0.256, *P*=0.006) and LDL-cholesterol (Pearson's *r*=0.223, *P*=0.016), but not for triglycerides.

Total cholesterol, triglycerides and lipoproteins in the child

Total cholesterol in the child was significantly associated with decreased methylation of *TNF*α (-5.8%, *P*=0.036) (Table 2). Additional adjustment for HDL-cholesterol in the child attenuated the effect (-4.9%, *P*=0.072).

HDL-cholesterol was associated with decreased methylation of both *TNF*α (-6.9%, *P*=0.013) and *LEP* (-3.4%, *P*=0.021) (Figure 1). Adjustment for maternal HDL-cholesterol slightly attenuated the association between HDL-cholesterol in the child, *TNF*α (-4.9%, *P*=0.078), and *LEP* (-3.5%, *P*=0.023). Additional adjustment for gestational age at birth, birth weight, sex, breastfeeding and educational level further attenuated the effects, *TNF*α (-6.1%, *P*=0.058) and *LEP* (-3.1%, *P*=0.041). *TNF*α methylation was not associated with sex, maternal educational level or duration of breastfeeding. *LEP* methylation was significantly associated with sex and duration of breastfeeding, but not with maternal educational level. Additional adjustments for ethnicity did not change the results. Triglycerides and LDL-cholesterol were not significantly associated with DNA methylation. Bonferroni correction attenuated to nonsignificant estimates.

Table 1 | Child and mother characteristics

	All children (n=120)	Boys (n=70)	Girls (n=50)	*P* value[c]
Child				
Age (months)	17.0 (15.1-18.6)	17.2 (15.5-18.8)	17.0 (15.0-18.3)	0.409
Birth weight[a] (g)	3455 (3115-3818)	3545 (3185-3917)	3348 (3066-3666)	0.044
Mean gestational age at birth (weeks)	39.6 (1.83)	39.6 (1.88)	39.6 (1.78)	0.927
Total cholesterol (mmol/L)	3.86 (0.80)	3.83 (0.76)	3.90 (0.86)	0.652
Triglycerides (mmol/L)	1.22 (0.70)	1.21 (0.65)	1.24 (0.76)	0.795
HDL-cholesterol (mmol/L)	1.01 (0.26)	1.21 (0.65)	0.99 (0.20)	0.591
LDL-cholesterol (mmol/L)	2.30 (0.70)	2.27 (0.67)	2.35 (0.73)	0.556
Leptin (mmol/L)	2.82 (0.64)	2.71 (0.44)	2.96 (0.80)	0.068
Overall mean methylation at study moment[b]				
TNFα	17.3 (0.50)	17.7 (0.60)	16.8 (0.70)	0.369
LEP	23.6 (0.30)	22.8 (0.50)	24.7 (0.50)	0.010
Mother				
Age at birth (years)	30.9 (21.5-42.8)	31.0 (22.5-39.4)	30.7 (21.5-42.8)	0.711
Periconception folic acid supplement use	34 (28)	20 (29)	14 (28)	0.945
Total cholesterol (mmol/L)	4.77 (0.88)	4.78 (0.95)	4.75 (0.80)	0.837
Triglycerides (mmol/L)	1.03 (0.51)	1.07 (0.97)	0.97 (0.40)	0.225
HDL-cholesterol (mmol/L)	1.47 (0.35)	1.44 (0.34)	1.51 (0.36)	0.243
LDL-cholesterol (mmol/L)	2.83 (0.77)	2.86 (0.80)	2.80 (0.74)	0.671

HDL, high-density lipoprotein; *LDL,* low-density lipoprotein; *TNFα,* tumor necrosis factor-alpha; *LEP,* leptin.

[a] Adjusted for gestational age.

[b] Overall mean (SE) is estimated from the linear mixed model adjusted for bisulphite batch and CpG dinucleotides, accounting for the correlation between individual CpG dinucleotides. Sex mean (SE) is estimated from the linear mixed model adjusted for sex, bisulphite batch and CpG dinucleotides, accounting for the correlation between individual CpG dinucleotides.

[c] *P* values for comparisons between boys and girls.

Table 2 | Serum lipid profiles in the child and mother and methylation of *TNFα* and *LEP*

		TNFα methylation in children			*LEP* methylation in children		
	SD[a]	% absolute methylation change (SE)[b]	% relative methylation change (SE)	*P* value	% absolute methylation change (SE)[b]	% relative methylation change (SE)	*P* value
Blood biomarkers child							
Total cholesterol	0.80 mmol/L	-1.0 (0.5)	-5.8 (2.7)	0.036	-0.6 (0.3)	-2.4 (1.5)	0.111
Triglycerides	0.70 mmol/L	+0.1 (0.5)	+0.8 (2.8)	0.773	+0.1 (0.4)	+0.6 (1.5)	0.709
HDL-cholesterol	0.26 mmol/L	-1.2 (0.5)	-6.9 (2.7)	0.013	-0.8 (0.3)	-3.4 (1.5)	0.021
LDL-cholesterol	0.70 mmol/L	-0.8 (0.5)	-4.5 (2.7)	0.100	-0.4 (0.3)	-1.7 (1.5)	0.249
Blood biomarkers mother							
Total cholesterol	0.88 mmol/L	-0.4 (0.5)	-2.4 (2.7)	0.371	-0.3 (0.3)	-1.3 (1.5)	0.380
Triglycerides	0.51 mmol/L	+0.3 (0.5)	+1.8 (2.6)	0.485	-0.1 (0.3)	-0.5 (1.5)	0.743
HDL-cholesterol	0.35 mmol/L	-1.5 (0.4)	-8.7 (2.6)	0.001[#]	-0.2 (0.3)	-1.0 (1.5)	0.497
LDL-cholesterol	0.77 mmol/L	+0.1 (0.5)	+0.6 (2.7)	0.822	-0.2 (0.3)	-0.9 (1.5)	0.550

TNFα tumor necrosis factor-alpha; *LEP* leptin; *HDL* high-density lipoprotein; *LDL* low-density lipoprotein.

[a] Z-scores have been calculated so the resulting estimated effect size indicates the methylation change per standard deviation change.

[b] Values are the regression coefficients (β (SE)) from a linear mixed model adjusted for bisulphite batch and CpG dinucleotides, accounting for the correlation between individual CpG dinucleotides.

[#] Significant after Bonferroni correction *P* value 0.006.

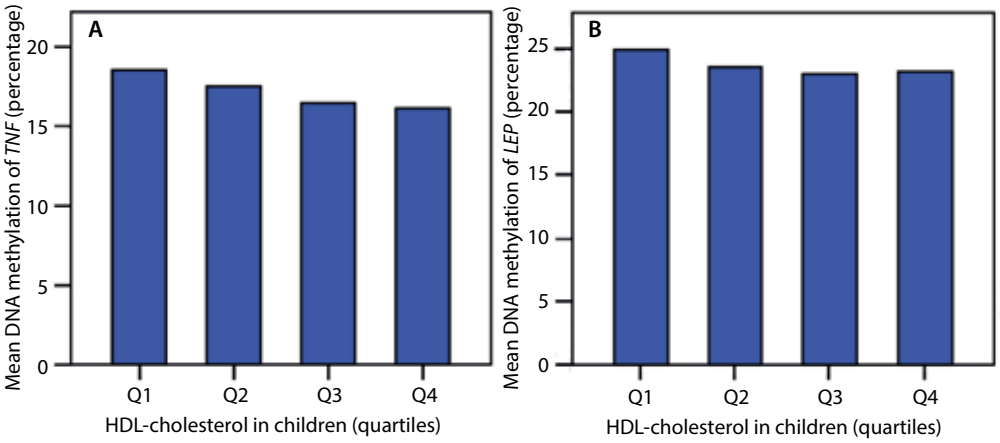

Figure 1 | HDL-cholesterol in children 17 months of age and DNA methylation in *TNFα* (A) and DNA methylation in *LEP* (B)

Total cholesterol, triglycerides and lipoproteins in the mother

HDL-cholesterol in the mother was associated with lower methylation of *TNF*α in the child (-8.7%, *P*=0.001) (Table 2). HDL-cholesterol in mothers and children was significantly correlated, but additional adjustment for HDL-cholesterol in the child did not substantially change the association with *TNF*α results (-7.5%, *P*=0.008).

Total cholesterol, triglycerides and LDL-cholesterol levels in mothers were not associated with DNA methylation in the child. Bonferroni correction did not significantly affect the association between HDL-cholesterol and *TNF*α methylation.

Discussion

In this cross-sectional study we observed in children at 17 months of age associations between HDL-cholesterol and decreased methylation of both *TNF*α and *LEP*, and between total cholesterol and decreased methylation of *TNF*α. Since total cholesterol consists of HDL-cholesterol, LDL-cholesterol and very low density lipoprotein-cholesterol and the association between total cholesterol and *TNF*α methylation diminished after adjusting for HDL-cholesterol, we conclude that the association between total cholesterol and DNA methylation is mainly driven by HDL-cholesterol.

Furthermore, we observed that maternal HDL-cholesterol was associated with decreased methylation of *TNF*α in the child, which did not modify the association between HDL-cholesterol in the child and *TNF*α methylation. Due to the cross-sectional design of our study we were not able to determine the direction of this association. Whether there is a link between maternal HDL and a child's HDL 17 months postnatal – as a result of a shared dietary pattern –or whether maternal HDL is a proxy for maternal HDL periconceptional entailing programming effects *in utero* remains to be elucidated.

We also observed that DNA methylation of *LEP* and *TNFα* was significantly correlated (Pearson's r=0.332, P=<0.001). It is suggested that *TNFα* is involved in the regulation of plasma leptin concentration in obese subjects, however evidence is inconclusive (178). *LEP* and *TNFα* methylation levels are previously described as epigenetic biomarkers and could predict in obese patients the response to a low-calorie diet, in which lower methylation levels predict more successful weight loss (179).

Hypercholesterolaemia in young children is associated with an increased risk of cardiovascular disease (173). Most research focuses mainly on the extremes as already pointed out by Gluckman et al. (180). However, here we report differences in epigenetic programming within the normal range of blood lipids. Since lower levels of leptin are related to cardiovascular and metabolic risks, such as obesity, it seems obvious that even within the normal range exposure for a long period of time to slightly higher HDL-cholesterol levels is associated with beneficially decreased *LEP* methylation. This will result in higher *LEP* expression and higher concentrations of leptin as shown by our group and others before (176). For *TNFα* the associations observed within the normal range of HDL-cholesterol seem to be different from those reported above this range (181). Since increased *TNFα* is associated with cardiovascular risk, we expected that higher HDL-cholesterol levels are associated with increased *TNFα* methylation and therefore lower expression of *TNFα*. However, this may not apply to HDL-cholesterol within the normal range of blood lipids. It has been reported that a complex nutriepigenomic network might regulate *TNFα* (182).

Our data are in line with current evidence for the effects of cardiovascular and metabolic risk factors on global and DNA hypomethylation. Hyperhomocysteinaemia has been associated with increased CVD risk, especially atherosclerosis (183). Hyperhomocysteinaemia has been related to global DNA hypomethylation, in which the methyl donor folate restores global DNA methylation to normal levels (184). Also umbilical cord blood homocysteine is inversely correlated with genome-wide methylation, assessed using mean *LINE-1* DNA methylation (185). With regard to lipids, Cash et al. showed that lower levels of *LINE-1* DNA methylation are associated with higher LDL-cholesterol and lower HDL-cholesterol levels in 355 adult Samoans (181). However, they dichotomised the data into risk levels of LDL-cholesterol and HDL-cholesterol. In our study group, only 5 children showed increased LDL-cholesterol and none of them decreased HDL-cholesterol levels according to the reference values used at the Erasmus MC clinical chemistry laboratory (normal range LDL 0.5-3.5 mmol/l and HDL 0.4-1.5 mmol/l).

Recent studies show that maternal cholesterol can be transported to the fetus via the placenta (186). It has been described that during pregnancy maternal total cholesterol concentrations were associated with birth weight, abdominal circumference and mid-upper arm circumference (187). Since both *LEP* and *TNFα* are non-imprinted genes which are demethylated postconceptional and remethylated from the early blastocyst stage onwards, epigenetic reprogramming in the early embryo under the influence of maternal lipid levels should also be considered. Maternal lipid levels 17 months after the index delivery as a proxy for periconceptional maternal lipid levels may reveal an epigenetic programming effect. Therefore, a programming effect of *in utero* exposure established at 17 months of age cannot be excluded. However, since DNA methylation is responsive to the environment in early postnatal life, also a direct programming effect of the child lipids should be considered.

In contrast to what has been observed by Tobi et al., methylation levels measured with the same technique and at the same laboratory in our very young children were lower for *LEP* (23.6% vs. 28.6%) and higher for *TNFα* (17.3% vs. 9.6%) (188). These differences may be explained by differences in age at the study moment and residual confounding by (un)known pre- and postnatal exposures. Differences in absolute levels are however not a problem when the associations between absolute differences remain the same. A limitation is that although we have previously shown the association between *LEP* methylation and leptin levels in the same study population of very young children, we were not able to measure the expression of *TNFα* (176).

Some strengths and limitations of this study need to be addressed. Due to ethical constraints we were not able to measure nonfasting blood biomarkers in children. However, small differences in lipid levels have been observed when measured in a fasting or nonfasting state (189). Moreover, a large variation in lipid levels has been observed across different age, sex, race and even over a period of time (190,191). Strength of our study is that we adjusted for these potential confounders which did not substantially affect the results. Also preterm birth is associated with less favorable lipid profiles (192). In our study population, 10 children were born premature. However, excluding these 10 children from the analyses showed comparable results (data not shown).

We measured DNA methylation in DNA derived from whole blood which is a heterogeneous collection of different cell types. Therefore, cell heterogeneity should be considered as a potential confounder of DNA methylation measurements (193). On the other hand, our genes of interest are housekeeping genes expressed in all tissues for which blood cell heterogeneity seems not a very important issue (113).

We describe several associations between lipid levels and altered DNA methylation, however, it is difficult to relate these findings to potential clinical consequences. The effects of alterations in DNA methylation are complex and biologic implications of small changes in DNA methylation are unknown. Unfortunately we were not able to measure gene expression. Furthermore, it has been reported that global DNA methylation levels persist within an individual from birth to age three which supports the belief that factors that influence global DNA methylation may confer long-term effects (194).

Finally, due to the cross-sectional design of the study we cannot infer causality and therefore also not exclude that methylation status influences serum lipid levels. Whether altered DNA methylation is induced through serum lipid levels *in utero* or postnatal remains to be elucidated.

In conclusion, our data support the developmental origins of health and disease hypothesis by showing that total cholesterol and HDL-cholesterol levels in very young children are associated with pre- or postnatally induced epigenetic metabolic programming, which may affect their cardiometabolic risk in later life. Due to the cross-sectional design of the study no causal relationship can be assessed and therefore the results have to be interpreted with caution. Further investigation and replication of these findings in both the normal and abnormal range of lipids is warranted to unravel potential mechanisms. Future health implications remain to be elucidated.

Supplementary material

Table S1 | Methylation of the CpG sites of *TNFα* and *LEP*

Locus	All children (n=120)	Boys (n=70)	Girls (n=70)
TNFα	**17.3 (0.50)**	**17.7 (0.70)**	**16.8 (0.70)**
#CpG 1.2.3	20.6 (0.61)	20.7 (0.86)	20.5 (0.84)
#CpG 5	28.0 (0.73)	28.6 (1.10)	27.1 (0.91)
#CpG 6	9.1 (0.49)	9.4 (0.68)	9.0 (0.71)
#CpG 9	17.9 (0.58)	18.2 (0.84)	17.5 (0.75)
#CpG 10	10.6 (0.60)	10.9 (0.76)	10.1 (0.96)
LEP	**23.6 (0.30)**	**22.8 (0.50)**	**24.6 (0.50)**
#CpG 1	25.1 (0.5)	24.5 (0.61)	25.9 (0.76)
#CpG 8	16.0 (0.6)	15.5 (0.64)	16.8 (1.04)
#CpG 16.17	15.6 (0.3)	14.7 (0.39)	16.86 (0.55)
#CpG 19.20.21	13.2 (0.3)	12.6 (0.36)	14.03 (0.40)
#CpG 22	45.1 (0.9)	43.9 (1.15)	46.9 (1.38)
#CpG 25	43.9 (0.8)	42.9 (1.06)	45.2 (1.26)
#CpG 27	6.3 (0.2)	6.1 (2.50)	6.4 (0.41)

Values are presented as mean (SE) per CpG site. The overall mean (SE) is estimated from the linear mixed model adjusted for bisulphite batch and CpG dinucleotides, accounting for the correlation between individual CpG dinucleotides. Sex mean (SE) is estimated from the linear mixed model adjusted for sex, bisulphite batch and CpG dinucleotides, accounting for the correlation between individual CpG dinucleotides.

Table S2 | Primer design

Gene Name	Genomic location CpG sites analysed	Primer forward Primer reverse
TNFα	chr6_qbl_hap2:2790712-2791113	GGGTATTTTTGATGTTTGTGTGTT
	CpG 1-3. 5. 6. 9. 10	CAATACTCATAATATCCTTTCCAAAAAA
LEP	chr7:127668290-127668646	GTTTTTGGAGGGATATTAAGGATTT
	CpG 1. 8. 16&17. 19-21. 22. 25. 27	CTACCAAAAAAAACCAACAAAAAAA

Chapter 8

General discussion

In this thesis we investigated CHD and CVD risk factors using periconception epidemiologic and epigenetic studies. This chapter discusses the methodological considerations, main findings and clinical implications. Finally, recommendations for future research are provided.

Methodological considerations

Study design

The majority of data used for this thesis were collected in the HAVEN study, a case-control study. In observational studies, a prospective cohort study is considered the gold standard. However, the relatively low prevalence of CHD, that is, 9 per 1000 live births makes a prospective preconception cohort study not feasible since a very large number of subjects is required. In order to study 250 case children with CHD almost 28.000 subjects should be included. Therefore, in case of a rare disease, a case-control study is an accepted valid alternative.

Since case-control studies are retrospective, they are susceptible to different types of bias. Selection bias is an important issue and may occur when cases are more likely to participate in the study. In the HAVEN study, the response rate was 74.7% for case families and 61.4% for control families. We had no permission from the medical ethical committee to collect data on non-responders and those who did not want to participate.

Information bias can arise from measurement error. Two common types are misclassification bias and recall bias (195). In order to minimise misclassification bias, children where included at a standardised study moment of 17 months of age. Most CHDs are detected and diagnosed during the first year of life. Therefore, the study moment in early childhood reduces the chance of misclassification. Case children were included in the period between 2003 and 2010. In the Netherlands, a standardised routine 20-week ultrasound scan ("SEO / Structureel Echografisch Onderzoek") has been introduced in 2007. From that moment the prenatal detection rate of CHD increased significantly resulting in both an increased total birth prevalence rate as well as a higher rate of terminated pregnancies (29). Since we included only a selection of eight different phenotypes, a selection of more severe phenotypes before 2007 is unlikely. Recall bias is minimised by blinding the study participants to the hypothesis. Furthermore, an empirical study on parental recall bias in a case-control study showed that parental recall is mostly nondifferential (196). This means that exposure misclassification is unrelated to the case status. However, it cannot be excluded that mothers with a child with a CHD are more likely to recall their exposure to potential risk factors. The study moment two years after the periconception period reflects the maternal biomarkers and nutritional status during the most sensitive window of CHD development (197). Finally, all self-administered questionnaires were checked by the researcher for completeness and consistency during the hospital visit. Also standardised anthropometric measurements including height, weight and blood pressure were performed in the hospital to limit measurement errors.

Study population

In this case-control family study children were enrolled together with their mother and father. Case children were recruited in collaboration with the departments of Paediatric Cardiology of the Erasmus MC, University Medical Centre in Rotterdam, Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam, the Netherlands. Although case children are recruited from four different university medical centres, there were only two paediatric cardiologists involved who diagnosed the children. Also, they were trained in the same medical centre. Diagnoses were confirmed by echocardiography and/or cardiac catheterisation and/or surgery after birth. Included CHD phenotypes comprised of pVSD, transposition of the great arteries, pulmonary valve stenosis, coarctation of the aorta, tetralogy of Fallot, atrioventricular septal defect, hypoplastic left heart syndrome and aortic valve stenosis. The selection of these phenotypes was based on experimental and epidemiological data that showed that hyperhomocysteinaemia and related gene-environment interactions are involved in the pathogenesis.

Control children were recruited in collaboration with the child health care centres of 'Careyn' in the Rotterdam area in the Netherlands. Child health care centres are part of the Dutch healthcare system were all newborns get a regular check-up by a physician at standardised moments on health, growth and development. Control children were eligible when they did not have a major congenital malformation or chromosomal abnormality according to the medical records and regular health checks by physicians of the public child health centres. To exclude the influence of a shared (epi) genetic background between family members cases and control families were unrelated.

Accuracy of the data

The accuracy of data refers to whether the measured value is the true value. Epigenome-wide DNA methylation was measured using the Illumina Infinium® HumanMethylation450 BeadChip (450k array). This array was released in 2011 and is an extension of the previous Illumina Infinium® HumanMethylation27 BeadChip (27k array). As a result of this extension, the 450K array uses two different types of chemical assays (Infinium Type I and Infinium Type II) (198). Since the 450K is relatively cheap and covers >480,000 CpG loci it is used by many researchers for epigenome-wide association studies. However, with an exponential increase in 450K data and more experience in analysing the 450K array over the last years, some disadvantages are reported. Type I and Type II probes are not directly comparable and correction for this probe-design is required (198). Multiple correction strategies have become available. We used the *dasen* procedure to normalise methylation data. This method involves background adjustment of the methylated and unmethylated intensities followed by separate quantile normalisation of methylated Type I, unmethylated Type I, methylated Type II and unmethylated Type II intensities (83). The 450K array covers 99% of RefSeq genes with an average of 17 CpG sites per gene region which makes it very comprehensive, however, within the rapid growing research field of epigenetics the 450K will soon be replaced by the Infinium® MethylationEPIC BeadChip which covers >850,000 CpG loci.

To increase accuracy of the methylation data in our candidate genes, DNA methylation was measured in triplicate using a mass spectrometry based method (EpiTYPER, Sequenom) in a laboratory with extensive experience. CpG loci containing fragments of too little or high mass for the mass spectrometer to resolve were excluded from the analysis.

Blood vs. Target tissue

For clinical settings, DNA methylation would be most relevant if it could be measured in easily accessible tissue such as leukocytes. Studies suggest that DNA methylation in blood is overall highly correlated with DNA methylation in internal tissues, however tissue-specific methylation patterns and differences in CpG loci across tissues are reported (112,199). However, in a human study using the Illumina Infinium® HumanMethylation27 and HumanMethylation450 BeadChip it was observed for certain CpG sites that methylation levels that are higher in the target tissue (such as heart tissue) than surrogate tissue (such as blood) in one individual, are on average also higher methylated in the target tissue than the surrogate tissue in other individuals (112). Also, the magnitude of such differences is similar across individuals.

Main results

Transgenerational effects and the risk of congenital heart diseases

In **Chapter 2** we observed that grandparents with CVD are at increased risk of having a grandchild with CHD. We propose that the underlying mechanism could be that CVD and CHD share variations in vascular and metabolic pathways in which poor nutrition and lifestyle (shared environment) will affect epigenetic (re)programming of the germ line (i.e. grandparents) and embryonic cardiac tissue (i.e. grandchild).

There are several epidemiologic studies providing evidence for transgenerational epigenetic inheritance, and also animal studies suggest that environmental exposures do have the ability to affect up to the fourth generation (200). Looking at transgenerational effects it is important to distinguish between intergenerational inheritance and true transgenerational inheritance. The impact of *in utero* exposure can affect not only the developing embryo but also its germline (which is the DNA blueprint for the grandchildren) (Figure 1) (201). Intergenerational effects are therefore defined as parental of grandparental exposures. Only when effects are shown in great-grandchildren (F3 generation) without direct exposure it could indicate transgenerational inheritance. Within our study we did not observe significant associations in the stratified analysis for great-grandparents and therefore could not show transgenerational effects. Hence, we can only conclude that the association observed between grandparents with CVD and grandchildren with CHD shows a pattern of intergenerational inheritance.

Periconception parental factors and the risk of congenital heart diseases

In **Chapter 3** we observed four parental conditions that increase the risk of CHD in the child. That is, maternal use of medication, advanced age and positive family history of CHD, and paternal exposure to phthalates. These risk factors – except a positive family history – have been associated with derangements of one-carbon metabolism which is an underlying mechanism of CHD. Multiple drug exposures have been linked to CHDs in the past (31). However, we were not able to verify our medication data in pharmacy records and could not exclude possible recall bias or type II errors. We consider medication use as a measure of health, although it can antagonise enzymes in folic acid

metabolism, or influence folic acid absorption or degradation and inhibit the folic acid methylation cycle, which in turn might increase the risk of CHD in the child.

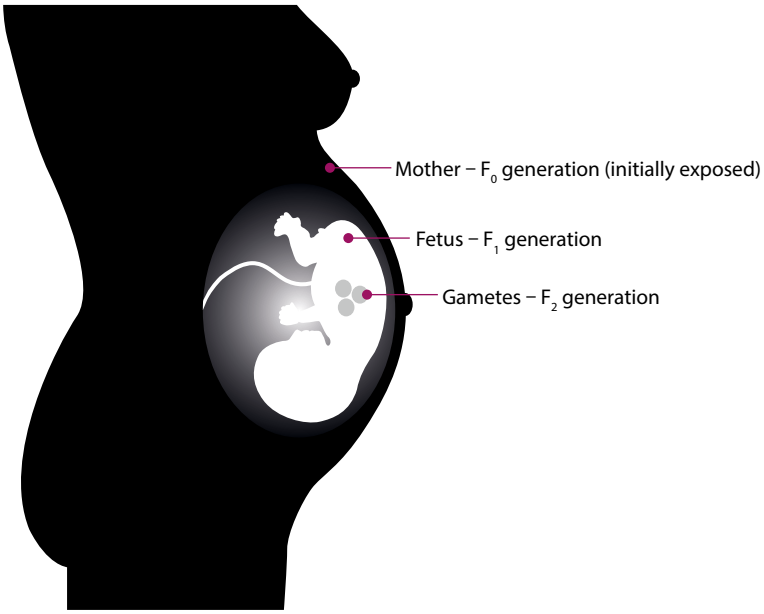

Figure 1 | Intergenerational effects: maternal exposure extending to herself, the fetus and the gametes

Advanced maternal age has been described before as a risk factor for CHD in a study of over 1 million subjects (202). Advanced maternal age can alter one-carbon metabolism and lead to moderate to severe hyperhomocysteinaemia by reducing the bioavailability of substrates, cofactors and intermediates, and that way increase the risk of CHD (35). Adkins et al. investigated the association between parental age and DNA methylation in the newborn by using the 27K methylation array and pyrosequencing replication. They found that levels of DNA methylation in umbilical cord blood are strongly correlated with maternal age with a general trend towards hypomethylation of CpG islands with increasing age. Proposed mechanisms underlying this differences are changes in DNA methylation of the oocytes which could influence disease risk in the child.

We have shown that paternal exposure to phthalates is associated with an increased risk of pVSD in the child. This has been described by Snijder et al. before (65). Occupations probably exposed to phthalates and therefore at risk are plastic workers, painters, electricians, hairdressers and printers. Recently, Wang et al. have described the same association (203). Evidence is growing that endocrine disrupting chemicals including phthalates have the ability to alter the human epigenome (204). It remains to be elucidated whether this will result in intergenerational or transgenerational disease risk.

DNA methylation in young children

The epigenetic studies described in this thesis include both candidate-gene studies as well as hypothesis-free studies. In **Chapter 4** and **Chapter 5** we explored epigenome-wide DNA methylation using a hypothesis-free approach to study novel epigenetic marks associated with nonsyndromic and syndromic pVSD. In **Chapter 7** we studied DNA methylation of candidate-genes in association with serum lipid levels.

The interpretation of EWAS data is complicated due to the complex interplay between genetic and environmental exposures (205). The differences in DNA methylation described in this thesis are small (<7%). Whether these differences will result in aberrant gene expression and have functional relevance has to be shown. It is thought that epigenetic mechanisms like DNA methylation contribute to the development of a "thrifty phenotype" (prenatal and early life exposures influence the development of adult diseases) and small epigenetic alterations (epigenetic fine-tuning) accumulatively affect metabolic pathways (206). Hence, in case of adverse exposures *in utero* it is feasible that the epigenetic modifications like DNA methylation are small. It is likely that CHD aetiology is the result of a combination of multiple small risk factors including CHD-associated epigenetic modifications.

Cardiovascular risk factors and embryonic growth

In **Chapter 6** we observed that mothers with a stronger CVD risk profile and conceiving after IVF/ICSI treatment had a significant higher risk of a smaller embryo during early pregnancy. Future research will have to demonstrate whether the observed differences in embryonic growth in our study will translate into differences in fetal growth and birth weight. The implications of impaired embryonic growth on both short and long term are currently unknown. It has been described that embryonic growth is positively associated with birth weight, which in turn is associated with health in later life (135,166,207). Following the developmental origins of health and disease paradigm linking birthweight to lifelong health, a smaller embryo is associated with lower birthweight and has an increased risk of chronic diseases like CVD (208).

Clinical implications and future perspectives

In order to move epigenetics from scientific research to clinical practice many steps have to be made. It should be noticed that aberrant DNA methylation can provide insides into many aspects of biology and pathophysiology, however, DNA methylation could very well function as a biomarker. That means that aberrant DNA methylation could predict disease risk, however it does not have to be causally involved in the disease (209). Within epigenetic epidemiology, a causal relationship between DNA methylation and the outcome cannot be proven. Animal studies could be helpful to further unravel the potential role of aberrant DNA methylation such as *PRDM16* in CHD pathophysiology, since it remains to be elucidated whether the described small methylation differences will result in aberrant gene expression and phenotype. It is crucial that our findings are replicated in other studies to eliminate false positive outcomes. Unfortunately, we were not able to find an independent study

with the same phenotype and methylation data available and could therefore not replicate our findings. (Epi)genetic manipulation of *PRDM16* in animal models is a suggested solution to provide more scientific evidence for the role of *PRDM16* in pVSD development.
Epigenetic epidemiology does often not provide the evidence that makes it directly useful in clinical practice in terms of diagnosis or treatment. However, valorisation of knowledge coming from an emerging field like epigenetic epidemiology is critical to improve perinatal health by pursuing primary prevention (210). Herein lies the responsibility of the scientific community to inform both health care providers as well as the target population. All couples planning pregnancy should have access to evidence-based preconception care in order to be informed about modifiable and non-modifiable risk factors, even though the attributable risk may seem small for the individual at first sight. In the Netherlands, this preconception care is available to all couples planning pregnancy through the mobile phone platform www.slimmerzwanger.nl ('Smarter Pregnancy') which provides an online personal coaching program focusing on diet and lifestyle habits (24).

General conclusion

To further improve clinical practice in CHDs, not only research and care, but also genetics and epigenetics should collaborate strongly. The complex relationship between epigenetic mechanisms such as DNA methylation and the pathophysiology of CHDs remains to be unravelled. This thesis is a new starting point for future research on CHD. We provide new DNA methylation loci associated with pVSD and propose that methylation of *PRDM16* is involved in pVSD pathophysiology in both nonsyndromic children and children with Down syndrome. Parental nongenetic risk factors are described, which enables health care professionals to provide better counselling on CHD risk factors. The observations in this thesis emphasise the importance of periconception care in relation to CHD and embryonic growth. The potential reversibility of epigenetics offers interesting possibilities for primary and secondary prevention and treatment.

Chapter 9

Summary

Samenvatting

Summary

Congenital heart diseases (CHD) are the most common birth defects worldwide with over 1 million children born each year. The origin is largely unknown and approximately only 15% can be traced to a known cause. The aims of this thesis were to study environmental and epigenetic determinants in association with CHD. Furthermore, a first approach was done to study the intergenerational effects of these exposures during early development. Furthermore, we investigated CVD risk factors which are common in women of reproductive age and can have an impact on pregnancy course and outcome.

In the introduction we provide the background for this thesis (**Chapter 1**). Most studies were conducted within the HAVEN study, a Dutch acronym of the case-control study designed to investigate determinants in the pathogenesis of CHDs and conducted in the western part of the Netherlands. The study described in **Chapter 6** is based on the Rotterdam Periconception Cohort (Predict study).

In the first part of this thesis we associate different determinants with the pathogenesis of CHDs.

In **Chapter 2** we investigated grandparents and their grandchildren. We showed that grandparents suffering from CVD have a significant increased risk of having a grandchild with a CHD. This risk was particularly increased in paternal grandfathers (father's father) with CVD. Having two or more grandparents with CVD was associated with an approximately threefold risk for CHD grandchildren.

In **Chapter 3** we investigated the association between nongenetic parental conditions and the risk of having a child with pVSD. Maternal use of medication, advanced age and a positive family history of CHD increased the risk of having a child with a pVSD. Paternal exposure to phthalates also increased this risk. Preconception counselling is an opportunity to identify these risk conditions which are partially modifiable and as such can contribute to the future prevention of pVSD.

In **Chapter 4** we present the results of a hypothesis-free epigenome-wide association study to investigate novel CpG loci in association with pVSD. We used a methylation microarray to study over 450,000 CpG loci. In leukocytes, we identified eight CpG loci with differential methylation in relation with pVSD. The most significant CpG was mapped to *PRDM16*. We validated this CpG with bisulphite pyrosequencing. Based on these findings and previous literature *PRDM16* is suggested to play a potential role in the pathogenesis of pVSD. *PRDM16* functions as a repressor of *TGFß* signaling which is crucial during embryonic development. Also the other CpG loci are mapped to genes involved in cardiogenesis and embryogenesis. Although this epidemiologic study cannot demonstrate the causality of these associations it provides enough evidence to substantiate these findings in future (animal) studies.

In **Chapter 5** we studied pVSD associated CpG loci from the previous epigenome-wide association study in children with pVSD and Down syndrome (DS) (trisomy 21). Approximately 50% of children born with DS have a CHD. Our data suggested that the CpG loci associated with nonsyndromic pVSD may also be involved in the pathogenesis of pVSD in DS. Furthermore, children with DS showed global hypermethylation in all autosomes except chromosome 21. This study suggests that the underlying mechanism of pVSD in children with and without DS share a common factor.

In the second part of this thesis we studied exposures to CVD risk factors during the periconception period.

In **Chapter 6** we investigated a maternal CVD risk profile including age, body-mass index, CVD in the family, diet and smoking in association with embryonic growth. Mothers with a strong CVD risk profile and a pregnancy conceived after IVF/ICSI treatment had a significant higher estimated risk of a smaller embryo during early pregnancy. This association was not found in women with a strong CVD risk profile and spontaneous conceived pregnancy. This dissimilarity might be explained by differences in pregnancy related factors such as sensitivity of endometrial, endothelial, placental and embryonic tissues.

In **Chapter 7** we investigated serum lipid levels in young children and their mothers and the relation with DNA methylation of the candidate-genes *TNFα* and leptin. We showed that total cholesterol and HDL-cholesterol in children is associated with aberrant DNA methylation of *TNFα* and leptin. In mothers, HDL-cholesterol was associated with decreased methylation of *TNF*α in the child. We suggest that early epigenetic metabolic programming in young children may affect their vulnerability of developing CVD in later life.

In the general discussion we interpret the findings of the studies described and their implications for clinical practice (**Chapter 8**). We also provide a starting point for future research including new DNA methylation loci associated with pVSD. The results reported in this thesis emphasise the importance of healthy periconception nutrition and lifestyle and suggest a potential role for epigenetic changes in CHD. Furthermore, maternal CVD risk factors seem to have an impact on the early development of the embryo conceived after IVF/ICSI treatment.

Samenvatting

Congenitale hartafwijkingen zijn de meest voorkomende aangeboren afwijking ter wereld en komen voor bij meer dan 1 miljoen pasgeboren kinderen per jaar. De oorzaak is meestal onbekend. In slechts ongeveer 15% van de kinderen kan de oorzaak worden achterhaald. Het doel van dit proefschrift was om verschillende omgevingsdeterminanten en epigenetische variaties in relatie tot congenitale hartafwijkingen (CHA) te onderzoeken. De intergenerationele effecten van deze blootstellingen gedurende de vroege ontwikkeling worden beschreven. Daarnaast worden factoren onderzocht die het risico op cardiovasculaire ziekten verhogen. Deze cardiovasculaire risicofactoren komen steeds vaker voor en kunnen bij vrouwen van vruchtbare leeftijd invloed hebben op het verloop en de uitkomst van de zwangerschap.

De achtergrond van dit proefschrift wordt beschreven in de introductie (**Hoofdstuk 1**). De meeste studies werden uitgevoerd binnen de HAVEN studie (Hart Afwijkingen, Vasculaire status, Erfelijkheid en Nutriënten), een patiënt-controle onderzoek uitgevoerd in de regio West Nederland. De studie beschreven in **Hoofdstuk 6** is gebaseerd op onderzoeksgegevens uit het Rotterdam Periconceptie Cohort (Predict studie).

In het eerste gedeelte van dit proefschrift wordt het onderzoek beschreven naar nieuwe oorzaken van CHA.

In **Hoofdstuk 2** zijn de grootouders en kleinkinderen onderzocht. Grootouders met cardiovasculaire ziekten lijken een significant hoger risico te hebben op het krijgen van een kleinkind met een CHA. Dit risico was het hoogst wanneer de paternale grootvader (vader van vader) cardiovasculaire ziekten had. Het risico op een kleinkind met een CHA was bijna driemaal verhoogd wanneer meer dan twee grootouders cardiovasculaire ziekten hadden.

In **Hoofdstuk 3** onderzochten we het verband tussen niet-genetische risicofactoren van de ouders en het risico op het krijgen van een kind met een pVSD. We toonden aan dat blootstelling van het kind tijdens de vroege ontwikkeling aan een viertal specifieke ouderlijke factoren het risico op een pVSD verhoogd. Een moeder die medicatie gebruikt, van gevorderde leeftijd is of met een positieve familie-anamnese voor CHA, heeft een hoger risico op het krijgen van een kind met een pVSD. Vaders die worden blootgesteld aan ftalaten (weekmaker) hebben ook een verhoogd risico. Deze risicofactoren zijn deels te vermijden door het aanbieden van preconceptionele counseling.

In **Hoofdstuk 4** presenteren we de resultaten van een hypothese-vrije epigenoom-wijde associatiestudie. Hiervoor werden meer dan 450,000 CpG loci getest in relatie tot het pVSD. In witte bloedcellen werden acht CpG loci gevonden die variaties vertoonden in DNA methylering en geassocieerd zijn met het pVSD. De meest significante CpG bevond zich in het *PRDM16* gen. Deze bevinding werd bevestigd met een andere meetmethode (bisulphite pyrosequencing). Op basis van deze nieuwe bevindingen en eerder onderzoek beschreven in de literatuur zou *PRDM16* mogelijk betrokken kunnen zijn bij het ontstaan van een pVSD doordat het onder andere het *TGFß* signaal onderdrukt, een cruciale cytokine tijdens de embryonale ontwikkeling. Ook de overige gevonden CpG loci bevinden zich in genen die op basis van eerder onderzoek betrokken lijken bij hartontwikkeling en embryogenese. De causaliteit van deze bevindingen kan niet worden

aangetoond op basis van dit epidemiologische onderzoek. Het is daarom noodzakelijk dat deze bevindingen worden bevestigd in andere (dier)studies.
In **Hoofdstuk 5** hebben we de eerder gevonden CpG loci uit Hoofdstuk 4 bestudeerd in kinderen met pVSD en het syndroom van Down (trisomie 21). Ongeveer 50% van de kinderen met het syndroom van Down wordt geboren met een hartafwijking. De eerder gevonden CpG loci in kinderen met een geïsoleerde (niet-syndromale) pVSD lijken ook geassocieerd te zijn met pVSD in het syndroom van Down. Opvallend was de globale hypermethylering in alle autosomen behalve chromosoom 21 in deze kinderen. Dit onderzoek suggereert overeenkomsten in het onderliggende ontstaansmechanisme van geïsoleerde pVSDs als dat bij kinderen met het syndroom van Down.

In het tweede gedeelte van dit proefschrift bestudeerden wij de invloed van blootstelling aan cardiovasculaire risicofactoren in de periconceptionele periode.
In **Hoofdstuk 6** werden vrouwen met een sterk cardiovasculair risicoprofiel geïdentificeerd met behulp van een maternale cardiovasculaire risicoscore gebaseerd op leeftijd, body-mass index, hart- en vaatziekten in de familie, dieet en roken. Moeders met een hoger cardiovasculair risicoprofiel die zwanger waren na een IVF/ICSI behandeling hadden een significant hoger risico op een kleiner embryo in de eerste weken van de zwangerschap. Dit was niet het geval bij moeders met een sterk cardiovasculair risicoprofiel en een spontane zwangerschap.
In **Hoofdstuk 7** onderzochten we het serumgehalte van lipiden in jonge kinderen en hun moeders en de relatie met DNA methylering van de kandidaatgenen *TNFα* en leptine. Dit onderzoek liet zien dat totaal cholesterol en HDL-cholesterol waarden in jonge kinderen geassocieerd lijken met veranderingen in DNA methylering van *TNFα* en leptine. Het HDL-cholesterol van de moeder was geassocieerd met minder methylering van *TNFα* in het kind. Deze epigenetische metabole programmering zou het kind meer kwetsbaar kunnen maken voor het ontwikkelen van cardiovasculaire ziekten later in het leven.

In de discussie gaan we in op de interpretatie van de belangrijkste bevindingen van dit proefschrift en de implicaties voor de klinische praktijk (**Hoofdstuk 8).** De gevonden CpG loci bieden aanknopingspunten voor toekomstig onderzoek naar CHA. De resultaten van dit proefschrift benadrukken het belang van gezonde periconceptionele voeding en leefstijl en suggereren dat epigenetische veranderingen een mogelijke rol spelen bij het ontstaan van CHA. Daarnaast zijn cardiovasculaire risicofactoren van de moeder van invloed op de vroege ontwikkeling en groei van het embryo.

Addendum

References

Authors and affiliations

List of abbreviations

Bibliography

PhD portfolio

About the author

Dankwoord

References

1. March of Dimes Birth Defects Foundation. *Global Report on Birth Defects. The Hidden Toll of Dying and Disabled Children*.White Plains, NY: March of Dimes Birth Defects Foundation, 2006.
2. van der Linde D, Konings EE, Slager MA, Witsenburg M, Helbing WA, Takkenberg JJ, et al. Birth prevalence of congenital heart disease worldwide: a systematic review and meta-analysis. J Am Coll Cardiol. 2011;58(21):2241-7.
3. Khairy P, Ionescu-Ittu R, Mackie AS, Abrahamowicz M, Pilote L, Marelli AJ. Changing mortality in congenital heart disease. J Am Coll Cardiol. 2010;56(14):1149-57.
4. Zomer AC, Vaartjes I, Uiterwaal CS, van der Velde ET, Sieswerda GJ, Wajon EM, et al. Social burden and lifestyle in adults with congenital heart disease. Am J Cardiol. 2012;109(11):1657-63.
5. Crossland DS, Jackson SP, Lyall R, Burn J, O'Sullivan JJ. Employment and advice regarding careers for adults with congenital heart disease. Cardiol Young. 2005;15(4):391-5.
6. Penny DJ, Vick GW, 3rd. Ventricular septal defect. Lancet. 2011;377(9771):1103-12.
7. Sommer RJ, Hijazi ZM, Rhodes JF, Jr. Pathophysiology of congenital heart disease in the adult: part I: Shunt lesions. Circulation. 2008;117(8):1090-9.
8. Carlson BM. Human Embryology and Developmental Biology. Third edition. Mosby, Inc.; 2004.
9. Botto LD, Correa A. Decreasing the burden of congenital heart anomalies: an epidemiologic evaluation of risk factors and survival. Progress in Paediatric Cardiology. 2003;18:111-21.
10. World Health Organization. Cardiovascular Disease. http://www.who.int/cardiovascular_diseases/en/.
11. Lloyd-Jones DM, Hong Y, Labarthe D, Mozaffarian D, Appel LJ, Van Horn L, et al. Defining and setting national goals for cardiovascular health promotion and disease reduction: the American Heart Association's strategic Impact Goal through 2020 and beyond. Circulation. 2010;121(4):586-613.
12. Centers for Disease Control and Prevention: Preventing and Managing Chronic Disease to Improve the Health of Women and Infants. http://198.246.124.29/reproductivehealth/WomensRH/PDF/ChronicDisease_FactSheet.pdf.
13. Barker DJ, Eriksson JG, Forsen T, Osmond C. Fetal origins of adult disease: strength of effects and biological basis. Int J Epidemiol. 2002;31(6):1235-9.
14. Steegers-Theunissen RP, Twigt J, Pestinger V, Sinclair KD. The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. Hum Reprod Update. 2013;19(6):640-55.
15. Czeizel AE, Dudas I, Vereczkey A, Banhidy F. Folate deficiency and folic acid supplementation: the prevention of neural-tube defects and congenital heart defects. Nutrients. 2013;5(11):4760-75.
16. Obermann-Borst SA, Vujkovic M, de Vries JH, Wildhagen MF, Looman CW, de Jonge R, et al. A maternal dietary pattern characterised by fish and seafood in association with the risk of congenital heart defects in the offspring. BJOG. 2011;118(10):1205-15.
17. Tai CG, Graff RE, Liu J, Passarelli MN, Mefford JA, Shaw GM, et al. Detecting gene-environment interactions in human birth defects: Study designs and statistical methods. Birth Defects Res A Clin Mol Teratol. 2015;103(8):692-702.
18. Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. Science. 1975;187(4173):226-32.
19. Riggs AD. X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet. 1975;14(1):9-25.
20. Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, Shen R, et al. Genome-wide DNA methylation profiling using Infinium(R) assay. Epigenomics. 2009;1(1):177-200.
21. Gibney ER, Nolan CM. Epigenetics and gene expression. Heredity (Edinb). 2010;105(1):4-13.
22. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. Science. 2001;293(5532):1089-93.
23. Saffery R. Epigenetic change as the major mediator of fetal programming in humans: Are we there yet? Ann Nutr Metab. 2014;64(3-4):203-7.

24. Steegers-Theunissen RPM, Steegers EAP. Embryonic health: new insights, mHealth and personalised patient care. Reproduction Fertility and Development. 2015;27(4):712-5.
25. A-Z of Public Health. Laverack G. Basingstoke: Palgrave Macmillan, 2014.
26. Hill AB. The Environment and Disease: Association or Causation? Proc R Soc Med. 1965;58:295-300.
27. Steegers-Theunissen RP, Verheijden-Paulissen JJ, van Uitert EM, Wildhagen MF, Exalto N, Koning AH, et al. Cohort Profile: The Rotterdam Periconceptional Cohort (Predict Study). Int J Epidemiol. 2015. [Epub ahead of print]
28. Thornburg KL. The programming of cardiovascular disease. Journal of developmental origins of health and disease. 2015;6(5):366-76.
29. World Health Organization, Fact sheet N°317 Cardiovascular Diseases, March 2013. http://www.who.int/mediacentre/factsheets/fs317/en/. Last accessed August 20, 2016.
30. March of Dimes Birth Defects Foundation. Global Report on Birth Defects. The Hidden Toll of Dying and Disabled Children. White Plains, NY: March of Dimes Birth Defects Foundation. 2006.
31. Jenkins KJ, Correa A, Feinstein JA, Botto L, Britt AE, Daniels SR, et al. Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American Heart Association Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. Circulation. 2007;115(23):2995-3014.
32. Steegers-Theunissen RP, Steegers EA. Nutrient-gene interactions in early pregnancy: a vascular hypothesis. Eur J Obstet Gynecol Reprod Biol. 2003;106(2):115-7.
33. Smedts HP, van Uitert EM, Valkenburg O, Laven JS, Eijkemans MJ, Lindemans J, et al. A derangement of the maternal lipid profile is associated with an elevated risk of congenital heart disease in the offspring. Nutr Metab Cardiovasc Dis. 2012;22(6):477-85.
34. Hobbs CA, Cleves MA, Melnyk S, Zhao W, James SJ. Congenital heart defects and abnormal maternal biomarkers of methionine and homocysteine metabolism. Am J Clin Nutr. 2005;81(1):147-53.
35. Verkleij-Hagoort AC, Verlinde M, Ursem NT, Lindemans J, Helbing WA, Ottenkamp J, et al. Maternal hyperhomocysteinaemia is a risk factor for congenital heart disease. BJOG. 2006;113(12):1412-8.
36. Fruchart JC, Nierman MC, Stroes ES, Kastelein JJ, Duriez P. New risk factors for atherosclerosis and patient risk assessment. Circulation. 2004;109(23 Suppl 1):III15-9.
37. Ingelsson E, Schaefer EJ, Contois JH, McNamara JR, Sullivan L, Keyes MJ, et al. Clinical utility of different lipid measures for prediction of coronary heart disease in men and women. JAMA. 2007;298(7):776-85.
38. Kaati G, Bygren LO, Edvinsson S. Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. Eur J Hum Genet. 2002;10(11):682-8.
39. Kaati G, Bygren LO, Pembrey M, Sjostrom M. Transgenerational response to nutrition, early life circumstances and longevity. Eur J Hum Genet. 2007;15(7):784-90.
40. Pembrey ME. Male-line transgenerational responses in humans. Hum Fertil (Camb). 2010;13(4):268-71.
41. Roseboom TJ, van der Meulen JH, Osmond C, Barker DJ, Ravelli AC, Schroeder-Tanka JM, et al. Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45. Heart. 2000;84(6):595-8.
42. Roseboom TJ, van der Meulen JH, Osmond C, Barker DJ, Ravelli AC, Bleker OP. Plasma lipid profiles in adults after prenatal exposure to the Dutch famine. Am J Clin Nutr. 2000;72(5):1101-6.
43. Jansen AC, van Aalst-Cohen ES, Tanck MW, Trip MD, Lansberg PJ, Liem AH, et al. The contribution of classical risk factors to cardiovascular disease in familial hypercholesterolaemia: data in 2400 patients. J Intern Med. 2004;256(6):482-90.
44. Centers for Disease Control and Prevention: National Health Interview Survey. http://www.cdc.gov/nchs/nhis/quest_data_related_1997_forward.htm. Last accessed July 2, 2013.
45. Verkleij-Hagoort AC, de Vries JH, Ursem NT, de Jonge R, Hop WC, Steegers-Theunissen RP. Dietary intake of B-vitamins in mothers born a child with a congenital heart defect. Eur J Nutr. 2006;45(8):478-86.
46. Steegers-Theunissen RP. Inaugural lecture: 'New life in a changing environment'. 2010.
47. van der Bom T, Zomer AC, Zwinderman AH, Meijboom FJ, Bouma BJ, Mulder BJ. The changing epidemiology of congenital heart disease. Nat Rev Cardiol. 2011;8(1):50-60.

48. Pierpont ME, Basson CT, Benson DW, Jr., Gelb BD, Giglia TM, Goldmuntz E, et al. Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American Heart Association Congenital Cardiac Defects Committee, Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. Circulation. 2007;115(23):3015-38.

49. Carone BR, Fauquier L, Habib N, Shea JM, Hart CE, Li R, et al. Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. Cell. 2010;143(7):1084-96.

50. Nafee TM, Farrell WE, Carroll WD, Fryer AA, Ismail KM. Epigenetic control of fetal gene expression. BJOG. 2008;115(2):158-68.

51. Hanson MA, Gluckman PD. Developmental origins of health and disease: new insights. Basic Clin Pharmacol Toxicol. 2008;102(2):90-3.

52. Moons P, Van Deyk K, Dedroog D, Troost E, Budts W. Prevalence of cardiovascular risk factors in adults with congenital heart disease. Eur J Cardiovasc Prev Rehabil. 2006;13(4):612-6.

53. Hopper JL, Bishop DT, Easton DF. Population-based family studies in genetic epidemiology. Lancet. 2005;366(9494):1397-406.

54. Li X, Li S, Mu D, Liu Z, Li Y, Lin Y, et al. The association between periconceptional folic acid supplementation and congenital heart defects: A case-control study in China. Prev Med. 2013;56(6):385-9.

55. Cresci M, Foffa I, Ait-Ali L, Pulignani S, Gianicolo EA, Botto N, et al. Maternal and paternal environmental risk factors, metabolizing GSTM1 and GSTT1 polymorphisms, and congenital heart disease. Am J Cardiol. 2011;108(11):1625-31.

56. van Beynum IM, Kapusta L, Bakker MK, den Heijer M, Blom HJ, de Walle HE. Protective effect of periconceptional folic acid supplements on the risk of congenital heart defects: a registry-based case-control study in the northern Netherlands. Eur Heart J. 2010;31(4):464-71.

57. Hoffman JI, Kaplan S. The incidence of congenital heart disease. J Am Coll Cardiol. 2002;39(12):1890-900.

58. Boneva RS, Botto LD, Moore CA, Yang QH, Correa A, Erickson JD. Mortality associated with congenital heart defects in the United States - Trends and racial disparities, 1979-1997. Circulation. 2001;103(19):2376-81.

59. Poelmann RE, Lie-Venema H, Gittenberger-de Groot AC. The role of the epicardium and neural crest as extracardiac contributors to coronary vascular development. Tex Heart Inst J. 2002;29(4):255-61.

60. van Driel LM, de Jonge R, Helbing WA, van Zelst BD, Ottenkamp J, Steegers EA, et al. Maternal global methylation status and risk of congenital heart diseases. Obstet Gynecol. 2008;112(2 Pt 1):277-83.

61. Rosenquist TH, Ratashak SA, Selhub J. Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid. Proc Natl Acad Sci U S A. 1996;93(26):15227-32.

62. Boot MJ, Steegers-Theunissen RP, Poelmann RE, van Iperen L, Gittenberger-de Groot AC. Cardiac outflow tract malformations in chick embryos exposed to homocysteine. Cardiovasc Res. 2004;64(2):365-73.

63. Sotres-Alvarez D, Siega-Riz AM, Herring AH, Carmichael SL, Feldkamp ML, Hobbs CA, et al. Maternal dietary patterns are associated with risk of neural tube and congenital heart defects. Am J Epidemiol. 2013;177(11):1279-88.

64. Patel SS, Burns TL. Nongenetic risk factors and congenital heart defects. Pediatr Cardiol. 2013;34(7):1535-55.

65. Snijder CA, Vlot IJ, Burdorf A, Obermann-Borst SA, Helbing WA, Wildhagen MF, et al. Congenital heart defects and parental occupational exposure to chemicals. Hum Reprod. 2012;27(5):1510-7.

66. Statistics Netherlands. Definition country of origin. Voorburg / Heerlen, the Netherlands, 1999.

67. Statistics Netherlands, The Dutch Standard Classification of Education. 2008; [http://www.cbs.nl/NR/rdonlyres/2D265545-433C-484B-849C-5BBCCAAF6229/0/2008thedutchstandardclassificationofeducationsoiart.pdf].

68. Singh S, Li SS. Epigenetic effects of environmental chemicals bisphenol a and phthalates. Int J Mol Sci. 2012;13(8):10143-53.

69. Hollier LM, Leveno KJ, Kelly MA, DD MC, Cunningham FG. Maternal age and malformations in singleton births. Obstet Gynecol. 2000;96(5 Pt 1):701-6.

70. Miller A, Riehle-Colarusso T, Siffel C, Frias JL, Correa A. Maternal age and prevalence of isolated congenital heart defects in an urban area of the United States. Am J Med Genet A. 2011;155A(9):2137-45.

71. Hamilton BE, Ventura SJ. Fertility and abortion rates in the United States, 1960-2002. Int J Androl. 2006;29(1):34-45.

72. Oyen N, Poulsen G, Boyd HA, Wohlfahrt J, Jensen PK, Melbye M. Recurrence of congenital heart defects in families. Circulation. 2009;120(4):295-301.

73. Cordell HJ, Bentham J, Topf A, Zelenika D, Heath S, Mamasoula C, et al. Genome-wide association study of multiple congenital heart disease phenotypes identifies a susceptibility locus for atrial septal defect at chromosome 4p16. Nat Genet. 2013;45(7):822-4.

74. Rasmussen S, Irgens LM. The effects of smoking and hypertensive disorders on fetal growth. BMC Pregnancy Childbirth. 2006;6:16.

75. Gittenberger-de Groot AC, Calkoen EE, Poelmann RE, Bartelings MM, Jongbloed MR. Morphogenesis and molecular considerations on congenital cardiac septal defects. Ann Med. 2014;46(8):640-52.

76. Zhong GW, Jiang P, Qiao WR, Zhang YW, Wei WF, Lu L. Protein Phosphatase 2A (PP2A) Regulatory Subunits ParA and PabA Orchestrate Septation and Conidiation and Are Essential for PP2A Activity in Aspergillus nidulans. Eukaryot Cell. 2014;13(12):1494-506.

77. Smedts HP, de Vries JH, Rakhshandehroo M, Wildhagen MF, Verkleij-Hagoort AC, Steegers EA, et al. High maternal vitamin E intake by diet or supplements is associated with congenital heart defects in the offspring. BJOG. 2009;116(3):416-23.

78. Smedts HP, Rakhshandehroo M, Verkleij-Hagoort AC, de Vries JH, Ottenkamp J, Steegers EA, et al. Maternal intake of fat, riboflavin and nicotinamide and the risk of having offspring with congenital heart defects. Eur J Nutr. 2008;47(7):357-65.

79. Rachfall N, Johnson AE, Mehta S, Chen JS, Gould KL. Cdk1 promotes cytokinesis in fission yeast through activation of the septation initiation network. Mol Biol Cell. 2014;25(15):2250-9.

80. Chowdhury S, Cleves MA, MacLeod SL, James SJ, Zhao W, Hobbs CA. Maternal DNA hypomethylation and congenital heart defects. Birth Defects Res A Clin Mol Teratol. 2011;91(2):69-76.

81. Obermann-Borst SA, van Driel LM, Helbing WA, de Jonge R, Wildhagen MF, Steegers EA, et al. Congenital heart defects and biomarkers of methylation in children: a case-control study. Eur J Clin Invest. 2011;41(2):143-50.

82. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, et al. High density DNA methylation array with single CpG site resolution. Genomics. 2011;98(4):288-95.

83. Pidsley R, CC YW, Volta M, Lunnon K, Mill J, Schalkwyk LC. A data-driven approach to preprocessing Illumina 450K methylation array data. BMC Genomics. 2013;14:293.

84. Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics. 2010;11:587.

85. Joubert BR, Haberg SE, Nilsen RM, Wang X, Vollset SE, Murphy SK, et al. 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. Environ Health Perspect. 2012;120(10):1425-31.

86. Reinius LE, Acevedo N, Joerink M, Pershagen G, Dahlen SE, Greco D, et al. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. PLoS One. 2012;7(7):e41361.

87. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13:86.

88. Alexa A, Rahnenfuhrer J, Lengauer T. Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. Bioinformatics. 2006;22(13):1600-7.

89. Chi J, Cohen P. The Multifaceted Roles of PRDM16: Adipose Biology and Beyond. Trends Endocrinol Metab. 2016;27(1):11-23.

90. Seale P, Kajimura S, Yang W, Chin S, Rohas LM, Uldry M, et al. Transcriptional control of brown fat determination by PRDM16. Cell metabolism. 2007;6(1):38-54.

91. Nagano G, Ohno H, Oki K, Kobuke K, Shiwa T, Yoneda M, et al. Activation of classical brown adipocytes in the adult human perirenal depot is highly correlated with PRDM16-EHMT1 complex expression. PLoS One. 2015;10(3):e0122584.

92. Harms MJ, Lim HW, Ho Y, Shapira SN, Ishibashi J, Rajakumari S, et al. PRDM16 binds MED1 and controls chromatin architecture to determine a brown fat transcriptional program. Genes Dev. 2015;29(3):298-307.

93. Arndt AK, Schafer S, Drenckhahn JD, Sabeh MK, Plovie ER, Caliebe A, et al. Fine mapping of the 1p36 deletion syndrome identifies mutation of PRDM16 as a cause of cardiomyopathy. Am J Hum Genet. 2013;93(1):67-77.

94. Bjork BC, Turbe-Doan A, Prysak M, Herron BJ, Beier DR. Prdm16 is required for normal palatogenesis in mice. Hum Mol Genet. 2010;19(5):774-89.

95. Hong KW, Lim JE, Kim JW, Tabara Y, Ueshima H, Miki T, et al. Identification of three novel genetic variations associated with electrocardiographic traits (QRS duration and PR interval) in East Asians. Hum Mol Genet. 2014;23(24):6659-67.

96. Pinheiro I, Margueron R, Shukeir N, Eisold M, Fritzsch C, Richter FM, et al. Prdm3 and Prdm16 are H3K9me1 methyltransferases required for mammalian heterochromatin integrity. Cell. 2012;150(5):948-60.

97. Aguilo F, Avagyan S, Labar A, Sevilla A, Lee DF, Kumar P, et al. Prdm16 is a physiologic regulator of hematopoietic stem cells. Blood. 2011;117(19):5057-66.

98. Arthur HM, Bamforth SD. TGFbeta signaling and congenital heart disease: Insights from mouse studies. Birth Defects Res A Clin Mol Teratol. 2011;91(6):423-34.

99. Bartram U, Molin DG, Wisse LJ, Mohamad A, Sanford LP, Doetschman T, et al. Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGF-beta(2)-knockout mice. Circulation. 2001;103(22):2745-52.

100. Warner DR, Horn KH, Mudd L, Webb CL, Greene RM, Pisano MM. PRDM16/MEL1: a novel Smad binding protein expressed in murine embryonic orofacial tissue. Biochim Biophys Acta. 2007;1773(6):814-20.

101. Chen Q, Chen H, Zheng D, Kuang C, Fang H, Zou B, et al. Smad7 is required for the development and function of the heart. J Biol Chem. 2009;284(1):292-300.

102. Glass C, Wuertzer C, Cui X, Bi Y, Davuluri R, Xiao YY, et al. Global Identification of EVI1 Target Genes in Acute Myeloid Leukemia. PLoS One. 2013;8(6):e67134.

103. Modlich U, Schambach A, Brugman MH, Wicke DC, Knoess S, Li Z, et al. Leukemia induction after a single retroviral vector insertion in Evi1 or Prdm16. Leukemia. 2008;22(8):1519-28.

104. Perkins AS, Mercer JA, Jenkins NA, Copeland NG. Patterns of Evi-1 expression in embryonic and adult tissues suggest that Evi-1 plays an important regulatory role in mouse development. Development. 1991;111(2):479-87.

105. Doyle AJ, Doyle JJ, Bessling SL, Maragh S, Lindsay ME, Schepers D, et al. Mutations in the TGF-beta repressor SKI cause Shprintzen-Goldberg syndrome with aortic aneurysm. Nat Genet. 2012;44(11):1249-54.

106. Turner J, Crossley M. Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators. EMBO J. 1998;17(17):5129-40.

107. Hinck L. The versatile roles of "axon guidance" cues in tissue morphogenesis. Dev Cell. 2004;7(6):783-93.

108. Sanchez-Castro M, Pichon O, Briand A, Poulain D, Gournay V, David A, et al. Disruption of the SEMA3D Gene in a Patient with Congenital Heart Defects. Hum Mutat. 2015;36(1):30-3.

109. Butler JE, Kadonaga JT. The RNA polymerase II core promoter: a key component in the regulation of gene expression. Genes Dev. 2002;16(20):2583-92.

110. Lagha M, Bothma JP, Esposito E, Ng S, Stefanik L, Tsui C, et al. Paused Pol II coordinates tissue morphogenesis in the Drosophila embryo. Cell. 2013;153(5):976-87.

111. Michels KB, Binder AM, Dedeurwaerder S, Epstein CB, Greally JM, Gut I, et al. Recommendations for the design and analysis of epigenome-wide association studies. Nat Methods. 2013;10(10):949-55.

112. Ma B, Wilker EH, Willis-Owen SA, Byun HM, Wong KC, Motta V, et al. Predicting DNA methylation level across human tissues. Nucleic Acids Res. 2014;42(6):3515-28.

113. Talens RP, Boomsma DI, Tobi EW, Kremer D, Jukema JW, Willemsen G, et al. Variation, patterns, and temporal stability of DNA methylation: considerations for epigenetic epidemiology. FASEB J. 2010;24(9):3135-44.

114. Liu Y, Aryee MJ, Padyukov L, Fallin MD, Hesselberg E, Runarsson A, et al. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. Nat Biotechnol. 2013;31(2):142-7.

115. Souren NY, Lutsik P, Gasparoni G, Tierling S, Gries J, Riemenschneider M, et al. Adult monozygotic twins discordant for intra-uterine growth have indistinguishable genome-wide DNA methylation profiles. Genome Biol. 2013;14(5):R44.

116. Steegers-Theunissen RP, Obermann-Borst SA, Kremer D, Lindemans J, Siebel C, Steegers EA, et al. Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. PLoS One. 2009;4(11):e7845.

117. American Academy of Pediatrics. Committee on G. American Academy of Pediatrics: Health supervision for children with Down syndrome. Pediatrics. 2001;107(2):442-9.

118. Sailani MR, Makrythanasis P, Valsesia A, Santoni FA, Deutsch S, Popadin K, et al. The complex SNP and CNV genetic architecture of the increased risk of congenital heart defects in Down syndrome. Genome Res. 2013;23(9):1410-21.

119. Kerkel K, Schupf N, Hatta K, Pang D, Salas M, Kratz A, et al. Altered DNA methylation in leukocytes with trisomy 21. PLoS Genet. 2010;6(11):e1001212.

120. Eckmann-Scholz C, Bens S, Kolarova J, Schneppenheim S, Caliebe A, Heidemann S, et al. DNA-methylation profiling of fetal tissues reveals marked epigenetic differences between chorionic and amniotic samples. PLoS One. 2012;7(6):e39014.

121. Jin S, Lee YK, Lim YC, Zheng Z, Lin XM, Ng DP, et al. Global DNA hypermethylation in down syndrome placenta. PLoS Genet. 2013;9(6):e1003515.

122. Bean LJ, Allen EG, Tinker SW, Hollis ND, Locke AE, Druschel C, et al. Lack of maternal folic acid supplementation is associated with heart defects in Down syndrome: a report from the National Down Syndrome Project. Birth Defects Res A Clin Mol Teratol. 2011;91(10):885-93.

123. Hobbs CA, Sherman SL, Yi P, Hopkins SE, Torfs CP, Hine RJ, et al. Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome. Am J Hum Genet. 2000;67(3):623-30.

124. James SJ, Pogribna M, Pogribny IP, Melnyk S, Hine RJ, Gibson JB, et al. Abnormal folate metabolism and mutation in the methylenetetrahydrofolate reductase gene may be maternal risk factors for Down syndrome. Am J Clin Nutr. 1999;70(4):495-501.

125. Coppede F. The genetics of folate metabolism and maternal risk of birth of a child with Down syndrome and associated congenital heart defects. Front Genet. 2015;6:223.

126. Rai V, Yadav U, Kumar P, Yadav SK, Mishra OP. Maternal methylenetetrahydrofolate reductase C677T polymorphism and down syndrome risk: a meta-analysis from 34 studies. PLoS One. 2014;9(9):e108552.

127. Pogribna M, Melnyk S, Pogribny I, Chango A, Yi P, James SJ. Homocysteine metabolism in children with Down syndrome: in vitro modulation. Am J Hum Genet. 2001;69(1):88-95.

128. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, et al. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics. 2013;29(2):189-96.

129. McArdle BH, Anderson MJ. Fitting multivariate models to community data: A comment on distance-based redundancy analysis. Ecology. 2001;82:290-7.

130. Ram G, Chinen J. Infections and immunodeficiency in Down syndrome. Clin Exp Immunol. 2011;164(1):9-16.

131. Serra-Juhe C, Cusco I, Homs A, Flores R, Toran N, Perez-Jurado LA. DNA methylation abnormalities in congenital heart disease. Epigenetics. 2015;10(2):167-77.

132. Sailani MR, Santoni FA, Letourneau A, Borel C, Makrythanasis P, Hibaoui Y, et al. DNA-Methylation Patterns in Trisomy 21 Using Cells from Monozygotic Twins. PLoS One. 2015;10(8):e0135555.

133. Marabita F, Almgren M, Lindholm ME, Ruhrmann S, Fagerstrom-Billai F, Jagodic M, et al. An evaluation of analysis pipelines for DNA methylation profiling using the Illumina HumanMethylation450 BeadChip platform. Epigenetics. 2013;8(3):333-46.

134. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. Lancet. 2010;376(9741):631-44.

135. Mook-Kanamori DO, Steegers EA, Eilers PH, Raat H, Hofman A, Jaddoe VW. Risk factors and outcomes associated with first-trimester fetal growth restriction. JAMA. 2010;303(6):527-34.

136. Misra C, Chang SW, Basu M, Huang N, Garg V. Disruption of myocardial Gata4 and Tbx5 results in defects in cardiomyocyte proliferation and atrioventricular septation. Hum Mol Genet. 2014;23(19):5025-35.

137. van Rossum CTM, Fransen HP, Verkaik-Kloosterman J, Buurma-Rethans EJM, MC O. Dutch National Food Consumption Survey 2007-2010: Diet of children and adults aged 7 to 69 years http://wwwrivmnl/bibliotheek/rapporten/350050006pdf. 2011.

138. Hammiche F, Laven JS, van Mil N, de Cock M, de Vries JH, Lindemans J, et al. Tailored preconceptional dietary and lifestyle counselling in a tertiary outpatient clinic in The Netherlands. Hum Reprod. 2011;26(9):2432-41.

139. Gelson E, Curry R, Gatzoulis MA, Swan L, Lupton M, Steer P, et al. Effect of maternal heart disease on fetal growth. Obstet Gynecol. 2011;117(4):886-91.

140. Bonamy AK, Parikh NI, Cnattingius S, Ludvigsson JF, Ingelsson E. Birth characteristics and subsequent risks of maternal cardiovascular disease: effects of gestational age and fetal growth. Circulation. 2011;124(25):2839-46.

141. Smith GC, Pell JP, Walsh D. Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129,290 births. Lancet. 2001;357(9273):2002-6.

142. Smith GD, Sterne J, Tynelius P, Lawlor DA, Rasmussen F. Birth weight of offspring and subsequent cardiovascular mortality of the parents. Epidemiology. 2005;16(4):563-9.

143. Cruz-Neira C SD, DeFanti T. Surround-screen projection-based virtual reality: the design and implementation of the CAVE (tm). Proceedings of the 20th Annual Conference on Computer Graphics and Interactive Techniques: ACM Press; 1993. p. 135-42.

144. Koning AH, Rousian M, Verwoerd-Dikkeboom CM, Goedknegt L, Steegers EA, van der Spek PJ. V-scope: design and implementation of an immersive and desktop virtual reality volume visualization system. Stud Health Technol Inform. 2009;142:136-8.

145. Verwoerd-Dikkeboom CM, Koning AH, Hop WC, Rousian M, Van Der Spek PJ, Exalto N, et al. Reliability of three-dimensional sonographic measurements in early pregnancy using virtual reality. Ultrasound Obstet Gynecol. 2008;32(7):910-6.

146. Verwoerd-Dikkeboom CM, Koning AH, Hop WC, van der Spek PJ, Exalto N, Steegers EA. Innovative virtual reality measurements for embryonic growth and development. Hum Reprod. 2010;25(6):1404-10.

147. Huijgen N, van de Kamp M, Twigt J, de Vries J, Eilers P, Steegers E, et al. The Preconception Dietary Risk score; a simple tool to assess an inadequate habitual diet for clinical practice. e-SPEN Journal 9 (2014) e13ee19e14.

148. D'Agostino RB, Sr., Vasan RS, Pencina MJ, Wolf PA, Cobain M, Massaro JM, et al. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. Circulation. 2008;117(6):743-53.

149. Goff DC, Jr., Lloyd-Jones DM, Bennett G, Coady S, D'Agostino RB, Gibbons R, et al. 2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. Circulation. 2014;129(25 Suppl 2):S49-73.

150. Robinson HP. Sonar measurement of fetal crown-rump length as means of assessing maturity in first trimester of pregnancy. Br Med J. 1973;4(5883):28-31.

151. Kleinbaum DG, Klein M. Logistic Regression. 2nd ed. New York, USA: Springer; 2002.

152. Blaas HG, Eik-Nes SH, Bremnes JB. The growth of the human embryo. A longitudinal biometric assessment from 7 to 12 weeks of gestation. Ultrasound Obstet Gynecol. 1998;12(5):346-54.

153. Rossavik IK, Torjusen GO, Gibbons WE. Conceptual age and ultrasound measurements of gestational sac and crown-rump length in in vitro fertilization pregnancies. Fertil Steril. 1988;49(6):1012-7.

154. Rousian M, Koning AH, Hop WC, van der Spek PJ, Exalto N, Steegers EA. Gestational sac fluid volume measurements in virtual reality. Ultrasound Obstet Gynecol. 2011;38(5):524-9.

155. Macklon NS, Brosens JJ. The human endometrium as a sensor of embryo quality. Biol Reprod. 2014;91(4):98.

156. Kermack AJ, Finn-Sell S, Cheong YC, Brook N, Eckert JJ, Macklon NS, et al. Amino acid composition of human uterine fluid: association with age, lifestyle and gynaecological pathology. Hum Reprod. 2015;30(4):917-24.

157. Zandstra H, Van Montfoort AP, Dumoulin JC. Does the type of culture medium used influence birthweight of children born after IVF? Hum Reprod. 2015;30(3):530-42.

158. Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, et al. Effect of in vitro culture of human embryos on birthweight of newborns. Human Reproduction. 2010;25(3):605-12.

159. Nelissen EC, Van Montfoort AP, Smits LJ, Menheere PP, Evers JL, Coonen E, et al. IVF culture medium affects human intrauterine growth as early as the second trimester of pregnancy. Hum Reprod. 2013;28(8):2067-74.

160. Bergen NE, Bouwland-Both MI, Steegers-Theunissen RP, Hofman A, Russcher H, Lindemans J, et al. Early pregnancy maternal and fetal angiogenic factors and fetal and childhood growth: the Generation R Study. Hum Reprod. 2015;30(6):1302-13.

161. Refsum H, Nurk E, Smith AD, Ueland PM, Gjesdal CG, Bjelland I, et al. The Hordaland Homocysteine Study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr. 2006;136(6 Suppl):1731S-40S.

162. van Mil NH, Oosterbaan AM, Steegers-Theunissen RP. Teratogenicity and underlying mechanisms of homocysteine in animal models: a review. Reprod Toxicol. 2010;30(4):520-31.

163. van Uitert EM, van der Elst-Otte N, Wilbers JJ, Exalto N, Willemsen SP, Eilers PH, et al. Periconception maternal characteristics and embryonic growth trajectories: the Rotterdam Predict study. Hum Reprod. 2013;28(12):3188-96.

164. van Uitert EM, van Ginkel S, Willemsen SP, Lindemans J, Koning AH, Eilers PH, et al. An optimal periconception maternal folate status for embryonic size: the Rotterdam Predict study. BJOG. 2014;121(7):821-9.

165. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med. 2008;359(1):61-73.

166. van Uitert EM, Exalto N, Burton GJ, Willemsen SP, Koning AH, Eilers PH, et al. Human embryonic growth trajectories and associations with fetal growth and birthweight. Hum Reprod. 2013;28(7):1753-61.

167. Jaddoe VW, de Jonge LL, Hofman A, Franco OH, Steegers EA, Gaillard R. First trimester fetal growth restriction and cardiovascular risk factors in school age children: population based cohort study. BMJ. 2014;348:g14.

168. Barker DJ, Gelow J, Thornburg K, Osmond C, Kajantie E, Eriksson JG. The early origins of chronic heart failure: impaired placental growth and initiation of insulin resistance in childhood. Eur J Heart Fail. 2010;12(8):819-25.

169. Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. Lancet. 1993;341(8850):938-41.

170. Barker DJ, Thornburg KL. The obstetric origins of health for a lifetime. Clin Obstet Gynecol. 2013;56(3):511-9.

171. El Hajj N, Schneider E, Lehnen H, Haaf T. Epigenetics and life-long consequences of an adverse nutritional and diabetic intrauterine environment. Reproduction. 2014;148(6):R111-R20.

172. Centers for Disease Control and Prevention. Prevalence of abnormal lipid levels among youths --- United States, 1999-2006. MMWR Morb Mortal Wkly Rep. 2010;59(2):29-33.

173. Rodrigues AN, Abreu GR, Resende RS, Goncalves WL, Gouvea SA. Cardiovascular risk factor investigation: a pediatric issue. Int J Gen Med. 2013;6:57-66.

174. Patti ME. Intergenerational programming of metabolic disease: evidence from human populations and experimental animal models. Cell Mol Life Sci. 2013;70(9):1597-608.

175. Willerson JT, Ridker PM. Inflammation as a cardiovascular risk factor. Circulation. 2004;109(21 Suppl 1):II2-10.

176. Obermann-Borst SA, Eilers PH, Tobi EW, de Jong FH, Slagboom PE, Heijmans BT, et al. Duration of breastfeeding and gender are associated with methylation of the LEPTIN gene in very young children. Pediatr Res. 2013;74(3):344-9.

177. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 1972;18(6):499-502.

178. Corica F, Allegra A, Corsonello A, Buemi M, Calapai G, Ruello A, et al. Relationship between plasma leptin levels and the tumor necrosis factor-alpha system in obese subjects. Int J Obes Relat Metab Disord. 1999;23(4):355-60.

179. Cordero P, Campion J, Milagro FI, Goyenechea E, Steemburgo T, Javierre BM, et al. Leptin and TNF-alpha promoter methylation levels measured by MSP could predict the response to a low-calorie diet. J Physiol Biochem. 2011;67(3):463-70.

180. Gluckman PD, Hanson MA, Beedle AS. Early life events and their consequences for later disease: a life history and evolutionary perspective. Am J Hum Biol. 2007;19(1):1-19.

181. Cash HL, McGarvey ST, Houseman EA, Marsit CJ, Hawley NL, Lambert-Messerlian GM, et al. Cardiovascular disease risk factors and DNA methylation at the LINE-1 repeat region in peripheral blood from Samoan Islanders. Epigenetics. 2011;6(10):1257-64.

182. Hermsdorff HH, Mansego ML, Campion J, Milagro FI, Zulet MA, Martinez JA. TNF-alpha promoter methylation in peripheral white blood cells: relationship with circulating TNFalpha, truncal fat and n-6 PUFA intake in young women. Cytokine. 2013;64(1):265-71.

183. Antoniades C, Antonopoulos AS, Tousoulis D, Marinou K, Stefanadis C. Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. Eur Heart J. 2009;30(1):6-15.

184. Ingrosso D, Cimmino A, Perna AF, Masella L, De Santo NG, De Bonis ML, et al. Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia. Lancet. 2003;361(9370):1693-9.

185. Fryer AA, Nafee TM, Ismail KM, Carroll WD, Emes RD, Farrell WE. LINE-1 DNA methylation is inversely correlated with cord plasma homocysteine in man: a preliminary study. Epigenetics. 2009;4(6):394-8.

186. Woollett LA. Review: Transport of maternal cholesterol to the fetal circulation. Placenta. 2011;32 Suppl 2:S218-21.

187. Kulkarni SR, Kumaran K, Rao SR, Chougule SD, Deokar TM, Bhalerao AJ, et al. Maternal Lipids Are as Important as Glucose for Fetal Growth: Findings from the Pune Maternal Nutrition Study. Diabetes Care. 2013.

188. Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet. 2009;18(21):4046-53.

189. Sidhu D, Naugler C. Fasting time and lipid levels in a community-based population: a cross-sectional study. Arch Intern Med. 2012;172(22):1707-10.

190. Brotons C, Ribera A, Perich RM, Abrodos D, Magana P, Pablo S, et al. Worldwide distribution of blood lipids and lipoproteins in childhood and adolescence: a review study. Atherosclerosis. 1998;139(1):1-9.

191. Dai S, Fulton JE, Harrist RB, Grunbaum JA, Steffen LM, Labarthe DR. Blood lipids in children: age-related patterns and association with body-fat indices: Project HeartBeat! Am J Prev Med. 2009;37(1 Suppl):S56-64.

192. Mathai S, Derraik JG, Cutfield WS, Dalziel SR, Harding JE, Biggs J, et al. Increased adiposity in adults born preterm and their children. PLoS One. 2013;8(11):e81840.

193. Adalsteinsson BT, Gudnason H, Aspelund T, Harris TB, Launer LJ, Eiriksdottir G, et al. Heterogeneity in white blood cells has potential to confound DNA methylation measurements. PLoS One. 2012;7(10):e46705.

194. Herbstman JB, Wang S, Perera FP, Lederman SA, Vishnevetsky J, Rundle AG, et al. Predictors and consequences of global DNA methylation in cord blood and at three years. PLoS One. 2013;8(9):e72824.

195. Rothman KJ. Epidemiology. An Introduction. New York: Oxford University Press Inc., 2002.

196. Infante-Rivard C, Jacques L. Empirical study of parental recall bias. Am J Epidemiol. 2000;152(5):480-6.

197. van Driel LMJW, Zwolle LJH, de Vries JHM, Boxmeer JC, Lindemans J, Steegers EAP, et al. The preconception nutritional status of women undergoing fertility treatment: Use of a one-year post-delivery assessment. e-SPEN, 5 (2010) e284-e291.

198. Dedeurwaerder S, Defrance M, Calonne E, Denis H, Sotiriou C, Fuks F. Evaluation of the Infinium Methylation 450K technology. Epigenomics. 2011;3(6):771-84.

199. Slieker RC, Bos SD, Goeman JJ, Bovee JV, Talens RP, van der Breggen R, et al. Identification and systematic annotation of tissue-specific differentially methylated regions using the Illumina 450k array. Epigenetics Chromatin. 2013;6(1):26.

200. Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science. 2005;308(5727):1466-9.

201. Heard E, Martienssen RA. Transgenerational epigenetic inheritance: myths and mechanisms. Cell. 2014;157(1):95-109.

202. Reefhuis J, Honein MA. Maternal age and non-chromosomal birth defects, Atlanta--1968-2000: teenager or thirty-something, who is at risk? Birth Defects Res A Clin Mol Teratol. 2004;70(9):572-9.

203. Wang C, Zhan Y, Wang F, Li H, Xie L, Liu B, et al. Parental occupational exposures to endocrine disruptors and the risk of simple isolated congenital heart defects. Pediatr Cardiol. 2015;36(5):1024-37.

204. Xin F, Susiarjo M, Bartolomei MS. Multigenerational and transgenerational effects of endocrine disrupting chemicals: A role for altered epigenetic regulation? Seminars in cell & developmental biology. 2015;43:66-75.

205. Rakyan VK, Down TA, Balding DJ, Beck S. Epigenome-wide association studies for common human diseases. Nat Rev Genet. 2011;12(8):529-41.

206. Stoger R. The thrifty epigenotype: an acquired and heritable predisposition for obesity and diabetes? Bioessays. 2008;30(2):156-66.

207. Salomon LJ, Hourrier S, Fanchin R, Ville Y, Rozenberg P. Is first-trimester crown-rump length associated with birthweight? BJOG. 2011;118(10):1223-8.

208. Barker DJ. The origins of the developmental origins theory. J Intern Med. 2007;261(5):412-7.

209. Schubeler D. Function and information content of DNA methylation. Nature. 2015;517(7534):321-6.

210. Steegers EA, Barker ME, Steegers-Theunissen RP, Williams MA. Societal Valorisation of New Knowledge to Improve Perinatal Health: Time to Act. Paediatr Perinat Epidemiol. 2016;30(2):201-4.

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List of abbreviations

1-C	One-carbon
3D US	Three-dimensional ultrasound scan
3′ UTR	Three prime untranslated region
5′ UTR	Five prime untranslated region
ATC	Anatomical Therapeutical Classification
AUC	Area under the receiver operating curve
AVSD	Atrioventricular septal defect
BMIQ	Beta Mixture Quantile dilation
BMP	Bone morphogenetic proteins
C14orf109	Chromosome 14 open reading frame 109
CDK4	Cyclin-dependent kinase-4
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CDMR	Cancer-specific differentially methylated region
CHD	Congenital heart disease
CMD	Colour magnitude diagram
CpG	5′-cytosine-phosphoguanosine
CRL	Crown-rump length
CTBP2	C-terminal-binding protein 2
CVA	Cerebrovasculair accident
CVD	Cardiovascular disease
DHS	DNase I hypersensitive
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
DS	Down syndrome
ESAM	Endothelial cell adhesion molecule
EVI1	Ectropic virus integration site 1
EWAS	Epigenome-wide association study
FDR	False discovery rate
GA	Gestational age
GO	Gene ontology
HDL	High-density lipoprotein
HGF	Hepatocyte growth factor
HIF3A	Hypoxia-inducible factor 3 alpha
HNRNPU	Heterogeneous nuclear ribonucleoprotein U
HSC	Hematopoietic stem cells

IC	Intermittent claudication
ICSI	Intracytoplasmic sperm injection
IVF	*In vitro* fertilisation
KS	Kolmogorov-Smirnov
LDL	Low-density lipoprotein
LEP	Leptin
LMP	Last menstrual period
MAF	Minor allele frequencies
MOAP1	Modulator of apoptosis 1
MTHFR	Methylenetetrahydrofolate reductase
NFIA	Nuclear factor I/A
NK	Natural killer
OR	Odds ratio
PC	Principle component
PCA	Principal component analysis
PDR	Preconception Dietary Risk
PERMANOVA	Permutational multivariate analysis of variance
PPFIA4	Protein-tyrosine phosphatase, receptor-type, F polypeptide-interacting protein alpha-4
PRDM16	PR domain containing 16
pVSD	Perimembranous ventricular septal defect
PWBC	Peripheral white blood cells
RDMR	Reprogramming-specific differentially methylated region
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	Standard deviation
SE	Standard error
SEM	Structural equation modelling
SGA	Small for gestational age
SKI	Sloan-Kettering Institute proto-oncogene
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor-alpha
TSS1500	Left 1500bp from transcription start site
TSS200	Left 200bp from transcription start site
VSD	Ventricular septal defect

Bibliography

This thesis

Chapter 2
Wijnands K.P.J., Obermann-Borst S.A., Sijbrands E.J., Wildhagen M.F., Helbing W.A., Steegers-Theunissen R.P.M. Cardiovascular diseases in grandparents and the risk of congenital heart diseases in grandchildren.
J Dev Orig Health Dis. 2014 Apr;5(2):152-8.

Chapter 3
Wijnands K.P.J., Zeilmaker G.A., Meijer W.M., Helbing W.A., Steegers-Theunissen R.P.M. Periconceptional parental conditions and perimembranous ventricular septal defects in the offspring.
Birth Defects Res A Clin Mol Teratol. 2014 Dec;100(12):944-50.

Chapter 4
Wijnands K.P.J., Chen J., Liang L., Verbiest M.M.P.J., Lin X., Helbing W.A., Gittenberger-de Groot A.C., van der Spek P.J., Uitterlinden A.G., Steegers-Theunissen R.P.M. Genome-wide methylation analysis identifies novel CpG loci for perimembranous ventricular septal defects in human.
Submitted for publication

Chapter 5
Wijnands K.P.J., Chen J., Liang L., Verbiest M.M.P.J., Lin X., Helbing W.A., Uitterlinden A.G., Steegers-Theunissen R.P.M. Genome-wide methylation analysis in perimembranous ventricular septal defects and Down syndrome.
Submitted for publication

Chapter 6
Wijnands K.P.J., van Uitert E.M., Roeters van Lennep J.E., Koning A.H.J., Mulders A.G.M.G.J., Laven J.S.E., Steegers E.A.P., Steegers-Theunissen R.P.M. The periconception maternal cardiovascular risk profile influences human embryonic growth trajectories in IVF/ICSI pregnancies.
Hum Reprod. 2016 Jun;31(6):1173-81.

Chapter 7
Wijnands K.P.J., Obermann-Borst S.A., Steegers-Theunissen R.P.M. Early life lipid profile and metabolic programming in very young children.
Nutr Metab Cardiovasc Dis. 2015 Jun;25(6):608-14.

Other publications

van Wijck C.K.I., **Wijnands K.P.J.**, Dohle G.R. Wat is de beste techniek om zaadcellen uit de testis te verkrijgen bij patiënten met een niet-obstructieve azoöspermie?
Nederlands Tijdschrift voor Urologie. 2009 Mei; 17(3): 69-73.

PhD Portfolio

1. PhD Training

Master of Science in Health Sciences, specialisation Clinical Epidemiology, NIHES 2008-2011

	Year	Workload (ECTS)
General courses		
Study Design	2008	4.3
Clinical Trials	2008	0.7
Case-control studies	2008	0.7
Methods of Clinical Research	2008	0.7
Introduction to Decision-making in Medicine	2008	0.7
Topics in Health and Diseases in the Elderly	2008	0.7
Principles of Research in Medicine and Epidemiology	2008	0.7
Introduction to Data-analysis	2009	1.0
Regression Analysis	2009	1.9
Topics in Meta-analysis	2009	0.7
Survival Analysis	2009	1.9
Methods of Public Health Research	2010	0.7
History of Epidemiologic Ideas	2010	0.7
Advanced courses		
Intervention Research and Clinical Trials	2009	0.9
Diagnostic Research	2009	0.9
Courses for the Quantitative Researcher	2009	1.4
Introduction to Clinical Research	2009	0.9
Prognosis Research	2009	0.9
Advanced Topics in Decision-making in Medicine	2009	1.9
Medical Demography	2010	1.1
Bayesian Statistics	2010	1.1
Missing Values in Clinical Research	2010	0.7
Pharmaco-epidemiology and Drug Safety	2011	1.9
Advanced Topics in Clinical Trials	2011	1.9
Advanced Analysis of Prognosis Studies	2011	0.9
Principles of Epidemiologic Data-analysis	2011	0.9
Genomics in Molecular Medicine	2011	1.4
Repeated Measurements	2012	1.4

	Year	Workload (ECTS)
General academic skills courses		
A First Glance at SPSS for Windows	2009	0.15
The Basic Introduction Course on SPSS (MOLMED)	2010	1.0
Working with SPSS for Windows	2010	0.15
Scientific Writing in English for Publication	2010	2.0
International training		
At Harvard T.H. Chan School of Public Health, Boston, MA, USA		
Introduction to Management of Health Care Organisations	2010	2.0
Ethical Basis of the Practice of Public Health	2010	2.0
Presentations		
Nederlandse Vereniging voor Toxicologie, sectie Teratologie en Reproductietoxicologie, Zeist, The Netherlands	2011	1.0
Bo Hjelt Foundation, Rotterdam, The Netherlands	2011	1.0
Research meeting, Department of Obstetrics and Prenatal Medicine, Rotterdam, The Netherlands	2011-2013	2.0
Annual Juriy Wladimiroff Research Meeting, Rotterdam, The Netherlands	2012	1.0
Science lunch cluster 12, Rotterdam, The Netherlands	2012-2013	2.0
Seminars and workshops		
Weekly research meetings, Department of Obstetrics and Prenatal Medicine, Rotterdam, The Netherlands	2008-2013	5.0
Annual Juriy Wladimiroff Research Meeting, Rotterdam, The Netherlands	2011-2013	0.4
Workgroup Epigenetic Epidemiology, Rotterdam, The Netherlands	2011	1.0
Symposium Jonge Zwangerschap, Rotterdam, The Netherlands	2012	0.2
Erasmus MC PhD Day, Rotterdam, The Netherlands	2012-2013	0.4
Advice to Young Scientists: How to build a career in Research, Rotterdam, The Netherlands	2013	0.2
(Inter)national conferences		
7th World Congress on Developmental Origins of Health and Disease, Portland, OR, USA	2011	1.0
59th Annual Meeting Society of Gynecological Investigations, San Diego, CA, USA	2012	1.0
2nd European Periconception Health Congress, Rotterdam, The Netherlands	2012	1.0
8th World Congress on Developmental Origins of Health and Disease, Singapore	2013	1.0
9th World Congress on Developmental Origins of Health and Disease, Cape Town, South Africa	2015	1.0

	Year	Workload (ECTS)
International work experience		
Harvard T.H. Chan School of Public Health, Department of Biostatistics, Boston, MA, USA (6 weeks)	2013	2.0

2. Teaching activities

Lecturing		
Curriculum Bachelor Medicine, Erasmus University, minor Congenital heart diseases, topic Gene-environment interactions	2011-2012	1.0
Supervising Master's theses		
Supervising elective research programs (21 weeks) of medical students:		
Gerdien Zeilmaker, Erasmus University, Rotterdam, The Netherlands	2011	1.5
Remco Visser, Erasmus University, Rotterdam, The Netherlands	2012	1.5
Fleur Bruinier, Erasmus University, Rotterdam, The Netherlands	2013	1.5
Other skills		
Preconception counselling of subfertile couples	2010-2012	3.0

About the author

Kim Wijnands werd geboren op 17 maart 1988 te Amersfoort, 12 minuten na haar tweelingbroer Ralph. Na het behalen van haar VWO diploma in 2006 op middelbare school 't Atrium te Amersfoort begon zij met haar studie Geneeskunde aan de Erasmus Universiteit te Rotterdam.
Vanaf het derde jaar van haar Geneeskunde opleiding startte zij ook met een Research Master aan het Netherlands Institute for Health Sciences. Haar masteronderzoek verrichtte zij op de afdeling Verloskunde & Gynaecologie onder supervisie van prof.dr. Régine Steegers-Theunissen. Als onderdeel van deze Research Master nam zij in 2010 deel aan de Summer School van Harvard T.H. Chan School of Public Health, Boston, Verenigde Staten. In 2011 behaalde zij haar doctoraal Geneeskunde en haar Master of Science graad in Health Sciences met een specialisatie in Clinical Epidemiology. Hierna startte zij in mei 2011 als promovendus binnen de HAVEN studie onder begeleiding van prof.dr. Régine Steegers-Theunissen (Verloskunde & Gynaecologie) en prof.dr. André Uitterlinden (Interne Geneeskunde). Gedurende haar onderzoek werkte zij onder meer op de afdeling Biostatistiek op Harvard T.H. Chan School of Public Health, Boston, Verenigde Staten, voor het uitvoeren van statistische analyses.
Na tweeënhalf jaar promotieonderzoek vervolgde zij in 2014 haar opleiding Geneeskunde en liep twee jaar co-schappen binnen de regio Rotterdam. In maart 2016 studeerde zij cum laude af als arts. Aansluitend is zij begonnen als arts-assistent (niet in opleiding tot specialist) Interne Geneeskunde in het Maasstad Ziekenhuis te Rotterdam.

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Kim