The origin of congenital heart defects and the epigenetic programming of the healthy child

Determinanten van aangeboren hartafwijkingen en de programmering van jonge kinderen

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I love the time and in between
The calm inside me
In the space where I can breathe
I believe there is a
Distance I have wandered
To touch upon the years of
Reaching out and reaching in
Holding out holding in

I believe
This is heaven to no one else but me
And I'll defend it as long as I can be
Left here to linger in silence

"Elsewhere" -SARAH McLACHLAN

Contents

Chapter 1

Introduction

Rationale

Birth defects are a global burden affecting 7% of births worldwide.[1] Congenital heart defects (CHD) are the most common congenital malformation with approximately 1 million children born each year.[2, 3] It is not only the most frequent group of birth defects in human, but also the leading cause of infant morbidity in the Western world.[4] Although the mortality of CHD has decreased in the last 20 years, the burden for the child, family, society, health care and insurances is enormous.[5] Also in adulthood having a CHD, even when mild, has a substantial negative impact on societal perspectives.Patients with CHD are more likely to have a lower education, are more often unemployed and less likely to be in a relationship.[6]

Decades of animal and human studies made it clear that the vast majority of birth defects have a multifactorial origin, with contributions from genetic and environmental factors.[7] Already in 1965 gene environment interactions factors have been recognized as the "Principle of Teratology" by Wilson and Warkany.[8] This is also true for CHD, in which 80-90% seems to result from complex interactions between subtle genetic variations and periconception maternal characteristics and environmental exposures. The periconception environment comprises not only the external milieu of the pregnant woman, but also includes her metabolic, endocrine, immunological and vascular state as an internal environment for the developing embryo, foetus and placenta.[9]

Periconception folate shortage is an important nutritional, but also environmental factor, which contributes to the development of several birth defects, including CHD. Randomized controlled trials have shown that the periconception use of a synthetic folic acid in tablets prevents neural tube defects by around 70%.[10] For that reason periconceptional folic acid in a dose of 400 microgram per day is being promoted to all women planning pregnancy.[11] Over the last decade, several campaigns have improved the awareness of the importance of periconception folic acid use. Furthermore, folic acid fortification of food has been introduced in the US, Canada and Chile. Since the implementation of these measures, significant decreases in the occurrence of neural tube defects, but also of CHD, orofacial clefts and diaphragmatic hernia are reported.[12, 13]

The mechanisms underlying the beneficial effects of periconceptional folic acid use are not yet clarified. We are most interested in the one carbon pathway, which is important in the periconceptional period for embryonic and placental growth and development, in which B vitamins including folate serve as methyl donors, substrates and cofactors. A shortage, in particular of folate, due to malnutrition, use of folate antagonists, metabolic derangements or polymorphisms in genes involved in the same pathway, i.e., methylenetetrahydrofolate reductase gene (*MTHFR*), deranges this pathway due to amongst others a decreased supply of methyl moieties. As a consequence of a folate shortage, mild to moderate hyperhomocysteinaemia and hypomethylation occur, and biological processes implicated in growth and development are impaired. This is due to the accompanying excessive oxidative stress and shortage of

methyl and other one carbon moieties, which derange the synthesis of proteins, lipids, DNA and RNA, DNA repair mechanisms and DNA methylation. In general, these derangements can be treated successfully using a healthy diet in combination with the administration of folic acid and/or vitamin B12.[14]

DNA methylation is an important mechanism of epigenetic programming essential for normal development.[15] It is associated with a number of key processes including genomic imprinting, X-chromosome inactivation, suppression of repetitive elements, and carcinogenesis. DNA methylation occurs predominantly, but not exclusively, at CpG dinucleotides, ('CpG' stands for cytosine and guanine, separated by a phosphate atom). These CpG islands are especially found in promoter regions and its methylation is associated in general with low gene activity.[16] We hypothesize that tissue specific derangements in DNA methylation may contribute to birth defects, including CHD, and global derangements in DNA methylation to impaired growth. Indeed, we have demonstrated before, and confirmed by others, that a deranged one carbon pathway with high maternal concentrations of total homocysteine (tHcy) and S-adenosylhomocysteine (SAH) and a low S-adenosylmethionine (SAM): SAH ratio is significantly associated with an increased risk of having a child with CHD, in particular a child with Down syndrome and CHD.[17-19] Moreover, cellular hypomethylation determined by biomarkers in blood and in DNA has been associated with vascular diseases, which is consistent with the vascular hypothesis of CHD. [9, 20]

Poor periconception nutrition is a risk factor for birth defects and impaired growth, an effect which might be mediated by DNA methylation.[21] Animal studies have shown that the dietary availability of methyl groups affects DNA methylation. Supplementation of mice with methyl donors before or in early pregnancy increases the level of DNA methylation and changes the phenotype of the offspring.[22, 23] A study in rats has shown that maternal dietary protein restriction during pregnancy, including low methionine, leads to a persistent decrease in the methylation of several genes. This pattern of hypomethylation is not present in rats supplemented with folic acid.[24] Furthermore, deficiencies of B-vitamins and methionine in the preconception diet in sheep lead to both hypomethylation and hypermethylation in the offspring's genome, accompanied by an unhealthy phenotype.[25]

In the past years, it has been shown that periconception environmental conditions are also associated with persistent changes of the human epigenome and phenotype.[26-29] The human epigenome is susceptible to dysregulation throughout life; however, it is thought to be most sensitive to environmental factors during early embryogenesis, which is a period of rapid cell division and epigenetic remodelling.[30]

Although nutrition is the most important environmental exposure, the environment is much more complex. Other and related periconception environmental factors, such as the socioeconomic status (SES) of the parents , breastfeeding and associated lifestyle factors, such as smoking, may effect the epigenome of the embryo, foetus and very young child directly or via the one-carbon pathway. While low SES is in general associated with major health inequalities in

child and adult life, it is also suggested that the impact of socioeconomic status already starts in the womb.[31] Thus, adverse lifestyle exposures in utero may lead to the induction of epigenetic marks, in a similar fashion as the disruption of the foetal epigenome by poor nutrition and diverse environmental chemicals.[32]

Objectives of the thesis

Against this background the topics to be addressed are, the investigation of:

1) periconception maternal nutrition and lifestyle and the risk of having a child with a CHD (Part I)
2) biomarkers of lipid metabolism and global cellular methylation in the child and the association with CHD (Part II)
3) periconception nutrition, lifestyle and breastfeeding in association with the epigenetic programming of the healthy child (Part III)

The studies in part I and II are conducted in the HAVEN study, a case-control family study on CHD. The studies described in part III were conducted in a subset of the control children of the same HAVEN study.

Thesis Outline

This thesis is based on the HAVEN-study (Hart Afwijkingen, Vasculaire status, Erfelijkheid en Nutriënten), a case-control triad 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 and the data has been collected from June 2003 until January 2010 at the Department of Obstetrics and Gynaecology/ Division of Prenatal Medicine of the Erasmus MC, University Medical Centre in Rotterdam, The Netherlands. Recruitment of cases was performed in collaboration with the Departments of Paediatric Cardiology of the Erasmus MC, Leiden University Medical Centre in Leiden, VU University Medical Centre and Academic Medical Centre in Amsterdam. Controls were recruited through the child health care centres of "Careyn" in the close proximity of Rotterdam.

In **Part I** of this thesis we investigate determinants of maternal nutrition and lifestyle in association with the risk of having a child with CHD. In **Chapter 2**, we focus on the interaction between the C3435T polymorphism in the *MDR1*-gene, folic acid use and medication use in the periconception period. In **Chapter 3**, we study the association between a periconception maternal dietary pattern related to biomarkers of methylation and the risk of CHD in the offspring.

Part II of this thesis addresses biomarkers of lipid metabolism and global cellular methylation in children with CHD. **Chapter 4** focuses on the association between concentrations of lipids in the children and CHD. In **Chapter 5**, we describe the associations between the biomarkers of the cellular methylation state and CHD. We evaluated the concentrations of SAM, SAH, tHcy, folate and vitamin B12 in case children and control children.

Part III of this thesis focusses on the epigenetic programming of the healthy child. We investigated maternal nutrition and lifestyle, breastfeeding and DNA methylation of several genes in the very young child. The studies in this part were conducted in a subset of the control children that were included in the HAVEN study. In **Chapter 6**, we investigated the association between periconception folic acid use and methylation of *IGF2* DMR in the child. In **Chapter 7**, we studied maternal and child characteristics and the associations with methylation of *INSIGF, IGF2R* and *IGF2* DMR. In **Chapter 8**, we investigate associations between maternal education, breastfeeding and methylation of *LEP* in the child. Finally, methodological considerations, main findings, inferences and suggestions for future research are presented in **Chapter 9**. A summary is provided in **Chapter 10**.

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Part I

The origin of congenital heart defects: determinants in the mother

If I could keep you underneath my wing
The sky above you, safe from everything
Under, under my wing
"Motherland" -HEATHER NOVA

Chapter 2

General maternal medication use, folic acid, the MDR1 C3435T polymorphism, and the risk of a child with a congenital heart defect

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Abstract

Studies in knockout mice have shown that deficiencies of the *MDR1* transporter in combination with a toxic exposure can lead to an increased risk of birth defects. The *MDR1* C3435T genotype in human is associated with a decreased expression of the *MDR1* transporter. The objective of this study was to study whether the maternal *MDR1* C3435T genotype in combination with periconception medication and or folic acid use is associated with the risk for congenital heart disease (CHD) in the offspring.

MDR1 3435CT genotyping was performed in 283 case triads (mother, father, child) and 308 control triads. Information on periconception medication and folic acid use was obtained through questionnaires.

Mothers with *MDR1* 3435CT/TT genotype and using medication showed a significant association with the risk of a child with CHD (odds ratio [OR] 2.4, 95% confidence interval [CI], [1.3– 4.3]) compared to mothers with *MDR1* 3435CC genotype not using medication. This risk increased without folic acid use (OR 2.8, 95% CI [1.2– 6.4]), and decreased in folic acid users (OR 1.7, 95% CI [0.8 –3.7]). Children carrying the *MDR1* 3435CT/TT genotype and periconceptionally exposed to medication without folic acid did not show significant risks.

In conclusion, mothers carrying the *MDR1* 3435T allele, seem more at risk for the teratogenic effects of periconception medication use. This effect can be prevented in part by the periconception use of folic acid.

Introduction

Worldwide around 1 million children are born every year with a congenital heart defect (CHD), which not only is the most frequent class of birth defects but also the leading cause of infant morbidity in the Western world.[1, 2] The aetiology of CHD is complex and believed to result from complex interactions between subtle genetic variations and periconception environmental exposures. As the mother serves as the environment of the developing child in utero, maternal disease and harmful lifestyles in conjunction with genetic susceptibilities can modulate the risk of CHD.[3] It is known that maternal medication use can exert teratogenic effects, and several medications have been linked to CHD, such as antihypertensives and anticonvulsants. [4-6]

Several transporters are responsible for an adequate efflux of medication and other toxins from the circulation. Of special interest are the ABC transporters, particularly ABCB1 or P-glycoprotein (P-gp), encoded by the *MDR1* gene.[7] Studies in *MDR1* knockout mice demonstrated that P-gp deficiency enhances the susceptibility to chemically induced birth defects.[8] The 3435C T synonymous single nucleotide polymorphism in *MDR1* is associated with an increased degradation of *MDR1* messenger RNA (mRNA) and, consequently, decreased P-gp expression.[9] This polymorphism may thus modify cellular exposures to several compounds.[10] A recent in vitro study showed that the cellular folate concentration also determines the function of the *MDR1* transporter; a folate-rich environment increases the efflux of *MDR1* substrates from the cell.[11] Therefore, carrier ship of the *MDR1* 3435C>T polymorphism in the mother and/or the child might perturb the detoxification pathway.

From this background we hypothesize that carrier ship of *MDR1* 3435C>T in combination with periconception maternal medication use increases the risk of CHD, in which folic acid in the periconception period acts as modifier. This hypothesis was investigated in a case-control family study of mother, father, and the child, in an ethnically homogeneous sample in the western part of The Netherlands.

Materials and Methods

Study population

This study is part of the HAVEN study, an on-going case-control family study designed to investigate the role of genetic and lifestyle factors in the pathogenesis and prevention of CHDs. From June 2003 onward, we recruited cases from four university medical centres and randomly recruited controls in collaboration with the child health care centres of Thuiszorg Nieuwe Waterweg Noord in the Rotterdam area. Child health care centres are part of the Dutch Health Care system where physicians specializing in child health care regularly check all newborns at standardized moments on health, growth, and development. Case and control

children were derived from the same domain population in the western part of The Netherlands. The materials and methods for this study have been described previously and are summarized below.[12]

All children were aged 11-18 months and of European origin. To exclude strong genetic factors, no familial relationship existed between cases and controls.[13]

The included CHD phenotypes (n = 283) were Tetralogy of Fallot (n = 31), transposition of the great arteries (n = 49), atrioventricular septal defect (n = 28), perimembranous ventricular septal defect (n = 75), coarctation of the aorta (n = 28), aortic valve stenosis (n = 5), pulmonary valve stenosis (n = 52), and hypoplastic left heart syndrome (n = 15). This selection of CHD phenotypes was based on experimental and epidemiological studies that showed that hyperhomocysteinemia and related gene-environment interactions are involved in the etiology.[12, 14-16] All CHDs were diagnosed by 2 paediatric cardiologists, both trained at the university medical centre in Leiden, using echocardiography and/or cardiac catheterization and/ or surgery data.

Control children (n = 308) had no major congenital malformations or chromosomal abnormalities according to the medical records and regular health checks by physicians of the child health centres. The Central Committee on Research involving Human Subjects and the institutional review boards of all participating hospitals approved the study protocol. All parents gave their written informed consent also on behalf of their child before participation.

Data collection

At the time of study, approximately 16 months after delivery of the index child, data were obtained by a self-administered questionnaire on sociodemographic characteristics, such as age, ethnicity, educational level, and periconception use of medication and folic acid. During the hospital visit the questionnaire was checked by the researcher for completeness and consistency.

We defined the periconception period as 4 weeks before conception until 10 weeks after conception. The use of folic acid in the periconception period was defined as the daily use of at least 400 μg of folic acid, either in a multivitamin preparation or as a single tablet during the complete period. Mothers who used folic acid only during a part of the periconception period were classified as nonusers. A positive family history of CHD was defined as the child having a third-degree, or closer, relative with CHD. We categorized education level as low (primary/lower vocational/intermediate secondary), intermediate (higher secondary/ intermediate vocational), or high (higher vocational/university) according to the Dutch classification.[17]

We defined medication use as any use in the periconception period. We classified the medication according to the Anatomical Therapeutic Chemical classification. At the time of study, blood or buccal swabs were obtained to extract DNA from all children and their parents.

For the current study we selected case and control families from whom DNA was available from at least the mother, i.e., case family: 257 children, 284 mothers, and 264 fathers; and control triads: 299 children, 309 mothers, and 292 fathers. Genotype data were checked for mendelian errors. Inconsistent families (1 case and 1 control) were excluded from analysis.

Blood sampling

DNA was isolated from EDTA blood with a total nucleic acid extraction kit on a MagNAPure LC (Roche Molecular Biochemicals, Mannheim, Germany). *MDR1* 3435CT genotyping on case and control triads was done using a Taqman allelic discrimination assay on the ABI Prism 7000 HT Sequence detection system (Applied Biosystems [ABI], Nieuwerkerk a/d IJssel, The Netherlands). The assay consisted of 2 allele specific minor groove binding probes designed by Applied Biosystems' Assayby-Design service; the probe sequence CCCTCACGATCTCTT was labelled with the fluorescent dye VIC and the probe sequence CCCTCACAATCTCTT with the fluorescent dye FAM. The primer sequence for the forward primer was ATGTATGTTGGCCTCCTTTGCT, and for the reverse primer GCCGGGTGGTGTCACA. The polymerase chain reaction was performed in a reaction volume of 10 μL, containing assay specific primers, allele-specific Taqman minor groove binding probes, Abgene Absolute QPCR Rox Mix, and genomic DNA (1 ng). The thermal profile consists of an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds, and annealing and extension at 60°C for 1 minute. Genotypes were scored by measuring allele-specific fluorescence using the SDS 2.2.2 software (Applied Biosystems) for allelic discrimination.

Statistical analyses

Sociodemographic and lifestyle characteristics were compared between cases and controls using a χ^2 test for categorical variables and a Mann-Whitney U test for continuous variables. All continuous variables are presented as medians with interquartile range, because some of them were skewed even after transformation. Deviations from Hardy-Weinberg expectations were tested with a χ^2 test. The frequencies of the alleles were compared between cases and controls, and odds ratios (ORs) were calculated with 95% confidence intervals (CIs), using the C allele as the reference. To test the association between the *MDR1* polymorphism and CHD risk, we used the Family Based Association Test (FBAT) software that looks for distortions in the transmission frequencies of a given allele, compared to the assumption of random transmission, and also incorporates control data.[18] We used a dominant model combining the CT and TT genotypes based on the functional effect of the polymorphism. Univariate logistic regression analysis was used to compute ORs and 95% CIs for the association between case-control status and dichotomous variables *MDR1* polymorphism, medicine and folic acid use. We coded separate categories for the risk of the genotype of mother or child in combination with periconception medication, separate categories for the risk of the genotype of mother

or child in combination with periconception medication and folic acid use. The reference category was considered the lowest risk category: *MDR1* CC carriers without periconception medication use and with the use of folic acid. The highest risk group consisted of *MDR1* CT/TT carriers with periconception exposure to medication and no use of folic acid. In addition, we computed ORs with 95% CI in a multi variable logistic regression model adjusting for the genotype of either child or mother. The p values for trend were calculated across the different subgroups with a linear-by-linear association test. Probability values of $p < 0.05$ were considered statistically significant; all tests were 2-sided. Analyses were performed with software (SPSS for Windows, version 15.0; SPSS Inc, Chicago, IL) or FBAT 3.2. Stratified analyses of the different CHD phenotypes were not feasible due to the limited sample size.

Results

Sociodemographic and lifestyle characteristics of mothers, fathers, and children are presented in Table 1. Periconception medication use was the only significant difference between case and control mothers. Case mothers used overall more often medication (OR 1.5; 95% CI 1.0 –2.1). According to the Anatomical Therapeutic Chemical classification, medication use comprised laxatives (A01 cases 0.4%), antacids (A02 cases 0.4% vs. controls 0.3%), spasmolytics (A03 controls 0.3%), insulin (A10 cases 0.4% vs. controls 0.6%), antihemorrhagics (B01 controls 0.3%), antithrombotic agents (B02 cases 0.4% vs. controls 0.3%), antihypertensive drugs (C02 cases 0.4% vs. controls 0.6%), beta blockers (C07 cases 0.8% vs. controls 0.3%), prolactin inhibitors (G02 cases 0.7%), sex hormones and modulators of the genital system (G03 cases 3.2% vs. 2.9%), thyreomimetics (H03 cases 1.8% vs. 1.0%), systemic antibacterials (J01 cases 3.5% vs. controls 3.2%), hormones and related agents (L02 cases 0.4%), prostaglandine-synthase blockers (M01 cases 0.1% vs. controls 0.6%), analgesics (N02 cases 5.3% vs. 4.5%), antiepileptics (N03 cases 0.4% vs. controls 0.6%), psycholeptics (N05 cases 1.4%), psychoanaleptics (N06 cases 1.8% vs. controls 1.6%), nasal preparations (R01 cases 0.4%), drugs for obstructive airway disease (R03 cases 2.8% vs. controls 1.3%), cough and cold preparations (R05 cases 0.4% vs. controls 0.3%), systemic antihistamines (R06 cases 5.3% vs. controls 1.9%), unspecified antibiotics/mycotics (cases 0.4% vs. controls 1.6%), and homeopathics (cases 0.7% vs. controls 1.0%). Except for a higher percentage of systemic antihistamines (cases n = 15 vs. controls n = 6; p = 0.028) and psycholeptics (cases n = 4 vs. controls n = 0; p = 0.036) in case mothers, medication use was not significantly different from control mothers.

Table 1 Sociodemographic and lifestyle characteristics

Characteristic	Cases	Control	p-value
Mothers	**n = 283**	**n = 308**	
Age at delivery index child, y[a]	31.6 (28.9-34.8)	31.4 (28.2-34.0)	0.15[b]
Educational level:			0.49[c]
Low[d]	57 (18.5)	63 (22.3)	-
Intermediate[d]	154 (50.0)	131 (46.3)	-
High[d]	97 (31.5)	89 (31.4)	-
Periconception:			
Medication[d]	83 (29.3)	68 (22.1)	0.04[c]
Tobacco[d]	53 (18.7)	64 (20.8)	0.53[c]
Alcohol[d]	121 (42.8)	113 (36.7)	0.15[c]
Folic acid[d,e]	152 (53.7)	183 (59.4)	0.16[c]
Fathers	**n = 283**	**n = 308**	
Age at birth index child, y[a]	33.5 (30.6-37.0)	34.0 (30.6-37.2)	0.74[b]
Educational level:			0.11[c]
Low[d]	67 (23.7)	69 (22.4)	-
Intermediate[d]	100 (35.3)	134 (43.5)	-
High[d]	116 (41.0)	105 (34.1)	-
Periconception:			
Medication[d]	43 (15.2)	44 (14.3)	0.76[c]
Tobacco[d]	91 (32.2)	107(34.7)	0.51[c]
Alcohol[d]	246 (86.9)	257 (83.4)	0.24[c]
Children	**n = 283**	**n = 308**	
Age, study moment, months[a]	16.3 (15.0-19.0)	16.0 (15.1-17.9)	0.09[b]
Male gender	159 (56.2)	164 (53.2)	0.47[c]
Birth weight, g[a,f]	3380 (2865-3700)	3545(3221-3900)	0.48[b]
Family history of CHD[b,g]	23 (8.1)	16 (5.2)	0.15[c]
Ethnicity[h]			0.76[c]
Dutch Natives	232 (90.6)	273 (91.6)	-
European others	24 (9.4)	25 (8.4)	-

[a] Data are presented as median and interquartile range; [b] Mann-Whitney *U* test for difference between cases and controls; [c] χ^2 test for difference between cases and controls; [d] Data are number (percentage); [e] Daily use of supplements containing folic acid with a minimum of 400 μg folic acid from 4 weeks before conception to 8 weeks thereafter; [f] Adjusted for gestational age; [g] Positive family history of CHD was defined as the child having a 3rd degree, or closer, relative with CHD; [h] Dutch Natives were defined as those of whom both parents and grandparents were born in the Netherlands, or one of the parents was born in another country, but both grandparents were born in the Netherlands. European others were defined as those of whom one of the parents or grandparents was born in a European country, Indonesia, or was from European origin and living in the USA or Australia.[12]

Table 2 presents the allele and genotype frequencies and risk estimates for the *MDR1* polymorphism for mothers, fathers, and children. The distribution of the T allele and genotypes were not significantly different among cases and control fathers and children. In contrast, more case mothers were carriers of the *MDR1* TT/CT genotypes, which were significantly associated with an increased risk for CHD offspring (OR 1.7; 95% CI [1.1-2.5]).

Table 2 Distribution and odds ratios of the *MDR1* C3435T polymorphism

MDR1	**Cases (%)**	**Controls (%)**	**OR[a] [95%CI][b]**	**OR[a][95%CI][b]**
Mothers	**n = 283**	**n = 308**		
T/T	81 (28.6)	82 (26.6)	1.6 [1.0-2.5]	1.7 [1.1-2.5]
C/T	151 (53.4)	144 (46.8)	1.7 [1.1-2.6]	
C/C	51 (18.0)	82 (26.6)	1 [ref]	1 [ref]
Hardy-Weinberg equilibrium P-value	0.18	0.25		
C Allele frequency	0.45	0.50		
T Allele frequency	0.55	0.50		
Fathers	**n = 263**	**n = 291**		
T/T	80 (30.4)	76 (26.1)	1.5 [0.9-2.4]	1.4 [0.9-2.1]
C/T	134 (51.0)	145 (49.8)	1.3 [0.9-2.0]	
C/C	49 (18.6)	70 (24.1)	1 [ref]	1 [ref]
Hardy-Weinberg equilibrium P-value	0.59	0.96		
C Allele frequency	0.44	0.49		
T Allele frequency	0.56	0.51		
Children	**n = 256**	**n = 298**		
T/T	73 (28.5)	69 (23.2)	1.2 [0.7-2.0]	1.2 [0.8-1.8]
C/T	132 (51.6)	162 (54.3)	0.9 [0.6-1.4]	
C/C	51 (19.9)	67 (22.5)	1 [ref]	1 [ref]
Hardy-Weinberg equilibrium P-value	0.53	0.13		
C Allele frequency	0.46	0.50		
T Allele frequency	0.54	0.50		

[a] OR, odds ratio; [b] CI, confidence interval.

Table 3 presents the results of the FBAT analysis. We analysed 185 informative case triads and 211 informative control triads. FBAT revealed no statistically significant association between the *MDR1* genotype and CHD risk.

The results of the interaction analysis of the maternal, paternal, and child *MDR1* CT/TT genotypes in conjunction with periconception parental medication use are shown in Table 4. Maternal medication use modified the risk of CHD offspring if the mother carried the CT or TT genotype (OR 2.4; 95% CI [1.3– 4.3]; p = 0.004; Bonferroni-adjusted p = 0.008). Without medication use these carriers showed a smaller risk (OR 1.7; 95% CI [1.1–2.7]; p = 0.046; Bonferroni-adjusted p = 0.092). Analysis of the genotypes of the fathers and children revealed no significant associations.

Table 3 FBAT[a] Analysis of the *MDR1* genotype in an additive model

	Allele	Frequency	Inf. Fam. [b]	p-value	p_{perm} [c]
Cases Only	C	0.447	185	0.899	0.860
	T	0.553	185	0.899	0.860
Cases and Controls	C	0.474	396	0.474	0.831
	T	0.526	396	0.474	0.831

[a] FBAT, family based association test; [b] Inf. Fam., Informative Families; [c] p_{perm}, Permutation p-value after 100,000 permutations.

Table 4 Odds ratios for the MDR1 C3435T polymorphism and periconception parental medication use

MDR-1	Cases (%)	Controls (%)	OR[a] [95%CI][b]	Medication	Cases	Controls	OR[a] [95%CI][b]
Mother	**n = 283**	**n = 308**		**Mother**	**n = 283**	**n = 308**	
T/T and C/T	232 (82)	226 (73.4)	1.6 [1.1-2.5][c]	yes	67	47	2.4 [1.3-4.3][c]
				no	165	179	1.7 [1.1-2.7][c]
C/C	51 (18)	82 (26.6)	1 [ref]	yes	16	21	1.3 [0.6-3.0][c]
				no	35	61	1 [ref]
						p-trend	0.001[d]
Father	**n = 263**	**n = 291**		**Father**	**n = 263**	**n = 291**	
TT/CT	214 (81.4)	221 (75.9)	1.4 [0.9-2.1][c]	yes	33	28	1.5 [0.8-3.0][c]
				no	181	193	1.2 [0.7-1.9][c]
C/C	49 (18.6)	70 (24.1)	1 [ref]	yes	6	11	0.6 [0.2-2.2][c]
				no	43	59	1 [ref]
						p-trend	0.106[d]
Child	**n = 256**	**n = 298**		**Mother**	**n = 256**	**n = 298**	
T/T and C/T	205 (80.1)	231 (77.5)	1.0 [0.7-1.5][e]	yes	63	45	1.4 [0.8-2.7][e]
				no	142	186	0.8 [0.5-1.4][e]
C/C	51 (19.9)	67 (22.5)	1 [ref]	yes	15	22	0.9 [0.4-1.9][e]
				no	36	45	1 [ref]
						p-trend	0.112[d]

[a] OR, odds ratio; [b] CI, confidence interval; [c] The risk estimates are adjusted for the genotype of the child; [d] p-value Linear by Linear association test; [e] The risk estimates are adjusted for the genotype of the mother.

In Table 5 we show the associations between the *MDR1* CT/TT genotypes in mothers and children, and maternal medication and folic acid use on the risk of CHD offspring. Mothers with the risky genotypes, not taking folic acid in the periconception period in combination with medication use showed an association with an increased risk of CHD offspring. The trend toward an increased risk was highly significant (p = 0.001). No folic acid use and using medication increased CHD risk almost 3-fold in mothers with the CT/TT genotypes (OR 2.8; 95% CI [1.2– 6.4]; p = 0.015; Bonferroni-adjusted p = 0.045). In the children, this trend was borderline significant (p = 0.05). The subgroups were too small to attain enough statistical power to test the risk for both mother and child having the CT/TT genotypes on the risk of CHD.

Discussion

This is the first study to investigate associations among the *MDR1* 3435C>T polymorphism, periconception medication and/or folic acid use, and CHD risk. Our data suggest that mothers carrying the CT or TT genotypes have an almost 3-fold increased risk (OR 2.8; 95% CI [1.2– 6.4]) for having a child with CHD when medication was used and no folic acid was taken during the periconception period. In mothers carrying the CT or TT genotypes, who used medication but also folic acid, this risk was lower albeit not significant (OR 1.7; 95% CI [0.8 –3.7]). The functional single nucleotide polymorphism in the *MDR1* gene is associated with an increased degradation of *MDR1* mRNA, thereby explaining the earlier described association of this polymorphism with decreased protein expression and decreased activity. [9, 10] A recent study demonstrated that the 3435C>T polymorphism can also influence the selectivity for transporter substrates, thereby indicating that mRNA stability does not completely explain the effect of the 3435C>T mutation on the function of the protein.[19, 20]

Studies in knockout mice demonstrated the importance of the *MDR1* gene in decreasing cellular toxicity by regulating efflux of medication in several protective barriers, including the blood-brain barrier, blood-nerve barrier, blood-testis barrier, and maternal-foetal barrier.[8, 21, 22] Studies from *MDR1* knockout mice have also provided insight into the role of the *MDR1* transporter; the 2 gene products of *MDR1*a and *MDR1*b are considered to be the analogues of the human *MDR1* transporter gene. When embryonic mice with *MDR1*a -/ - were exposed to avermectin, a medicine, all mice developed cleft palate; *MDR1*a +/- mice were less sensitive; and *MDR1*a +/ + mice did not show malformations.[8] Furthermore, it was revealed that, in general, the genotype of the mouse foetus is important in limiting the transfer of toxins.[23] The concentrations of P-gp substrates in the foetus were measured after intravenous administration to the mother. This resulted in significantly increased concentrations in *MDR1*a -/- foetuses. In mice exposed to Dilantin, hydrocortisone and 6-aminonicotinamide, a protective effect of the foetus' genotype against defects was observed.[21] Given these findings in mice, we expected to find a similar effect in human beings.

Table 5 Odds ratios for the MDR1 C3435T polymorphism, periconception maternal medication and folic acid use

MDR1 Mother	Cases (%) n = 283	Controls (%) n = 308	ORa (95% CI) b	Medication Mother	Cases n = 283	Controls n = 308	ORa (95%CI)b	Folic acid	Cases	Controls	ORa (95%CI)b
T/T and C/T	232 (82)	226 (73.4)	1.6 [1.1-2.5][c]	yes	67	47	2.4 [1.3-4.3][c]	No	31	19	2.8 [1.2-6.4][c]
								Yes	36	27	1.7 [0.8-3.7][c]
				no	165	179	1.7 [1.1-2.7][c]	No	79	71	1.7 [0.9-3.2][c]
								Yes	86	108	1.3 [0.7-2.5][c]
C/C	51 (18)	82 (26.6)	1 [ref]	yes	16	21	1.3 [0.6-3.0][c]	No	10	11	1.6 [0.6-4.8][c]
								Yes	6	10	0.9 [0.3-3.0][c]
				no	35	61	1 [ref]	No	11	24	0.8 [0.3-1.9][c]
								Yes	24	37	1 [ref]
						p-trend	0.001[d]			p-trend	0.000[d]
Child	**n = 256**	**n = 298**		**Mother**	**n = 256**	**n = 298**			**n = 256**	**n = 298**	
T/T and C/T	205 (80.1)	231 (77.5)	1.0 [0.7-1.5][e]	yes	63	45	1.4 [0.8-2.7][e]	No	26	21	1.4 [0.6-3.3][e]
								Yes	37	24	2.5 [1.1-6.0][e]
				no	142	186	0.9 [0.5-1.4][e]	No	66	76	1.6 [0.7-3.5][e]
								Yes	76	110	0.8 [0.4-1.7][e]
C/C	51 (19.9)	67 (22.5)	1 [ref]	yes	15	22	0.9 [0.4-1.9][e]	No	12	9	2.5[0.8-7.6][e]
								Yes	3	13	0.3 [0.1-1.0][e]
				no	36	45	1 [ref]	No	14	16	1.1 [0.4-2.8][e]
								Yes	22	29	1 [ref]
						p-trend	0.112[d]			p-trend	0.051[d]

[a] OR, odds ratio; [b] CI, confidence interval; [c] The risk estimates are adjusted for the genotype of the child; [d] p-value Linear by Linear association test; [e] The risk estimates are adjusted for the genotype of the mother.

We showed previously that the *MDR1* 3435C>T polymorphism was associated with the risk of cleft lip/palate in the offspring.[24] Mothers carrying the 3435TT genotype and using medication showed a 6.2-fold (95% CI [1.6 –24.2]) increased risk of a child with cleft lip/palate compared to mothers carrying the 3435CC genotype and not using medication. Mothers carrying the 3435TT genotype, using medication and not taking folic acid showed the highest risk estimate (OR 19.2; 95% CI [1.0 –369.2]). In children carrying the *MDR1* 3435CT and 3435TT genotypes in combination with periconception medication use by the mother, increased risks were observed, although not significantly (i.e., OR 3.9; 95% CI [0.9 –16.1], and OR 2.5; 95% CI [0.7–9.5]). Herewith in line is our finding of an increased risk for CHD in children carrying the CT/TT genotype exposed to maternal medication use. In both our studies, periconception folic acid use seems to counteract the detrimental effect of medication use in the risky genotypes. The underlying mechanism, however, is unknown. Based on these findings, we suggest that when a mother carries the 3435CT/TT genotypes and takes medication, she provides an environment in which the developing embryo is exposed to higher levels of toxins, which may result in different CHD dependent on the exposure window.

It is known that folic acid contributes to the prevention of CHD, while the use of folate antagonists, dihydrofolate reductase inhibitors, or antiepileptics increases CHD risk.[25-30] Folate antagonists were not predominantly used by mothers in our study group; of the case mothers only 1 case mother used the folate antagonists valproic acid and phenobarbital vs. 3 control mothers who used trimethoprim, carbamazepine, and both carbamazepine and valproic acid. The studied CHDs by Hernandez-Diaz et al comprised transposition of the great vessels, Tetralogy of Fallot, ventricular septal defects, patent ductus arteriosus, atrial septal defects, coarctation of the aorta, and pulmonary-valve anomalies.[29] They reported an increased risk of 3.4 (95% CI [1.8 – 6.4]) for CHD in the offspring of mothers who used dihydrofolate reductase inhibitors. After maternal use of antiepileptic medication the risk was 2.2 (95% CI [1.4 –3.5]). In this study it was also shown that the use of multivitamins containing folic acid reduced the risk estimates of dihydrofolate reductase inhibitors. Although dihydrofolate reductase inhibitors and antiepileptic drugs are not a known substrate of *MDR1* P-gp, it would be very interesting to study the use of folate antagonists in a larger data set in combination with the *MDR1* genotype and CHD risk. In addition, these data are in line with our previous finding that children carrying the nicotinamide N-methyltransferase A allele face additional CHD risk in combination with periconception exposure to medicines and/or a low dietary nicotinamide intake.[31]

The frequency of adequate folic acid use starting preconceptionally was very high in both case and control mothers. In the general Dutch population unplanned pregnancy is very low, i.e., 15%, whereas unplanned pregnancy is the most important risk factor for inadequate use of folic acid in the periconception period (OR 9.5; 95% CI [7.2–12.4]; p = 0.001).[32, 33] Education and ethnicity are also strong determinants of adequate folic acid use. The high preconception usage of vitamins with folate of 50% in our study compared to the United States

and other countries, therefore, can be also explained by the relatively high percentages of case and control women with an intermediate to high education and Dutch ethnicity.

We did not distinguish mothers who started using preconceptionally or postconceptionally folic acid. We classified mothers as users when they used folic acid during the complete periconception period and nonusers when they used no folic acid or only during a part of this period. Therefore, recall bias of the start of the vitamins before or after conception is not an issue. However, recall bias of adequate use of folic acid in the periconception period cannot be excluded.

In our study, mothers were considered medication users when taking any medication. The detrimental effect of the 3435CT/TT genotype might be larger if only known substrates of this transporter are taken into account. Unfortunately, of many medications it is still unknown whether they are *MDR1* substrates.[24] A limitation of the study is that due to ethical constraints we were not able to verify the self-reported use of medication through the pharmacist. Others have reported frequencies of 45-48% on the periconception use of prescribed medication after ascertainment by pharmacies.[34,35] Underreporting of medication use might be an issue in this study. Nevertheless, the demonstrated interaction between medication use and *MDR1* CT/TT genotypes cannot be explained by recall bias or confounding by indication. Mothers were not aware of their genotype when they provided information on medication and folic acid use. A limitation of the study is also that due to the small numbers we are not able to give the risks for the separate medicines. Although maternal use of antihistamines in early pregnancy has not shown to be harmful with respect to birth defects, our finding of a higher reported use in case mothers is interesting and worthwhile to study in further detail when larger numbers are included.[36]

To minimize recall bias of exposure by the mother (medication and folic acid) and father (medication), we used a standardized study moment when both case and control children were approximately 16 months of age. Differential recall bias could therefore be of concern. An important study on the validity of parental reporting in case-control studies on different childhood diseases showed, however, that in case-control studies focusing on exposures in relation to disease, recall bias is mostly nondifferential.[37] Another strength of our study is the homogeneity of the CHD group, because we selected CHD phenotypes that share common pathways. It will be interesting, however, to study in future the phenotypes separately after enlarging the study populations. Homogeneity with regard to ethnicity was also reached with regard to the *MDR1* allele frequencies, which is in line with other studies.[7, 38]

In conclusion, we show that children of mothers carrying the *MDR1* 3435 CT/TT genotypes are probably more exposed to the teratogens derived of the medication used, which seem to be prevented in part by folic acid use. Our results strongly emphasize the importance to limit medication use by women in the periconception period and provide a first set of data against which future studies with larger sample sizes can be compared.

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Chapter 3

A maternal dietary pattern characterised by fish and seafood in association with the risk of congenital heart defects in the offspring

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Abstract

Derangements in the maternal one-carbon pathway increase the risk of congenital heart defects in the offspring and may be linked to diet. This study aims to identify dietary patterns related to biomarkers of methylation of this pathway and to investigate associations with CHD risk in the offspring.

From 179 case mothers of a child with CHD and 231 control mothers food intake was assessed from food frequency questionnaires (FFQ) collected 16 months after the index-pregnancy as a proxy of the periconceptional maternal dietary intake. In maternal blood concentrations of biomarkers of methylation were determined, i.e., S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). Folate, homocysteine, vitamin B12 and vitamin B6 were determined for validation of the dietary patterns. Food groups were summarized from individual FFQ items which were entered as predicting variables in the reduced rank regression (RRR) method while the methylation biomarkers served as response measures. The RRR calculated combinations of food groups, e.g., dietary patterns, which predict the methylation biomarkers. Linear and logistic regression models were used to asses associations between dietary patterns, biomarkers and risk of CHD. Two dietary patterns were identified. The one-carbon-poor dietary pattern, comprising high intake of snacks, sugar-rich products and beverages, was associated with SAH ($\beta = 0.92$, $p < 0.001$). The one-carbon-rich dietary pattern comprising of high fish and seafood intake was associated with SAM ($\beta = 0.44$, $p < 0.001$) and inversely with SAH ($\beta = -0.08$, $p < 0.001$). Strong adherence to this dietary pattern resulted in higher serum ($p < 0.05$) and red blood cell ($p < 0.01$) folate and a reduced risk of CHD in offspring: (odds ratio [OR] 0.3, 95% confidence interval [CI], [0.2–0.6]). In conclusion, the one-carbon-rich dietary pattern is associated with a reduced risk of CHD, comparable to the effects of periconceptional folic acid use.

Introduction

Worldwide each year around eight million children are born with a congenital malformation, one million of whom are affected by a congenital heart defect (CHD).[1] Its complex aetiology is characterised by an interplay between subtle genetic variations and harmful environmental, nutritional and lifestyle factors.[2] The importance of adequate maternal nutrition in the periconception period is well established. It has been shown that folic acid fortification of foods reduces significantly the prevalence of neural tube defects.[3] Evidence of its preventative effect against CHD, however, is still controversial.[4, 5]

Folate, methionine and choline are substrates and several B vitamins serve as cofactors in the one-carbon pathway. The methyl groups provided by folate, methionine and choline are ultimately important for the transmethylation of S-adenosylmethionine (SAM) into S-adenosylhomocysteine (SAH), after which homocysteine is formed. Methyl groups are needed for the methylation of lipids, proteins, chromatin and DNA. Evidence from animal studies has shown that the methylation of DNA by methyl groups is important in the epigenetic programming of cells and tissues during the periconception period and embryogenesis.[6-8] This is supported by our recent study in humans, showing that the maternal use of a low dose of folic acid in the periconception period increases significantly the methylation of the imprinted *IGF2* gene in the child.[9]

The concentrations of SAM and SAH in blood plasma and their ratio are frequently used as markers of global DNA methylation potential.[10] Low SAM and/or high SAH may result in cellular hypomethylation, which is associated with chromosomal instability and abnormal chromosomal segregation.[11] In previous work, we have demonstrated that a maternal state of global hypomethylation, that is low SAM/SAH ratio and high SAH concentration, is associated with a significantly increased risk of CHD and Down syndrome in the offspring.[12]

From this background, it was our aim to: (1) identify dietary patterns related to the biomarkers of methylation of the one-carbon pathway; (2) to validate the identified dietary patterns with nutrient intakes and other biomarkers of this pathway; and (3) to investigate associations between adherence to the identified maternal dietary patterns and the risk of CHD in the offspring.

Materials and Methods

The case and control groups were all enrolled in the HAVEN study, a Dutch acronym of the case–control family study designed to investigate determinants in the pathogenesis and prevention of CHD.[13] This study was conducted in the western part of the Netherlands. At a fixed study moment of around 16 months after delivery of the case or control child, the families visited the hospital for the standardised collection of information on exposures, food intake,

general characteristics and outcomes. Between June 2003 and August 2008, 351 case children with CHD and both parents were enrolled from four university medical centres. The CHD phenotypes were selected on the basis of a mutual aetiology with regard to the involvement of gene– environment interactions.[13-16] CHD was diagnosed after birth by two paediatric cardiologists with echocardiography and/or cardiac catheterisation and/or surgery. Control children (n = 466) were randomly recruited from the child healthcare centres of 'Thuiszorg Nieuwe Waterweg Noord', the area from which the cases arose. Child healthcare centres are part of the Dutch healthcare system, where up to 95% of newborns are regularly checked in a standardised manner during physical examinations directed towards health, growth and development by physicians specialised in child health care. Controls without a major congenital malformation were eligible for participation. Case and control children were eligible for inclusion if they were singletons. Controls were not eligible if they displayed a congenital malformation ascertained by their physician at the child healthcare centre or they were related to a case child. Only biological parents could participate and they had to be familiar with the Dutch language (writing and reading). All children were between 11 and 18 months of age.

At the study moment of 16 months after delivery of the case or control child, mothers filled out a food frequency questionnaire (FFQ) referring to the dietary intake of the previous month to reflect the dietary intake during the periconception period.[17]

The study protocol was approved by the Central Committee on Research Involving Human Subjects and the Institutional Review Boards of all participating hospitals. Informed consent was obtained from all parents.

Questionnaires

At the study moment of approximately 16 months after birth of the index child, mothers completed a validated FFQ at home that covered the food intake of the previous 4 weeks. The FFQ was developed by the Division of Human Nutrition, Wageningen University and validated for the intake of energy, B vitamins and fatty acids.[18, 19]

Moreover, at the same time, a self-administered general questionnaire was completed by the mother. The extracted data comprised educational level, ethnicity, and alcohol, tobacco, medication and vitamin B use at the study moment and during the periconception period of the index pregnancy. The periconception period was defined as 4 weeks before until 8 weeks after conception. The use of folic acid in the periconception period was defined as the daily use of at least 400 μg of folic acid, in either a multivitamin preparation or as a single tablet during the whole period. We defined medication use as any prescribed use of medication during the periconception period. Educational level and ethnicity were classified according to the definition of Statistics Netherlands.[20]

Also at 16 months after the index birth, all case and control mothers and children were invited for a hospital visit, at which time the FFQ and general questionnaires were checked in a standardised manner for completeness and consistency by the researcher. Furthermore,

standardised anthropometric measurements were performed, including maternal height and weight (anthropometric rod and weighing scale; SECA, Hamburg, Germany). The body mass index (BMI) was calculated by weight divided by the squared height.

Assays of biomarkers

During the hospital visit, fasting venous blood samples were drawn from case and control mothers in parallel during the whole study period for the measurement of the biomarkers of methylation in plasma (SAM, SAH and total homocysteine [tHcy]), and red blood cell (RBC) and serum folate and vitamin B12.

Immediately after blood sampling, an ethylenediaminetetraacetic acid (EDTA) tube was placed on ice and a serum separator tube was kept at room temperature. Both tubes were centrifuged at 4000 • g for 10 minutes at 4 °C and separated within 1 hour. All samples were stored at -80 °C and measured anonymously, that is without a knowledge of the case or control state, in batches within 5 months of collection. Thus, the samples from cases and controls were analysed over similar time frames. To determine SAM and SAH, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS; Waters acquity UPLC premier XE, Milford, MA, USA) as described previously.[12] tHcy was also determined using LC-MS/MS. Serum folate and vitamin B12 were routinely determined by immunoelectrochemiluminescence immunoassay (ECLIA) on a Roche Modular E170 (Roche Diagnostics GmbH, Mannheim, Germany). RBC folate was measured in the haemolysate of whole blood with ascorbic acid for stabilisation. The RBC folate concentration was calculated according to the following formula: (nmol/l haemolysate folate x 10/haematocrit) - (nmol/l serum folate x [1 - haematocrit]/haematocrit) = nmol/l RBC folate. The inter-assay coefficients of variation (CV) were 4.4% at 70.8 nmol/l, 4.4% at 100.8 nmol/l and 4.8% at 143.2 nmol/l for SAM, 4.2% at 24.2 nmol/l for SAH, 5.9% at 15.3 µmol/l and 3.4% at 39.3 µmol/l for tHcy, 9.5% at 8.3 nmol/l and 3.2% at 20.2 nmol/l for folate, and 5.1% at 125 pmol/l and 2.9% at 753 pmol/l for vitamin B12.

Statistical and food frequency analysis

Before analysis, we excluded mothers whose nutritional state could have been influenced by a non-European ethnicity, a reported dietary difference at the study moment compared with the periconception period, breastfeeding, new pregnancy and/or the use of folic acid at the study moment. Maternal age and log-transformed energy intake are presented as the mean with interquartile range (25th–75th percentile, p25–p75). BMIs at the study moment and over the duration of pregnancy showed skewed distributions even after log transformation and are presented as the median with interquartile range (p25–p75). Whether or not the differences between cases and controls were substantial was tested with Student's t-test for age and energy intake, Mann–Whitney U-test for the remaining continuous variables and χ^2 test for categorical variables. The Kruskall–Wallis U-test was used to test differences in BMI and duration of

pregnancy. The χ^2 test for linear association was used to investigate differences in educational level, medication use, smoking, alcohol use and vitamin B intake.

We chose to perform dietary pattern analysis to identify dietary patterns as predictors of the biomarker concentrations of methylation: SAM and SAH. In the first step, we reduced the number of 200 food items in the FFQ to 22 predefined food groups, which are similar in nutrient content and comparable with the grouping schemes reported in the literature.[21] The food groups were adjusted for energy intake according to Willett et al.[22] In the second step, the group of control mothers was used to identify the dietary patterns, because these dietary patterns will reflect the dietary habits of Dutch mothers of reproductive age. In the third step, dietary pattern analysis was performed with the reduced rank regression (RRR) method.[23] The analysis starts with the selection of the 22 energy-adjusted food groups as independent or exposure variables, followed by the correlation with the remaining food groups. This is followed by the choice of the biomarker concentrations of SAM and SAH as dependent or response measures following centring, standardising and log transformation. This is followed by the application of RRR with CANOCO (Canonical Community Ordination, version 4.5) software for Windows.[24] The number of factors identified by the RRR method equals the number of response variables. The two factors, further referred to as dietary patterns, explained the largest proportion of variation in the biomarkers of methylation: SAM and SAH. The overall p value for the explained variance of the dietary pattern analysis was tested using the permutation method.

The relationship between the 22 food groups and the identified dietary patterns (factors) was denoted by factor loadings, which represent the correlation coefficients between the food groups and the dietary patterns. These correlation coefficients are given by β estimates from a linear regression model, where the 22 food groups were simultaneously and independently entered with the respective dietary pattern as the dependent variable. This allowed us to investigate the independent effect of each food group adjusted for all other food groups. In the next analysis, all case and control mothers were assigned a score of adherence to the two identified dietary patterns, calculated as the product of the food group value and its factor loading and summed across the food groups. The case and control mothers were divided into tertiles according to the degree to which their individual nutritional intakes agreed with the dietary pattern derived from the control group. This resulted in the classification of mothers into low, intermediate or high adherence to the respective dietary pattern. The association between the degree of adherence to the dietary pattern and the concentrations of the other biomarkers, that is tHcy, folate and vitamin B12, was examined by linear regression analysis.

Finally, we investigated whether the maternal dietary pattern was associated with the risk of CHD in the offspring. A logistic regression model was applied in which the occurrence of CHD was chosen as the outcome. The degree of maternal adherence to the respective dietary pattern was chosen as the independent variable, in which a low adherence served as a reference. The estimates are given by odds ratios [OR] and 95% confidence intervals [95% CI].

In an additional logistic regression analysis, we adjusted the crude CHD risk estimates for the potential confounders: maternal age, BMI, educational level, periconception folic acid/multivitamin use, smoking, alcohol and medication use.

Results

We excluded from the analysis mothers with the following: (1) missing biomarker concentrations (cases n = 2; controls n = 2); (2) a non-European ethnicity (cases n = 54; controls n = 100); (3) an altered diet compared with the diet used in the periconception period (cases n = 26; controls n = 35); (4) pregnancy at the study moment (cases n = 26; controls n = 25); (5) breastfeeding at the study moment (cases n = 10; controls n = 6); and (6) the current use of folic acid supplements at the study moment (cases n = 54; controls n = 67). This resulted in the further analysis of 179 case mothers and 231 control mothers for dietary pattern analysis. The CHD phenotypes (n = 179) comprised Tetralogy of Fallot (n = 25), transposition of the great arteries (n = 37), atrioventricular septal defect (n = 16), perimembranous ventricular septal defect (n = 41), coarctation of the aorta (n = 21), aortic valve stenosis (n = 6), pulmonary valve stenosis (n = 26) and hypoplastic left heart syndrome (n = 7). The CHDs were divided into 128 isolated and 51 nonisolated cases. The latter group was further divided into nonsyndromic CHD (n = 20), Down syndrome (n = 19), 22q11 deletion syndrome (n = 5), insertion 1 > 3 (n = 1), and Noonan (n = 1), Turner (n = 1), Alagille (n = 1), Saethre–Chotzen (n = 1), an acronym for Coloboma, Heart defect, Atresia of the choanae, Retardation of growth and development, Genital and urinary abnormalities, Ear abnormalities and/or hearing loss (CHARGE) (n = 1) and Beckwith–Wiedemann (n = 1) syndrome. Seventeen also had other defects that could not be traced to a known syndrome, such as hip dysplasia, anus atresia, hydronephrosis, deafness and hydrocele.

The general characteristics of case and control mothers are shown in Table 1. Case mothers had a higher BMI ($p = 0.029$), showed a lower SAM concentration ($p = 0.025$), a slightly shorter duration of pregnancy ($p = 0.002$) and used more medication in the periconception period ($p = 0.005$), such as antibiotics, anticonvulsants, antiinflammatory medicines, hormones and antimycotics. The RRR analysis of the control mothers revealed two factors or dietary patterns explaining 10.6% of the variance in SAH ($p < 0.001$) and 4.2% of the variance in SAM ($p < 0.001$). The strong positive association between the first dietary pattern and SAH ($\beta = 0.92$, $p < 0.001$) reflects a diet poor in one-carbon donors, and was therefore labelled as the 'one-carbon-poor diet'. This diet was also positively, albeit marginally, associated with SAM ($\beta = 0.03$, $p < 0.001$). This one-carbon-poor dietary pattern contained, in particular, a high intake of snacks and sugar-rich products and beverages (Table 2), which were not significantly correlated with nutrient intake (Table 3) or other biomarkers across the tertiles of degree of adherence (Table 4).

The second dietary pattern showed a positive association with SAM ($\beta = 0.44$, $p < 0.001$) and an inverse, albeit marginal, association with SAH ($\beta = -0.08$, $p < 0.001$). This one-carbon-rich dietary pattern contained, in particular, a high intake of fish and other seafood (Table 2). Adherence to this dietary pattern was significantly correlated with a high intake of total protein, the B vitamins B1, B2, B3, B6 and B12, the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and zinc (Table 3). Adherence to the one-carbon-rich dietary pattern was associated with higher levels of serum folate ($p = 0.026$) and RBC folate ($p = 0.005$; Table 4).

In contrast with the one-carbon-poor dietary pattern, strong adherence to the one-carbon-rich dietary pattern significantly reduced the CHD risk (crude OR 0.59; 95% CI [0.37–0.96]). After adjustment for maternal age, BMI, educational level, periconception use of folic acid, alcohol, medication and smoking, the risk estimate became even more prominent (OR 0.32; 95% CI [0.18–0.59]). Only BMI was associated with adherence to both the one-carbon-poor ($p = 0.012$) and one-carbon-rich ($p = 0.001$) dietary pattern.

Table 1 General characteristics of Mothers of a Child with a CHD and control mothers

	Case mothers (n=179)	Control mothers (n=231)	p-value
At the conception of index pregnancy:			
Maternal age, (y)*	33.1 (30.2-35.5)	32.4 (29.3-35.4)	0.142
Duration of pregnancy, (wk)†	39.3 (38.1-40.6)	40.0 (38.7-41.0)	0.002
At the study moment:			
BMI, (kg/m2)†	24.9 (22.1-28.6)	24.1 (21.9-26.8)	0.029
Energy intake (kJ/d)*	8856 (7474-9991)	9041 (7248-10543)	0.393
High education‡	127 (29.1)	64 (27.7)	0.216
Medication use	33 (18.4)	44 (19.0)	0.899
Smoking	32 (17.9)	44 (19.0)	0.798
Alcohol	100 (55.9)	143 (61.9)	0.225
Biomarkers†			
SAM (nmol/L)	77.0 (70.9-86.4)	79.7 (73-88.4)	0.025
SAH (nmol/L)	14.3 (12.5-16.5)	14.3 (12.2-16.2)	0.800
tHcy (µmol/L)	10.9 (9.1-13.3)	10.3 (8.6-12.5)	0.101
Vitamin B-12 (pmol/L)	270 (203-360)	244 (198-335)	0.139
Folate serum (nmol/L)	14.2 (11.6-17.7)	13.3 (11.8-17.5)	0.496
Folate RBC (nmol/L)	620 (501-748)	607 (516-743)	0.885
In the periconception period:			
Medication	46 (25.7)	33 (14.3)	0.005
B-vitamins	98 (54.7)	133 (57.6)	0.616
Smoking	35 (19.6)	41 (17.7)	0.701
Alcohol	70 (39.1)	81 (35.1)	0.470

BMI, body mass index; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine, tHcy, total homocysteine.
Values are *mean (P25-p75), †median (P25-p75) or n (%).
‡ Categorized as high when the education level was 'higher vocational' or 'university'.

Table 2 Association between food groups and 'One carbon-poor' and 'One carbon-rich' dietary patterns, performed with RRR with food groups as predictor variables for SAM and SAH concentrations in blood of 231 control mothers

	SAM/SAH predictive dietary pattern*			
	Factor 1 'One carbon-poor'		Factor 2 'One carbon-rich'	
Biomarkers	**β (s.e)**	**p**	**β (s.e.)**	**p**
SAM	0.03 (0.01)	<.001	0.44 (0.01)	<.001
SAH	0.92 (0.01)	<.001	-0.08 (0.01)	<.001
Food Groups				
Alcoholic Drinks	-0.01 (0.01)	0.376	0.03 (0.02)	0.216
Breakfast Cereals	0.08 (0.08)	0.312	-0.05 (0.13)	0.667
Butter	1.19 (1.08)	0.271	0.25 (1.70)	0.885
Dairy Products	-0.01 (0.04)	0.797	0.11 (0.06)	0.079
Eggs	-0.03 (0.07)	0.647	-0.03 (0.10)	0.809
Fish and other Seafood	0.12 (0.21)	0.582	0.96 (0.33)	0.004
Fruits	0.00 (0.02)	0.889	-0.02 (0.03)	0.519
Legumes	0.09 (0.09)	0.278	0.11 (0.13)	0.426
Margarine	1.09 (0.77)	0.156	1.28 (1.20)	0.290
Mayonnaise	0.07 (0.21)	0.742	0.20 (0.33)	0.541
Meat products	0.06 (0.16)	0.682	0.17 (0.25)	0.479
Non-alcoholic beverages	0.01 (0.01)	0.034	0.01 (0.01)	0.538
Nuts	0.22 (0.11)	0.051	0.35 (0.18)	0.051
Refined Grains	0.06 (0.05)	0.268	-0.05 (0.09)	0.551
Potatoes	0.01 (0.03)	0.604	0.02 (0.04)	0.651
Sauces	0.00 (0.19)	0.999	0.17 (0.29)	0.553
Snacks	0.16 (0.06)	0.006	0.04 (0.09)	0.651
Soup	0.01 (0.01)	0.133	0.01 (0.01)	0.368
Sugar and Confectionary	0.27 (0.12)	0.023	0.01 (0.18)	0.939
Vegetable Oils	0.76 (1.30)	0.547	0.73 (2.00)	0.714
Vegetables	0.00 (0.03)	0.989	-0.05 (0.05)	0.314
Whole Grains	0.03 (0.04)	0.432	0.01 (0.06)	0.860

*Regression coefficients β (s.e.) and p - value have been analyzed in a linear regression model. All food groups were simultaneously independently entered in one model. The linear regression coefficients represent the independent effect of each food group adjusted for all other food groups

Table 3 Pearson correlation coefficients between the energy adjusted macro- and micronutrient intakes and the dietary patterns in 231 control mothers

Nutrients	Factor 1 'One carbon-poor' dietary pattern*	p	Factor 2 ' One carbon-rich' dietary pattern*	p
Total fat (g/d)	-0.064	0.331	-0.077	0.241
Saturated fats (g/d)	-0.041	0.536	-0.103	0.118
MUFA (g/d)	-0.032	0.632	-0.072	0.277
PUFA (g/d)	-0.087	0.188	0.003	0.968
LA (g/d)	-0.036	0.584	0.037	0.574
ALA (g/d)	0.044	0.503	0.033	0.617
EPA (g/d)	0.109	0.100	0.131	0.047
DHA (g/d)	0.085	0.196	0.131	0.047
Cholesterol (mg/d)	0.013	0.849	0.046	0.484
Total protein (g/d)	0.055	0.403	0.164	0.013
Total carbohydrates (g/d)	0.078	0.238	-0.017	0.801
Fiber (g/d)	0.042	0.527	0.065	0.327
Alcohol (g/d)	-0.103	0.119	0.046	0.484
Vitamin B 6 (mg/d)	-0.016	0.814	0.138	0.036
Vitamin B 12 (µg/d)	0.125	0.057	0.183	0.005
Folate (µg/d)	0.053	0.425	0.129	0.051
Zinc (mg/d)	0.071	0.285	0.145	0.027
Retinol (µg/d)	0.060	0.361	0.045	0.494
Thiamin (mg/d)	0.090	0.171	0.130	0.048
Riboflavin (m(g/d)	0.025	0.701	0.175	0.008
Nicotinamide (mg/d)	0.109	0.099	0.183	0.005
Vitamin C (mg/d)	-0.012	0.852	0.065	0.328
Vitamin E (mg/d)	-0.124	0.061	-0.024	0.714

Data presented as correlation coefficients.

Discussion

In this study, we identified one-carbon-poor and one- carbon-rich dietary patterns in mothers, reflecting the bioavailability of one-carbon donors. The one-carbon-rich dietary pattern, characterised by a high intake of fish and seafood, provided, in particular, total protein, vitamin B1, B2, B3, B6 and B12, zinc, EPA and DHA. Strong adherence to this dietary pattern was reflected in higher RBC and serum folate levels, and associated with a 70% reduced risk of CHD in the offspring. This estimate is much higher than the 20% reduction after the periconception use of a low dose of synthetic folic acid.[25]

The one-carbon-poor dietary pattern, characterised by a high intake of snacks, sugar-rich foods and beverages, resembles more closely a Western dietary pattern, and did not affect the biomarker levels and CHD risk. It has been shown previously that this dietary pattern related

Table 4 Correlations between the biomarkers and adherence to the dietary pattern in 231 control mothers

	Factor 1 'One carbon-poor' dietary pattern*				Factor 2 'One carbon-rich' dietary pattern*			
Biomarkers	Low (n = 74)	Intermediate (n = 79)	High (n = 78)	p†	Low (n=68)	Intermediate (n=78)	High (n=85)	p†
SAM (nmol/L)	74.7 (69.2-81.4)	82 (76.5-88.7)	84.2 (75.5-91.9)	<.001	69.5 (67.3-71.8)	78.7 (76.3-81.3)	90.4 (87- 95.7)	<.001
SAH (nmol/L)	11.5 (10.8-12.2)	14.2 (13.4.-14.7)	17.3 (16.2-19)	<.001	13.0 (11.4- 16.5)	13.95 (12.4-15.6)	15.2 (13.3-17.1)	<.001
tHcy (µmol/L)	10 (8.5-11.3)	10.5 (8.5- 12.4)	10.9 (9.2-13.8)	0.097	10.3 (8.7-11.7)	10.65 (8.7-12.6)	10.4 (8.5-12.8)	0.105
Vitamin B 12 (pmol/L)	260 (207-338)	237 (179-318)	246 (199-342)	0.723	241.5 (205- 340)	259 (201-359)	239 (194-313)	0.785
Folate serum (nmol/L)	13.0 (11.4-17.6)	13.5 (11.8-17.4)	13.45 (12.1-17.8)	0.623	13.4 (11.4-15.5)	13 (11.7-17.9)	14.1 (12.1-19.1)	0.027
Folate RBC (nmol/L)	627 (522-765)	588 (509-731)	593 (514- 747)	0.926	592 (516-707)	599 (517-731)	637 (518-808)	0.005

SAM. S-adenosylmethionine; SAH, S-adenosylhomocysteine; tHcy, total homocysteine.
Data are median (p25-p75).
* Grouping in low, intermediate or high adherence to the dietary pattern was based on tertiles calculated by summing of intake of foodgroups weighted by their factor loadings.
† Calculated in a linear regression model on log-transformed continuous biomarker variables.

Table 5 The association between adherence to the dietary patterns and the risk of having offspring with CHD

Dietary pattern*	Case mothers (n = 179)	Control mothers (n = 231)	OR [95% CI] Crude	OR [95% CI] Adjusted†
Factor 1 'One carbon-poor'				
Low	63 (35.2)	74 (32.0)	1.00 [Ref]	1.00 [Ref]
Intermediate	58 (32.4)	79 (34.2)	0.86 [0.54 - 1.39]	0.96 [0.56 - 1.64]
High	58 (32.4)	78 (33.8)	0.87 [0.54 - 1.41]	0.81 [0.47 - 1.40]
Factor 2 'One carbon-rich'				
Low	69 (38.5)	68 (29.4)	1.00 [Ref]	1.00 [Ref]
Intermediate	59 (33.0)	78 (33.8)	0.75 [0.46 - 1.20]	0.64 [0.37 - 1.11]
High	51 (28.5)	85 (36.8)	0.59 [0.37 - 0.96]	0.32 [0.18 - 0.59]

CHD, congenital heart defect; OR, odds ratio; CI, confidence interval.
Data are n (%) or odds ratio [95% confidence interval].
* Grouping in low, intermediate or high adherence to the dietary pattern was based on tertiles calculated by summing of the intake of food groups weighted by their factor loadings.
†Adjusted for maternal age at index-pregnancy, BMI, educational level, periconception folic acid/multivitamin use, smoking, alcohol intake and medication use.

to low SAM and high SAH is often accompanied by hyperhomocysteinaemia, which is a risk factor for CHD and neural tube defects.[12-14, 16, 26] In line with Shoob et al. who showed that a methionine-rich diet is associated with a reduced risk of neural tube defects, we now demonstrate an association between strong adherence to the one-carbon-rich diet and a reduction in CHD risk.[27] This finding substantiates our earlier observation that adherence to a Mediterranean dietary pattern, also rich in one-carbon groups and correlated with high serum and RBC folate levels, is associated with a reduced risk of spina bifida in the offspring.[28]

The relationship between fish and seafood intake during pregnancy and pregnancy outcome is not well understood. Researchers have suggested that, in particular, omega-3 fatty acids—as present in oily fish, such as salmon and tuna—have a positive effect on fetal growth and brain development.[29] In contrast, other studies have shown associations between maternal seafood intake during pregnancy and poor verbal skills and behavioural problems in children.[30] These harmful effects have been attributed to the contamination of seafood with mercury and other pollutants.

Our study supports the beneficial effects of maternal fish intake, in particular the reduced risk of CHD in the offspring. Fish intake in the Netherlands is generally very low, that is 7 g/day for women of reproductive age. The Netherlands Health Council recommends eating fish twice a week, that is approximately 350 g. The one-carbon-rich dietary pattern of the mothers in our study contained, on average, 91 g of fish per week. Therefore, it is possible that the observed beneficial effect of this dietary pattern is attributable to the essential nutrients in fish without reaching harmful concentrations of mercury and other pollutants.

The underlying mechanisms by which derangements in the one-carbon pathway, induced

by compromised dietary intakes of its substrates and cofactors, contribute to the prevention of birth defects are largely unknown. Nutrition plays a key role in epigenetic programming of embryonic growth and development, in which one-carbon groups, in particular methyl groups, play a significant role in DNA methylation and histone modification.[6, 31] Animal studies have shown that the dietary availability of methyl groups affects gene methylation. Supplementation of mice with methyl donors before or in early pregnancy increases the level of DNA methylation and changes the phenotype of the offspring.[7, 32] A study in rats has shown that maternal dietary protein restriction during pregnancy, including low methionine, leads to a persistent decrease in the methylation of several genes. This pattern of hypomethylation was not present in rats supplemented with folic acid.[33] Furthermore, Sinclair et al. demonstrated, in sheep, that deficiencies of B vitamins and methionine in the preconception diet lead to both hypomethylation and hypermethylation of the offspring's genome, accompanied by an unhealthy phenotype.[34] These findings underline the importance of periconceptional adherence to the one-carbon-rich dietary pattern as determinant of future health and disease risk in the offspring, thereby substantiating the developmental origin hypothesis of health and disease.[31]

The one-carbon-rich dietary pattern provides, in particular, B vitamins. This supports our earlier finding that a high intake of these nutrients reduces CHD risk in the offspring.[35, 36] Furthermore, this dietary pattern was also positively correlated with zinc, which is a cofactor of c-glutamylhydrolase, involved in the absorption of folate and of methionine synthase implicated in the conversion of homocysteine into methionine.[37] The positive correlation with the omega-3 fatty acid (EPA and DHA) intake may be explained by their homocysteine-reducing effect, and contribute to the discussion of the beneficial effects of fish intake on pregnancy outcome.[38-40]

Previously, we have investigated associations between lifestyle factors and the biomarkers of methylation, SAM and SAH, in the same HAVEN study among the control mothers.[41] It was revealed that BMI is a strong determinant of SAM and, to a lesser extent, of SAH. A high maternal BMI, however, is a known risk factor for CHD in the offspring, and is, in our study, independently associated with an increased risk of CHD in the offspring (BMI > 30 kg/m2 versus BMI < 30 kg/m2; OR 2.5; 95% CI [1.4–4.6]).[42] Randomised controlled clinical trials are necessary to exclude residual confounding of the relationship between maternal dietary patterns and the risk of CHD in the offspring.

Methionine, choline and betaine are also important donors of methyl groups, and their intake is associated with a significant decrease in plasma SAH and an increase in SAM:SAH, methionine and glutathione:GSSG (oxidised glutathione). It has been suggested that foods rich in these compounds may also prevent congenital malformations.[43, 44] Unfortunately, the FFQ used in our study is not validated for the precise assessment of the intake of these nutrients. Unfortunately, the FFQ used in our study is not validated for the precise assessment of the intake of these nutrients. Therefore, it would be interesting to study in future the effects of these nutrients in association with CHD risk.

The case mothers had a higher BMI, and reported a lower energy intake, which introduces the concern of differential under-reporting between obese and nonobese women. It has been shown that nutritional under-reporting increases with an increase in BMI.[45] We tested this potential confounder by estimating the mean basal metabolic rate (BMR) using the new Oxford equation for women aged 30–60 years: BMR (MJ/day) = 0.0407 X weight (kg) + 2.90. The physical activity level (PAL) was calculated by dividing the mean reported energy intake (EI) by the mean BMR.[46] A PAL cut-off value of 1.35 was used to evaluate under-reporting; both case and control mothers had a higher PAL: 1.39 and 1.44, respectively. Additional trend analysis revealed a significant trend for a decrease in PAL with an increase in BMI. The PAL values for case and control mothers with BMI > 25 kg/m2 were 1.31 and 1.27, respectively, which may suggest under-reporting. This association, however, is independent of the case or control status, and thus nondifferential, and therefore does not distort the validity of our results.

The main advantage of RRR is that all food groups are used to predict the global methylation status by SAM and SAH biomarkers. This sophisticated method includes all dietary information in which no foods have been omitted. Nevertheless, restricting the model to the prediction of methylation biomarker levels means that other pathways between diet and disease that may be involved are disregarded a priori.

Some strengths and limitations of this study need to be addressed. We used a standardised study moment of approximately 16 months after the index pregnancy. We consider this to be one of the main methodological strengths of our study compared with the variable study moments after birth (from weeks to years) used by others in studies on congenital malformations.[17] The study moment is within 2 years after birth of the index child to minimise recall bias concerning periconception exposures. Periconception exposures were comparable overall between case and control mothers, with the exception of the more frequently reported use of medication by case mothers. We hypothesise that children exposed periconceptionally to anticonvulsants and nonsteroidal anti-inflammatory medication, in particular, are at increased risk for CHD. However, because of the small sample size and absence of verification of medication use by the pharmacist, it cannot be excluded that a degree of differential recall bias could have occurred between case and control mothers. Moreover, we prevented the misclassification of cases and controls as much as possible by choosing the study moment of 16 months after delivery, as most CHDs are diagnosed during the first year of life. However, for ethical reasons and feasibility, echocardiography or catheterisation was not performed in control children. Therefore, misclassification of controls cannot be excluded completely, but this would have led to an underestimation of the observed associations.

The validated FFQ was completed at the study moment and covered the intake of the previous 4 weeks, whereby day-to-day variability of food intake is minimised. The study moment is 2 years after conception of the index pregnancy in the same season as the periconception period. Thus, the seasonal influences on food intake are comparable between these periods.

In addition, we have shown previously that the maternal nutritional status assessed more than 1 year after delivery can be used as a valid estimate of the preconception maternal nutritional status.[17] This is also supported by others, showing that every individual adheres to a habitual dietary pattern which is only influenced by periods of illness, diet, pregnancy, breastfeeding and growth spurts. After adjustment of the analysis for these factors, as performed here, dietary patterns are very stable during life.[47-49] An advantage of our study population is that we excluded non-European mothers, thereby increasing homogeneity with regard to nutritional habits and lifestyles, which are also determinants of dietary patterns.[50] Methodological limitations in the case–control study concern the degree to which the measured maternal biomarkers and BMI at the study moment truly reflect the periconception status. Because differences are thought to be nondifferential between cases and controls, they do not pose a threat to the validity of our findings.

We conclude that adherence to a one-carbon-rich dietary pattern, mainly reflected by a high intake of fish and seafood, is associated with a reduced CHD risk in the offspring. This finding may be the first link between periconception maternal nutrition and epigenetic programming of embryonic development and growth, but should be further substantiated by a prospective randomised intervention trial. However, we also wish to emphasise that, for many reasons, dietary intervention trials are very difficult to perform, especially in the target population of (pre)pregnant women.

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Part II

The origin of congenital heart defects: determinants in the child

Deck the halls, I'm young again
I'm you again, racing turtles
The grapefruit is winning
“Space Dog” -TORI AMOS

Chapter 4

Elevated triglyceride levels in very young children are associated with congenital heart defects

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Abstract

Mild dyslipidaemia in women is associated with an elevated risk of having a child with a congenital heart defect (CHD) and cardiovascular disease (CVD) in later life. The goal of this study was to investigate whether the lipid profiles in very young children are associated with CHD. We conducted a case-control study among 141 children with CHD and 178 healthy children at the age of 17 months. Serum concentrations of triglycerides, total cholesterol, and HDL-cholesterol were measured. LDL-cholesterol values were calculated using the Friedewald formula. Differences in concentrations were tested with Student's t-test. The risk for CHD was calculated in a logistic-regression model with quintiles of the lipid levels in the control population as cut-off points. Mean triglyceride levels were significantly higher in the total CHD group compared to healthy controls (1.44 vs. 1.04 mmol/L; $p < 0.001$) and above the upper reference value of 1.3 mmol/L. After stratification into isolated and non-isolated CHD, triglyceride concentrations were highest in the isolated CHD subgroup. Total cholesterol, HDL-cholesterol and LDL-cholesterol levels between cases and controls were not significantly different. The highest risk of CHD was observed in children with a triglyceride level in the upper quintile (>1.62 mmol/L; odds ratio [OR] 5.5, 95% confidence interval [CI], [2.3– 13.2]).

In conclusion, in very young children with CHD, in particular isolated CHD, the mean triglyceride level is almost 40% higher compared to control children and above the upper reference value. This may suggest that children with CHD are more at risk for the development of cardiovascular disease in later life.

Introduction

Congenital heart defects (CHD) are the most common congenital malformations in newborns, of which worldwide the birth prevalence rates are rising.[1] Although the mortality of CHD has decreased in the last 20 years, the burden for the patient, family, society, health care and insurances is enormous.[2]

Last decade several periconceptional maternal risk factors for having a child with CHD have been identified, of which many are also associated with cardiovascular disease risk, such as hyperhomocysteinaemia, obesity and diabetes.[3-5] Recently, we reported that a mild maternal dyslipidaemia, i.e., elevated levels of total cholesterol, LDL-cholesterol and apolipoprotein-B, is associated with a 2-fold increased risk of CHD in the offspring.[6] This finding could partially be explained by a higher intake of a diet rich in saturated fats by the mothers of a child with CHD.[7] During embryogenesis lipids are important building blocks of the cellular membranes, provide energy, and serve as transcription factors. Maternal nutrition during pregnancy is of major importance in determining future cardiovascular health risk in the offspring.[8-10] This is substantiated by animal studies showing that a prenatal maternal diet rich in fats leads to an increased risk of abnormalities in lipid levels and endothelial cell dysfunction in the offspring.[11, 12] Evidence in human from the Dutch Hunger Winter study also showed that prenatal under nutrition leads 6 decades later to higher cholesterol and triglycerides levels and risk of the development of cardiovascular diseases.[13, 14] Foetal programming through epigenetics may be one of the mechanisms that explain this increased risk.[15] In line with the Developmental Origin of Health and Disease hypothesis, we hypothesize that in utero exposure to harmful maternal traits may increase both the risk of CHD in the offspring as well as the development of cardiovascular disease in these children in later life.[16, 17]

From this background, we aimed to investigate lipid levels in very young children in association with CHD.

Materials and Methods

Study population

The design of the HAVEN study has been published previously. Briefly, this case-control family study is conducted from 2003 onwards in the Western part of the Netherlands at the Department of Obstetrics and Gynaecology of the Erasmus MC, Rotterdam, The Netherlands.[3] Children with CHD and both parents are recruited in collaboration with the Departments of Paediatrics of the Erasmus MC in Rotterdam, Leiden University Medical Centre in Leiden, and VU University Medical Centre and Academic Medical Centre in Amsterdam. Control children and their parents are enrolled in collaboration with child health centres where each child in The Netherlands is regularly checked on growth and development.

Case and control children were eligible for inclusion if they were singletons, around 17 months of age, their parents were familiar with the Dutch language in reading and writing, and there was no familial relationship between cases and controls. Children with CHD were diagnosed in the first year of life by two paediatric cardiologists using ultrasound and/or cardiac catheterization and/or surgery. In the randomly selected control children, however, recruited at the same age and without congenital malformations according to the medical records and regular health check by the physician of the child health centre, those assessments have not been made. The selection of CHD phenotypes is based on experimental and epidemiological studies that showed that hyperhomocysteinaemia and folic acid use are associated with CHD.[18, 19] The included CHD (n = 141) phenotypes were Tetralogy of Fallot (TOF; n = 15), transposition of the great arteries (TGA; n = 28), atrioventricular septal defect (AVSD; n = 7), peri-membraneous ventricular septal defect (pVSD; n = 43), coarctation of the aorta (CoA; n = 15), valvular aortic stenosis (AS; n = 5), valvular pulmonary stenosis (PS; n = 26) and hypoplastic left heart syndrome (HLHS; n = 2). Between June 2003 and March 2005, we included 210 cases and 203 controls for a hospital visit. From 141 case children and 178 control children enough blood was available for the determination of total cholesterol, HDL-cholesterol and triglycerides.

The Central Committee on Research involving Human Subjects and the Institutional Review Boards of all participating hospitals approved the study protocol, the research described in this report was conducted according to the Declaration of Helsinki. All parents gave their written informed consent also on behalf of their child before participation.

Sampling and Analytical Methods

All parents filled out a general questionnaire at home, which was checked during a hospital visit by the researcher for completeness and consistency. We extracted data on sociodemographic characteristics, such as age, ethnicity, educational level, family history of CHD, and periconception exposures. Ethnicity and educational level were classified according to the definitions of Statistics Netherlands.[20] The use of medication and vitamin supplements by the child at the study moment was recorded, because of potential interference with the lipid levels.

We defined the periconceptional period as 4 weeks before conception until 8 weeks after conception. The use of a folic acid supplement in the periconceptional period was defined as the daily use of at least 400μg folic acid, either in a multivitamin preparation or as a single tablet during the complete period. Mothers who used folic acid only during a part of the periconception period were classified as non-users. We defined medication use as any use of medication during the periconception period. A positive family history of CHD was defined as the child having a 1st, 2nd or 3rd degree relative with CHD.

Standardized anthropometric measurements of the mother were performed, including height and weight (anthropometric rod and weighing scale; SECA, Hamburg, Germany). Body mass index (BMI) was defined as weight divided by the square of the height.

At the hospital visit, venous blood samples were drawn from the children. Immediately after blood sampling, serum separator tubes were kept at room temperature and centrifuged at 1,500-x g for ten minutes at 4°C, and separated within one hour. All samples were stored at -80°C and measured anonymously in batches within five months after collection. Serum concentrations of total cholesterol, HDL-cholesterol and triglycerides were analysed using routine methods on a Roche Modular P (Roche, Almere, The Netherlands). All assays were analysed on a Cobas Mira auto analyser. LDL-cholesterol values were calculated using the Friedewald formula.

Statistical Methods

Child characteristics were compared between cases and controls using a chi-square test for categorical variables. Normal distributions of continuous variables were tested with the Kolmogorov-Smirnov test. Non-parametric tests for comparison were used when variables were not normally distributed. All continuous variables are presented as medians with interquartile range, because some of them were skewed even after transformation. The normal distributions of the levels of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides are presented as means and compared with the unpaired Student's t-test. The risk for CHD was expressed by odds ratios (OR) and 95% confidence intervals (95% CI) in a logistic-regression model, with the quintiles of the levels in the control population as cut-off points. Levels lower than the 20th percentile of the controls were considered as reference category. In a multivariable logistic regression model, we adjusted the association between lipid levels and case/control status for the potential confounders birth weight (adjusted for gestational age), family history of CHD, the child's use of vitamins and maternal periconceptional alcohol use.
Probability values of $p < 0.05$ were considered statistically significant; all tests were two-sided. All analyses were performed with SPSS for Windows software (version 15.0; SPSS Inc., Chicago, IL, USA).

Results

Characteristics of the case and control children and their mothers are summarized in Table 1. The case children had more often a CHD family history and used slightly less vitamin supplements compared to control children. There were no other significant differences between the groups.

In 23 cases other anomalies next to CHD were present. Of these 15 were part of a syndrome, including Down syndrome (n = 7), 22q11 deletion syndrome (n = 3), 22q13 duplicate (n = 1), insertion chromosome 1>3 (n = 1), Turner (n = 1), CHARGE syndrome (n = 1), and Alagille syndrome (n = 1). Subgroup comparisons with the controls revealed that the 118 children in the isolated CHD group showed significantly more often a positive CHD family

history (p = 0.030), used significantly less vitamins (p = 0.021), and mothers used more often alcoholic drinks (p = 0.044) in the periconceptional period. The non-isolated CHD group had a lower birth weight adjusted for gestational age (p = 0.026) and used more medication (p < 0.001) at the study moment.

Mean serum lipid levels are presented in Table 2 and stratified for isolated and non-isolated CHD. The mean concentrations of triglycerides were 38% higher in case children as

Table 1 General characteristics of children with CHD, controls and mothers

Characteristic	CHD n = 141	Control n = 178	p-value
Child			
At birth:			
Male gender (%)	88 (62.4)	99 (55.6)	0.241
Birth weight in gram, median (IQ)	3304 (54)	3492 (45)	0.064[a]
Ethnicity			
Dutch native (%)	109 (22.3)	134 (75.3)	0.765[b]
European others (%)	10 (7.1)	11 (6.2)	
Non-European (%)	22 (15.6)	33 (18.5)	
At the study moment			
Outflow tract defectc	96 (86)	-	N.A.
Non-outflow tract defectd	45 (14)	-	N.A.
Family history of CHD (%)	18 (12.8)	10 (5.6)	0.024[b]
Age, months	17.0 (4.9)	17.3 (3.5)	0.490[e]
Vitamin use (%)	83 (58.9)	132 (74.2)	0.009[b]
Vit A, D, E and/or C (%)	81 (57.4)	131 (73.5)	
Multivitamin (%)	2 (1.4)	1 (0.6)	
Medication use (%)	29 (20.6)	25 (14.5)	0.123[b]
Mother			
At the study moment			
Age in years, median (IQ)	32.8 (0.4)	32.4 (0.4)	0.431
Body Mass Index, median (IQ))	24.4 (6.9)	24.1 (4.8)	0.307[e]
Nulliparity (excluding this child)	44 (31.2)	67 (37.6)	0.736
In the periconceptional period:			
Medication (%)	35 (24.8)	36 (20.2)	0.112[b]
Smoking (%)	27 (19.1)	43 (24.2)	0.471[b]
Alcohol (%)	58 (41.1)	57 (32.0)	0.878[b]
Folic acid (%)	73 (51.8)	93 (52.2)	0.240[b]

Independent t-test unless otherwise stated, N.A. not applicable
[a] adjusted for gestational age
[b] χ^2 test
[c] outflow tract defects; pVSD, TOF, PS, AVSD, AS
[d] non-outflow tract defects; TGA, CoA, HLHS
[e] Mann-Whitney *U* test

Table 2 Mean serum lipid levels in the children

	CHD n = 141		Isolated CHD n = 118		Non-isolated CHD n = 23		Control n = 178
	Mean (SE)	p-value[a]	Mean (SE)	p-value[b]	Mean (SE)	p-value[c]	Mean (SE)
Triglycerides (mmol/L)	1.44 (0.06)	<0.001	1.46 (0.06)	<0.001	1.37 (0.02)	0.103	1.18 (0.05)
Cholesterol (mmol/L)	3.93 (0.06)	0.373	3.87 (0.06)	0.929	4.25 (0.20)	0.494	3.90 (0.06)
HDL-C (mmol/L)	1.03 (0.03)	0.864	1.04 (0.03)	0.783	1.02 (0.07)	0.482	1.02 (0.02)
LDL-C (mmol/L)	2.25 (0.05)	0.551	2.18 (0.05)	0.720	2.62 (0.17)	0.479	2.34 (0.05)

p-values calculated in a logistic regression model with CHD as outcome variable
[a] Compared with control, adjusted for CHD family history and vitamin use
[b] Compared with control, adjusted for CHD family history, child's vitamin use and maternal periconceptional alcohol use
[c] Compared with control, adjusted for birth weight (gestational age), child's medication use

compared to the control children (1.44 vs. 1.04mmol/L; p < 0.001), and highest in isolated CHD (1.46mmol/L; p < 0.001). When stratified for isolated and non-isolated CHD, the triglyceride level was only significantly higher in the isolated subgroup. Total-cholesterol, HDL and LDL-cholesterol were comparable between groups.

In a linear regression model, triglyceride levels were significantly associated with case or control status (Table 3). After adjustment for birth weight, gestational age, CHD family history, vitamin use and periconceptional maternal alcohol use this association remained significant. The highest risk of CHD was observed in children with a triglyceride level above p80 (>1.62mmol/L: OR 5.5, 95% CI [2.3-13.2]; p < 0.001)).

Table 3 Associations between lipid levels and CHD in a univariate and multivariable logistic-regression model

		CHD / control status			
		Bèta	**Exp(B)**	**95% CI**	**p-value**
Triglycerides (mmol/L)	Crude	0.952	2.6	[1.6-4.2]	<0.001
	Adjusted[a]	0.989	2.7	[1.6- 4.4]	<0.001
Cholesterol (mmol/L)	Crude	0.062	1.1	[0.8- 1.4]	0.688
	Adjusted[a]	0.106	1.1	[0.8- 1.5]	0.514
HDL-C (mmol/L)	Crude	0.097	1.1	[0.5-2.3]	0.800
	Adjusted[a]	-0.001	1.0	[0.5-2.2]	0.980
LDL-C (mmol/L)	Crude	-0.221	0.8	[0.6-1.1]	0.211
	Adjusted[a]	-0.155	0.9	[0.6-1.2]	0.209

[a] Adjusted for birth weight (gestational age), mother's periconceptional alcohol use, family history of CHD and the child's use of vitamins.

Discussion

We showed that very young children at the mean age of 17 months with a CHD have almost 40% higher levels of triglycerides than control children. The increased mean triglyceride level of 1.44 mmol/L is above the upper reference value for children at this age (0.3-1.3 mmol/L) of our Clinical Chemistry laboratory. When stratified for isolated and non-isolated CHD, the triglyceride levels were only significantly higher in children with an isolated CHD. We cannot exclude, however, that this is due to the small non-isolated CHD subgroup. There were no significant differences in total cholesterol, HDL and LDL-cholesterol levels between case and control children.

It has been suggested before that the lipid pathway is disturbed in CHD. A case report of an adult with Tetralogy of Fallot and several metabolic abnormalities reported chronic

systemic hypoxia as a cause of the associated hypertriglyceridemia.[21] Lundell et al suggested that an altered lipid metabolism is probably not the cause but a result of CHD, and stated that children with cyanotic CHD might have a decreased fatty acid oxidation capacity and this could be the underlying mechanism for the dyslipidemia.[22] This mechanism, however, would likely lead to a lower triglyceride level and therefore cannot explain our finding. Moreover, the period of hypoxemia is relatively short, because most children with a cyanotic heart defect undergo surgery within the first days or months of their life.

Little is known about the predictive value of slightly increased triglyceride levels in early life on the risk of cardiovascular disease.[23, 24] Therefore, the finding of the almost 40% increased triglyceride level in these children with CHD at 17 months of age could be important for their cardiovascular health in later life.[9] This finding is substantiated by a recent study of Morrison et al showing that higher levels of triglycerides at young age were independently associated with cardiovascular disease risk in later life (HR 5.4; 95%CI [1.7-20]).[25]

There are still many inconclusive reports on the association between birth weight and lipid concentrations. However, children with CHD in general have a lower birth weight than healthy children, and low birth weight is associated with higher cholesterol and as such with a higher risk for cardiovascular disease.[26-28] Therefore, we adjusted the associations between the lipid levels and CHD for birth weight and gestational age from which revealed that birth weight did not confound our finding. Catch up growth in the first year of life might also affect the lipid levels; unfortunately, we do not have postnatal growth data.[29]

Gluckman et al stated that maternal environmental modulation of gene expression and programming of metabolic pathways in offspring may be more important than heritable risk alone.[16] In line with these findings we hypothesize that a derangement in the intrauterine programming of the lipid pathway in the child by the maternal environment is involved in the pathogenesis of CHD. While much research focuses on the effects of prenatal under nutrition and the risk of cardiovascular disease in adulthood,[30, 31] evidence is rising that excessive energy supply via the mother during pregnancy to the foetus also could have adverse effects. The relationship between nutrition and cardiovascular risk may be U-shaped, instead of linear. Children of obese women are at a greater risk for developing metabolic disorders themselves, even during childhood.[32] Earlier studies showed that higher maternal intake of fat or higher apolipoprotein-A levels are not only risk factors for cardiovascular diseases but are also associated with CHD in the offspring.[7,33] The Developmental Origin of Health and Disease is further illustrated by the associations between maternal hyperhomocysteinaemia and the elevated risk of CHD and cardiovascular disease in later life, substantiated by studies in mice and chicken embryo´s.[18, 34]

In general, increased triglyceride levels can be due to a combination of genetic and/or lifestyle factors.[35,36] Therapeutic measures preferably target causes with dietary interventions to improve the lipid profile. More research is necessary before lipid measurements are advised to be part of routine management in children with CHD. However, important to notice is that

the American Heart Association advices an aggressive risk evaluation for cardiovascular risk factors in especially children with CHD, more specifically when they have obstructive lesions of the left ventricle and aorta (CoA and AS), since these anatomical changes seem to make them already more vulnerable for atherosclerosis.[37] Moreover, some children with (repaired) CHD have limitations in their ability to exercise, which is also an independent risk factor for cardiovascular diseases.

Prospective studies with CHD as outcome are not feasible. Therefore, the best alternative to minimize potential bias is to conduct a case-control study with a carefully chosen fixed study moment in cases and controls, i.e., 24 months after the periconception period of the index child.[38] At that time, the risk of misclassification of cases and controls is minimal, because the majority of congenital malformations is diagnosed in the first year of life. A limitation is that we did not collect data on the postnatal diet of the children, which may affect the lipid levels. Due to ethical constraints, we were not able to measure the fasting lipid levels. Although this may seem a limitation, data suggest there is no difference in the strengths of associations between fasting and nonfasting values.[23] We do not have data on metabolic functions; therefore, it cannot be excluded that factors such as liver function may have distorted the association between CHD and elevated levels of triglycerides. Furthermore, due to limited sample sizes, we were not able to study the CHD phenotypes separately.

This study demonstrated that children with CHD have higher triglyceride levels, which may in future also increase their risk of cardiovascular disease. This finding may justify earlier screening for abnormal lipid profiles in children with CHD. However, first we suggest a prospective study of lipid levels in a prospective cohort of children with CHD. More knowledge can also be gained in the more than 1 million adults living with CHD, a growing population due to the increased survival rates.[39] Further research is also needed to elucidate whether the increased triglyceride level is a result of deranged in utero metabolic programming by excessive exposure to the increased maternal trait or is a consequence of the CHD.

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Chapter 5

Congenital heart defects and biomarkers of methylation in children: a case-control study

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Abstract

Derangements in the maternal methylation pathway, expressed by global hypomethylation and hyperhomocysteinaemia, are associated with the risk of having a child with a congenital heart defect (CHD). It is not known whether periconception exposure to these metabolic derangements contributes to chromosome segregation and metabolic programming of this pathway in the foetus. In a Dutch population-based case–control study of 143 children with CHD and 186 healthy children, we investigated S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), total homocysteine (tHcy), the vitamins folate and B12 and the functional single nucleotide polymorphisms in the folate gene MTHFR 677C>T and 1298A>C. Comparisons were made between cases and controls adjusting for age, medication, vitamin use and CHD family history.

In the overall CHD group, the median concentrations of SAM ($p = 0.011$), folate in serum ($p = 0.021$) and RBC ($p = 0.030$) were significantly higher than in the controls. Subgroup analysis showed that this was mainly attributable to complex CHD with higher SAM ($p < 0.001$), SAH ($p = 0.012$) and serum folate ($p = 0.010$) independent of carrier ship of MTHFR polymorphisms. Highest concentrations of SAM, SAH and folate RBC were observed in complex syndromic CHD. The subgroup of children with Down syndrome, however, showed significantly higher SAH ($p = 0.037$) and significantly lower SAM:SAH ratio ($p = 0.034$) compared with other complex CHD, suggesting a state of global hypomethylation.

High concentrations of methylation biomarkers in very young children are associated with complex CHD. Down syndrome and CHD may be associated with a global hypomethylation status, which has to be confirmed in tissues and global DNA methylation in future studies.

Introduction

More than 1 million children are born with a congenital heart defect (CHD) each year.[1] Although the aetiology remains largely unknown, several studies have shown the beneficial effects of the maternal use of synthetic folic acid and the harmful effects of a diet low in vitamin B12 in the periconception period on the risk of CHD in the offspring.[2-5]

Folate and vitamin B12 are both involved in the folate-dependent homocysteine pathway, in which they serve as substrate and cofactor in the remethylation of homocysteine into the essential amino acid methionine. A low availability of those vitamins caused by malnutrition, metabolic derangements and polymorphisms in the methylenetetrahydrofolate reductase gene (*MTHFR*) 677C>T and 1298 A>C cause a mild- to-moderate hyperhomocysteinaemia, which can be treated successfully by the administration of synthetic folic acid and / or vitamin B12.[6]

Evidence is accumulating that the association between periconception hyperhomocysteinaemia and the 3–4 fold increased risk of CHD may be due to upstream alterations in the methylation biomarkers in the mother, i.e. S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and the SAM / SAH ratio.[7] A state of global hypomethylation, reflected by a high total homocysteine (tHcy), a high SAH and low SAM / SAH ratio, is significantly associated with an increased risk of having a child with CHD, and more specific with CHD and Down syndrome.[8, 9] Moreover, global hypomethylation in blood and DNA has been associated with vascular diseases, which is consistent with the vascular hypothesis of CHD.[10, 11] So far, however, there are no reports on the global methylation status in children with CHD.

The biomarker SAM is the most important methyl donor for the methylation of DNA, RNA, proteins, phospholipids, hormones and neurotransmitters. Of this, DNA-methylation is the best-characterized epigenetic mechanism to explain interactions between nutrients and genes implicated in intrauterine programming of growth and development.[12] Alterations in the global methylation status in blood and other tissues during pregnancy and post-weaning can derange DNA methylation and thereby modify embryonic, foetal and metabolic development.[13, 14] From this background, we hypothesize that children with CHD have an altered global methylation status due to periconception exposure to maternal hyperhomocysteinaemia and global hypomethylation. The aim of this study was to investigate associations between biomarkers of the global methylation status in blood of very young children and CHD.

Materials and Methods

Study design

This study was embedded in the HAVEN study, a case–control family study of the child, mother and father, conducted in the western part of the Netherlands to investigate gene–environment

interactions in the pathogenesis and prevention of CHD, as described in detail elsewhere.[15] In summary, participants were non-related case and control children born between October 2003 and March 2005 the parents of whom were familiar with the Dutch language in writing and reading. For the present study, we focused on 143 case children with CHD and 186 healthy control children. Children with CHD were recruited from four university medical centres. The CHD phenotypes were selected on the basis of a mutual aetiology with regard to the involvement of gene-environment interactions and are listed in Table 1.[16] We divided the CHD cases into isolated CHD (n = 119) and complex CHD (n = 24). The complex CHD (n = 24) consisted of non-syndromic CHD (n = 8) and syndromic CHD (n = 16), i.e. Down syndrome with CHD (n = 8) and other syndromic CHD (n = 8), i.e. 22q11 deletion syndrome (n = 3), 22q13 duplicate (n = 1), insertion 1 > 3 (n = 1), Turner (n = 1), CHARGE syndrome (n = 1), and Alagille syndrome (n = 1). CHD was diagnosed, after birth, by two paediatric cardiologists with echocardiography and/or cardiac catheterization and/or surgery. Control children were randomly recruited from the child health care centres of 'Thuiszorg Nieuwe Waterweg Noord'. Child health care centres are part of the Dutch Health Care system where physicians specialized in child health care regularly check all newborns at standardized moments on health, growth and development. The study was approved by the Central Committee on Research involving Human Subjects, and the Institutional Review Boards of all participating hospitals. A written informed consent was obtained from the parents of all children.

At the standardized hospital visit around 17 months of age, we collected data of the children and their mothers, comprising demographics, gender, family history of CHD defined as the child having a relative to the 3rd degree with CHD, periconception exposures and, if applicable, CHD phenotype of the child. For reasons of potential confounding, the use of medication and vitamin supplements of the child at the study moment was recorded.

Clinical chemistry

At the hospital visit, venous blood samples were drawn from the children to measure the concentrations of SAM, SAH, homocysteine (tHcy), serum and red blood cell (RBC) folate, serum vitamin B12 and the *MTHFR* 677C>T and 1298A>C polymorphisms. EDTA-blood was kept on ice and centrifuged within 2 h after withdrawal at 4 °C, plasma aliquots were stored at -80 °C until analysis. To determine SAM and SAH, we used liquid chromatography tandem mass spectrometry (LC-MS / MS; Waters acquity UPLC premier XE, Milford, MA, USA).[9] The plasma total homocysteine (tHcy) was determined using HPLC with fluorescence detection.[17] The folate and vitamin B12 concentrations were measured using electrochemiluminescence immunoassay (Elecsys 2010; Roche GmbH, Mannheim, Germany).[15]

To determine the *MTHFR* 677C>T and *MTHFR* 1298A>C polymorphism, 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); these data are previously described in a subgroup of CHD and controls.[18]

Table 1 CHD phenotypes in the case children, stratified in subgroups*

	CHD; n = 143	Isolated CHD; n = 119	Complex CHD; n = 24	
			Non-syndromic; n = 8	Syndromic; n = 16*
Tetralogy of Fallot; n (%)	15 (10.5)	12 (10.1)	1 (12.5)	2 (12-5)
Transposition of the great arteries; n (%)	28 (19.6)	26 (21.8)	2 (25.0)	-
Atrioventricular septal defect; n (%)	8 (5.6)	2 (1.7)	1 (12.5)	5 (31.3)
Perimembraneous ventricular septal defect; n (%)	43 (30.1)	35 (29.4)	1 (12.5)	7 (43.8)
Coarctation of the aorta; n (%)	15 (10.5)	14 (11.8)	-	1 (6.3)
Aortic valve stenosis; n (%)	5 (3.5)	5 (4.2)	-	-
Pulmonary valve stenosis; n (%)	27 (18.9)	24 (20.2)	2 (25.0)	1 (6.3)
Hypoplastic left heart syndrome; n (%)	2 (1.4)	1 (0.8)	1 (12.5)	-

*For a more detailed description of the syndromic CHD subgroup, see the section Study Design

Statistical analyses

Age of the child, the biomarkers of methylation and vitamins in blood are presented as medians and ranges, because of skewness even after transformation. Age was compared by Mann–Whitney U test. Demographics and exposures presented in frequencies were tested using the χ^2 test. We tested deviation of the *MTHFR* genotype frequencies from those expected under Hardy–Weinberg equilibrium for cases and controls separately using the χ^2 test. Allele and genotype frequencies were compared between cases and controls. The associations between the biomarker concentrations were correlated using Spearman's correlation coefficient. Multivariable logistic regression analysis was performed to test for differences in biomarker concentrations between groups with adjustment for confounders. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS software version 15.01 (SPSS Inc., Chicago, IL, USA).

Results

General characteristics

Children with CHD showed more often a positive family history of CHD (p = 0.020) and used significantly less vitamin preparations than healthy controls (p = 0.001), i.e. vitamin A, C, D and E (Table 2). Only two cases and one control used a multivitamin containing folic acid. The general characteristics stratified for the CHD subgroups showed that complex CHD compared with controls were almost 1 month older at the study moment (18.1 [15.2–27.0] vs. 17.3 [13.0–24.9], p = 0.034), and used more medication (58.3% vs. 14.5%, p < 0.001). In isolated CHD, a positive family history of CHD was more often observed than in controls (12.7% vs. 5.4%, p = 0.024). After adjusting for multiple testing with the Bonferroni method, only medication use remained significant in complex CHD (not shown).

Biomarkers of global methylation

All comparisons are adjusted for the child's age, positive family history of CHD, and medication and vitamin use at the study moment (Table 3). These were considered possible confounders based on significant differences between cases and controls. The overall CHD group showed significantly higher concentrations of SAM (p = 0.011), serum folate (p = 0.021) and RBC folate (p = 0.030) than the control group. The CHD subgroup analyses revealed that only complex CHD showed significantly higher SAM (p < 0.001), SAH (p = 0.012), serum folate (p = 0.010) and a borderline significant higher RBC folate (p = 0.059) compared with controls. The concentrations of SAM (p < 0.001), SAH (p = 0.009) and serum folate (p = 0.047) in complex CHD were also significantly higher compared with isolated CHD.

We observed a significant correlation in the overall CHD group between SAM and SAH (r = 0.283, p = 0.001), SAM and RBC folate (r = 0.227,p = 0.007), SAH and serum folate (r = 0.307, p < 0.001), and serum and RBC folate (r = 0.432, p < 0.001). In the controls, we

Table 2 Characteristics of 143 children with CHD and 186 healthy control children at the study moment

	CHD	Controls	p†
Age (months)*	17.0 (11.4-27.0)	17.3 (13.0-24.9)	0.534
Sex; males: n (%)	87 (60.8)	104 (55.9)	0.248
CHD family history‡; n (%)	18 (12.6)	10 (5.4)	0.020
Use of medication; n (%)	29 (20.3)	27 (14.5)	0.168
Use of vitamins; n (%)	83 (58.0)†	139 (74.7)	0.001
Ethnicity§;			0.825
Dutch native: n (%)	110 (76.9)	141 (75.8)	
European others: n (%)	10 (7.0)	11 (5.9)	
Non European: n (%)	23 (16.1)	34 (18.3)	

*Data for age is median (min-max)
†P for CHD vs. controls by Mann-Whitney-U test for age, $\chi 2$ test for categorical data.
‡Any congenital heart disease of family members in the first, second or third degree.
§Dutch Natives were defined as individuals of whom both parents and grandparents were born in the Netherlands, or one of the parents was born in another country, but both grandparents were born in the Netherlands. European others were defined as those of whom one of the parents or grandparents was born in a European country, Indonesia, or was from European origin and living in the USA or Australia. Non-Europeans were defined as all others.

Table 3 Biomarkers of methylation and vitamins in blood of children with CHD, after stratification in CHD subgroups, and controls

	CHD; n = 143	Isolated CHD; n = 119	Complex CHD; n = 24	Controls; n = 186
SAM (nmol/L)	107.5 (53.5-224.8)*	106.1 (53.5-221.8)	130.0 (99.8-224.8)†	104.4 (50.8-164.0)
SAH (nmol/L)	16.8 (7.6-51.8)	16.3 (7.6-51.8)	20.9 (10.6-39.6)*	15.9 (8.9-76.4)
SAM:SAH	6.5 (1.0-13.5)	6.6 (1.0-13.5)	6.4 (3.2-99)	6.5 (0.7-13.0)
tHcy (µmo/L)	6.2 (3.9-12.3)	6.2 (4.0-12.3)	6.3 (3.9-87)	6.2 (3.7-12.1)
Folate, serum (nmol /L)	32.0 (11.3-113.7)*	30.6 (11.3-113.7)	37.5 (15.1-100.3)*	28.5 (8.4-99.6)
Folate, RBC (nmol/L)	1032 (397-2353)*	1031 (397-2353)	1097 (561-2351)‡	916 (342-2460)
Vitamin B12 (pmol/L)	511 (149-1147)	508 (149-1147)	521 (201-1104)	481 (135-1232)

Data are median (range)
*$p < 0.05$ vs. controls, †$p < 0.001$ vs. controls ‡$p < 0.1$ vs. controls; Multivariable logistic regression analysis with outcome (CHD/control) as dependant variable was used to denote statistical significance for differences in biomarker concentrations between groups adjusted for age, CHD family history, medication and vitamin use at the study moment.

observed a significant correlation between tHcy and SAM / SAH ratio ($r = -0.153$, $p = 0.038$), tHcy and serum folate ($r = -0.252$, $p = 0.001$), tHcy and RBC folate ($r = -0.171$, $p = 0.023$), and between serum and RBC folate ($r = 0.605$, $p < 0.001$).

In complex non-syndromic CHD, SAM was significantly higher ($p = 0.026$) than in the controls. In complex syndromic CHD significantly higher SAM ($p = 0.002$), SAH ($p = 0.006$) and serum folate ($p = 0.010$) concentrations were established. These concentrations were the highest in comparison with all other CHD subgroups (Table 4).

Table 4 Biomarkers of methylation and vitamins in blood of children with complex CHD and controls

	Complex CHD		
	Nonsyndromic; n = 8	Syndromic; n = 16	Controls; n = 186
SAM (nmol/L)	111.9 (100.3-156.5)*	1321 (99.8-224.8)†	104.4 (50.8-164.0)
SAH (nmol/L)	17.0 (10.6-24.1)	22.1 (12.8-39.6)†	15.9 (8.9-76.4)
SAM:SAH	6.8 (6.0-9.9)	6.2 (3.2-9.0)	6.5 (0.7-13.0)
tHcy (µmo/L)	5.5 (3.9-8.7)	6.3 (4.8-8.3)	6.2 (3.7-12.1)
Folate, serum (nmol /L)	32.4 (15.1-79.8)	43.7 (23.4-100.3)*	28.5 (8.4-99.6)
Folate, RBC (nmol/L)	1084 (561-2213)	1097 (752-2351)	916 (342-2460)
Vitamin B12 (pmol/L)	581 (201-1104)	420 (278-911)	481 (135-1232)

Data are median (range)
*$p < 0.05$ vs. controls, † $p < 0.01$ vs. controls; Multivariable logistic regression analysis with outcome (CHD/control) as dependant variable was used to denote statistical significance for differences in biomarker concentrations between groups adjusted for age, CHD family history, medication and vitamin use at the study moment.

The group of complex other syndromic CHD showed a significantly higher SAM ($p = 0.033$) and serum folate concentration ($p = 0.014$) compared with the controls. The highest concentrations of SAM ($p = 0.004$) and SAH ($p = 0.002$), RBC folate ($p = 0.041$) and lowest SAM:SAH ratio, tHcy and vitamin B12 compared with the controls were demonstrated in children with Down syndrome (Table 4). Compared with the children with other complex CHD, children with Down syndrome revealed a significantly higher SAH ($p = 0.037$) and lower SAM:SAH ratio ($p = 0.034$). After adjusting for multiple testing, SAM and SAH in complex CHD, complex syndromic CHD and CHD with Down syndrome remained significant. Further subgroup comparisons between cases and controls were repeated after removal of outliers, which we defined as biomarker concentration above or below the subgroup or control mean ± 3SD, after which significance of results remained similar.

MTHFR polymorphisms

MTHFR polymorphisms were successfully measured in 139 CHD and 183 control children. Genotype distributions in the overall CHD and control groups were in Hardy–Weinberg equilibrium ($p > 0.05$) and the genotype frequencies were not significantly different between CHD and controls. In the CHD group, the frequencies for the *MTHFR* 677 CC / CT / TT genotypes were 46% / 47.5% / 6.5% and in controls, 50.3% / 41.5% / 8.2% respectively. In CHD, the frequencies for the *MTHFR* 1298 AA / AC / CC genotypes were 49.6% / 41% / 9.4% and 41% / 49.2% / 9.8% in controls. In the CHD-group, nine children carried the *MTHFR* 677TT genotype of which eight had an isolated CHD. The nine *MTHFR* 677TT carriers showed a significantly lower vitamin B12 concentration compared with *MTHFR* 677CC carriers (419 pmol/L [192–490] vs. 512 pmol/L [149–1147], $p = 0.022$). In the CHD group, 13 children carried the *MTHFR* 1298CC genotype with significantly lower tHcy (5.7 µmol/L [4.1–7.3] vs. 6.3

μmol/L [4.0–11.7], p = 0.031) and significant higher vitamin B12 (686 pmol/L [380–1147] vs. 497 pmol/L [149–971], p = 0.039) than *MTHFR* 1298 AA carriers.

Discussion

The data presented here show that young children with complex CHD, in particular syndromic CHD, have higher concentrations of biomarkers of methylation reflected by increased SAM, SAH and folate in blood. Despite the highest concentrations of these biomarkers in children with Down syndrome, they showed the lowest SAM:SAH ratio of all groups. Although only significant when compared with the group of other complex CHD, this is an interesting finding because it is similar to the global hypomethylation status observed in the mothers of these children with Down syndrome, as previously reported.[9]

It appeared that the correlations between the biomarkers of methylation in CHD children and control children were different, which may further suggest a change in regulation or programming of the methylation pathway. The Developmental Origins Hypothesis of Health and Disease states that exposure to malnutrition and metabolic derangements during pregnancy lead to phenotypic and metabolic derangements in later life, partly due to changes in metabolic imprinting.[19] Thus, assuming that the embryo was exposed to the maternal global hypomethylation status, the global hypomethylation state in the children might be explained by heritability and intrauterine adaptations with consequences for cardiogenesis.[14, 20]

Of interest is the significantly higher folate concentration in the complex CHD and subgroup of syndromic CHD, in particular, Down syndrome. After adjustment for a positive family history of CHD, age, medication and vitamin supplement use, the folate concentrations remained significantly higher and confirm our previous finding in a smaller group of children with the same CHD phenotypes[15]. For reasons of the significant correlations between SAM and SAH and folate status, it is likely that the high folate concentrations contribute to the increased biomarker concentrations. This is substantiated by others.[21]

Comparisons of the biomarker concentrations of methylation and vitamins in children with adults reveal that except for tHcy, the concentrations are overall much higher in children, especially in CHD. Age effects on concentrations of folate, vitamin B12 and tHcy have been previously reported[22-24]; associated changes in nutrition and lifestyle, and maturation of the metabolic organs i.e. the liver and kidney, may explain the different levels.

The *MTHFR* 677C>T and 1298A>C polymorphisms are known to reduce significantly the activity of the *MTHFR* enzyme resulting in a slightly lower folate status and higher tHcy and SAH concentrations. Our finding of the lower tHcy in 13 case children with *MTHFR* 1298CC is opposite to the expected high tHcy, but could be a result of the higher vitamin B12 concentration also present in this group. The higher folate status in children with CHD, however, cannot be explained by differences in *MTHFR* 677CC and / or 1298AA genotype

distributions, as the frequency of these genotypes was lower compared with controls.

Although associations have been reported between the maternal *MTHFR* genotypes and the risk of Down syndrome, the aberrant genotypes were not more prevalent in the children with Down syndrome in this study.[25] In eight of nine carriers of the *MTHFR* 677TT, the genotype with the strongest effect on folate and tHcy in blood, the child had an isolated CHD. This contributes to the lower folate status in isolated versus complex CHD. However, differential carriership of the aberrant *MTHFR* polymorphisms cannot explain the global hypermethylation status in complex CHD.

Differences in vitamin and medication use at the study moment cannot explain the biomarker differences observed between cases and controls, all analyses are adjusted for these possible confounding factors as numbers are too small to study every drug and vitamin group separately. However, we would have expected lower folate and higher tHcy concentrations in CHD due to lower nutritional intake and more medication use. Minot et al. reported that in healthy breastfed children with a median age of 12 weeks, tHcy concentrations were higher and vitamin B12 concentrations were lower compared with formula-fed children.[26] The investigation of breast and formula feeding, however, did not show a higher frequency of breastfeeding or folic acid-fortified formula or tube feeding in the case group.

Physical activity affects the methylation pathway by increasing the turnover of methionine into SAM, SAH and homocysteine in healthy persons.[27] As the physical activity is likely to be lower in case children than in controls, it cannot be excluded that this feature contributes to the methylation status in children with complex CHD, in particular Down syndrome, but needs further investigation.

In complex non-syndromic CHD, median tHcy was lowest, although not significant, compared with controls and the other CHD subgroups. This is likely due to the high vitamin B12 and high folate concentrations, which both reduce tHcy. Elaborating on the role of vitamin B12 in methylation, in our previous study, we observed a nearly 2-fold increased risk between low maternal vitamin B12 intake and vitamin B12 blood concentrations and CHD risk.[3, 15] In contrast, in the case children, we observed an increased, even though not significant, vitamin B12 concentration. This may substantiate our hypothesis that maternal global hypomethylation may influence the metabolic imprinting of the levels of folate and vitamin B12 as part of the methylation pathway in the child.

Although we cannot state that the biomarkers of global methylation and vitamins are an accurate reflection of tissue-specific DNA methylation, several studies indicate that the concentrations of SAM and SAH determined in blood very well reflect the tissue concentrations, as well as global DNA methylation.[28-30] Thus, if the SAM and SAH ratio is considered the golden standard, the hypomethylation in Down syndrome compared with controls and other CHD subgroups is the only difference, although not significantly. This is a very interesting finding considering that high maternal age is a risk factor for Down syndrome offspring; ageing is correlated with a loss of methylation and maternal global hypomethylation was shown to be a risk factor for Down syndrome.[9, 31, 32]

We studied a population-based large group of children with a related CHD phenotype and controls; all data were collected in a standardized manner thereby maximizing the validity of the data. All biomarkers in cases and controls were precisely and randomly measured in the same manner. Despite the limitation of the relatively small subgroups of CHD phenotypes, the difference in biomarkers remained significant. In future, larger groups of the separate CHD phenotypes should be studied. We selected CHD phenotypes on the basis of evidence from experimental and epidemiological studies showing associations with maternal hyperhomocysteinaemia and related gene-environment interactions thereby improving homogeneity of the aetiology and CHD phenotypes.[33-35] The standardized study moment is important because it reduces misclassification of CHD and controls, as CHD is mostly diagnosed within the first year of life. A prospective preconception study would be the first choice. However, this is not feasible because of the large population needed to include enough CHDs due to its low prevalence rate and the accompanied high costs.

In conclusion, we show that increased concentrations of the biomarkers of methylation in very young children are associated with complex CHD, in particular, complex syndromic CHD. Moreover, the previously observed global hypomethylation state in mothers of a child with Down syndrome and now also the global hypomethylation state in their child with Down syndrome and CHD suggest heritability of the global methylation state and a possible role in the segregation of chromosomes.

Further research is needed to elucidate whether the increased biomarker concentrations of methylation and folate are due to derangements in intrauterine metabolic programming of the methylation pathway.

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Part III

The epigenetic programming of the healthy child

Het begin is het belangrijkste deel van het werk.
PLATO, Grieks filosoof 427 v.C. - 347 v.C.

Chapter 6

Periconceptional maternal folic acid use of 400 μg per day is related to increased methylation of the *IGF2* Gene in the very young child

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Abstract

Countries worldwide recommend women planning pregnancy to use daily 400 μg of synthetic folic acid in the periconceptional period to prevent birth defects in children. The underlying mechanisms of this preventive effect are not clear, however, epigenetic modulation of growth processes by folic acid is hypothesized. Here, we investigated whether periconceptional maternal folic acid use and markers of global DNA methylation potential (S-adenosylmethionine and S-adenosylhomocysteine blood levels) in mothers and children affect methylation of the insulin-like growth factor 2 gene differentially methylation region (*IGF2* DMR) in the child. Moreover, we tested whether the methylation of the *IGF2* DMR was independently associated with birth weight.

In 120 children aged 17 months (SD 0.3) methylation was measured of 5 CpG dinucleotides covering the DMR using a mass spectrometry-based method. Periconception folic acid was used by 86 mothers. Children of mother who used folic acid had a 4.5% higher methylation of the *IGF2* DMR than children who were not exposed to folic acid (49.5% vs. 47.4%; $p = 0.014$). *IGF2* DMR methylation of the children also was associated with the S-adenosylmethionine blood level of the mother but not of the child (+1.7% methylation per SD S-adenosylmethionine; $p = 0.037$). Finally, we observed an inverse independent association between *IGF2* DMR methylation and birth weight (-1.7% methylation per SD birth weight; $p = 0.034$). In conclusion, periconceptional folic acid use is associated with epigenetic changes in *IGF2* in the child that may affect intrauterine programming of growth and development with consequences for health and disease throughout life.

Introduction

Every year around 8 million children are born with a serious birth defect worldwide. Folate deficiency during conception up to the third month of gestation, i.e., periconceptional period, is an etiological factor in several birth defects. Randomized controlled trials have shown that periconceptional synthetic folic acid use prevents neural tube defects.[1] For that reason periconceptional folic acid in a dose of 400 μg per day has been promoted to all women planning pregnancy.[2]

Over the last decade, several campaigns were started to improve the awareness of the importance of periconceptional use of synthetic folic acid in tablets. Furthermore, folic acid fortification of food has been introduced in the US, Canada and Chile.[3] Since the implementation of these measures, a significant decrease in birth rates of neural tube defects, orofacial clefts, congenital heart defects and diaphragmatic hernia has been reported.[4-7] However, periconceptional folic acid use has also been reported to have adverse effects including an elevated risk of pyloric stenosis, obstructive urinary tract defects, obesity, insulin resistance and colon cancer.[7-9]

The mechanisms underlying the beneficial and adverse effects of periconceptional folic acid use are largely unclear. Following current thinking about the developmental origins of health and disease, an altered epigenetic regulation of growth processes induced by periconceptional folic acid may contribute to both the immediate effects and chronic disease associations in later life.[10, 11] Epigenetic regulation determines the potential of a genomic region to become transcribed.[12] The best understood epigenetic mechanism is the methylation of cytosine-guanine (CpG) dinucleotides in the DNA of mammals.[11, 12] Methyl donors, including folic acid, are required to establish and maintain DNA methylation. Methyl groups for DNA methylation reactions are supplied by demethylating the activated form of methionine into S-adenosylmethionine (SAM), to form S-adenosylhomocysteine (SAH) and homocysteine. In agreement with their crucial role in methylation reactions, in human SAM and SAH plasma levels and the SAM/SAH ratio are frequently used markers of global DNA methylation potential.[13, 14]

Direct evidence that the availability of methyl donors during gestation is required to establish and maintain DNA methylation patterns comes from experiments in the yellow Avy agouti mice. Supplementing the diet of pregnant dams with methyl donors, including folic acid, results in silencing of the agouti gene due to DNA methylation resulting in offspring with a mainly brown coat colour and a lower tendency for obesity, cancer and diabetes.[15] We recently observed that similar mechanisms may play a role in humans. Periconceptional exposure to famine during the Dutch Famine at the end of WWII was associated with a persistently lower methylation of the maternally imprinted insulin-like growth factor 2 (*IGF2*) gene.[16] *IGF2* is an embryonic growth factor that is expressed in most tissues and regulated in rats by periconceptional nutrient intake.[17] Complete loss of methylation at the *IGF2* differentially methylated region (DMR) results in biallelic expression of *IGF2* and is associated

with an increased risk of colorectal adenoma.[18] *IGF2* imprinting defects also underlie Beckwith-Wiedemann syndrome which is characterized by overgrowth.[19] Here, we hypothesize that periconceptional folic acid use by the mother may have consequences for *IGF2* DMR methylation of the child with a subsequent effect on intrauterine growth as reflected in birth weight.

Materials and Methods

Ethics statements

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

Study design and population

In a cross-sectional study mothers and children between 12 and 18 months of age were enrolled via public health centers in Rotterdam, the Netherlands between October 2003 and January 2007. The Dutch health care system includes a standardized and regular check up of all newborns for health, growth and development by physicians trained in child health care. Children were eligible as controls if they did not have a major congenital malformation or chromosomal defect according to the medical records from the regular check up at the child health center. These mothers and their healthy nonmalformed child served as controls in the previously described HAVEN-study.[20, 21] Mothers and children were studied at the standardized study moment of around 17 months after delivery of the index child, at which both blood samples for DNA methylation and folate in serum and red blood cells of the child and questionnaire data via the mother on periconceptional exposures, such as folic acid use, were obtained. For 186 mother-child pairs biomarkers of global methylation and blood samples for DNA methylation and folate in serum and red blood cells were available. Because we aimed to show an effect of periconceptional folic acid use, in particular an extreme effect, the 48 mothers who used partially folic acid during this period were excluded. Fourty mothers had completely refrained from periconception folic acid use and 98 reported the use of folic acid according to the Dutch recommendation of a daily intake of a folic acid containing preparation of 400 mg from at least 4 weeks before until 8 weeks after conception. Six mother-child pairs of whom the mother had not used folic acid were excluded because insufficient genomic DNA was available of the child for bisulfite treatment resulting in 34 unexposed mother-children pairs eligible for the current epigenetic study. For technical reasons 86 mother-child pairs were randomly selected from the exposed group, so that the total number of mothers and children studies was 120.

The questionnaires providing information on general traits, folic acid use and birth weight filled out by the mother at home were checked for completeness and consistency at the hospital visit during the standardized study moment by the researcher. We extracted data on maternal age and folic acid use, and age, gender, birth weight and gestational age at delivery of the child. The standardization of the blood sampling, plasma handling, extraction of genomic DNA and measurement of SAM, SAH and folate were described previously and are reported as control data in the comparison of cases with a congenital heart defect.[20, 21]

DNA Methylation

DNA methylation measurements were performed on genomic DNA extracted from whole blood samples obtained from the children. Bisulphite treatment was carried out on 0.5 mg genomic DNA using the EZ 96-DNA methylation kit (Zymo Research). The 120 samples were blinded as to exposure status and split into two equal groups with a similar distribution in exposure status thus preventing possible batch effects. Subsequently, to assess *IGF2* DMR methylation 5 CpG dinucleotides of the *IGF2* DMR (chr11:2,126,035-2,126,372 in NCBI build 36.1) was measured in triplicate using a mass spectrometry-based method (Epityper, Sequenom).[22] The quantitative accuracy and concordance with clonal polymerase chain reaction bisulphite sequencing is well- established. Two CpG dinucleotides confounded by SNPs were discarded so that the current study reports on CpG dinucleotides located at positions 41 (CpG #1), 57 and 60 (#2&3; adjacent CpGs that could not be resolved individually, 202 (#4) and 251 (#5) bp in the amplicon targeting the *IGF2* DMR.

Statistical analyses

Differences in quantitative traits in mothers and in children according to maternal periconception folic acid use were tested using an independent t-test. Differences in gender distribution were tested using a χ^2 test. *IGF2* DMR methylation was assessed by measuring multiple CpG sites that are correlated.[22] Raw methylation data can still be used (instead of for example averaging over the various CpG dinucleotides that differ in DNA methylation level) by applying linear mixed models. Linear mixed models may be viewed as an extension of a t-test that accounts for the correlation between methylation of CpG dinucleotides and methylation data missing at random.[16, 22] When applied to the analysis of single CpG dinucleotides without adjustment for covariates, a linear mixed model and a t-test yield identical results. For the analysis of the complete *IGF2* DMR, methylation data all CpG dinucleotides were entered in the model with the study subject identifier as a random effect to account for the correlation between CpG dinucleotides. The CpG dinucleotide identifier, the exposure status and other traits (e.g., SAM concentration or birth weight) or covariates (e.g., bisulfite plate) were entered as fixed effects. When testing single CpG dinucleotides, the study subject and CpG nucleotide identifiers were removed from the linear mixed model. Before testing independent associations of quantitative traits with *IGF2* DMR methylation, Z-scores were calculated so

that the resulting estimated effect size indicates the methylation change per standard deviation (SD) change in the parameter tested. Z-score does not affect a variable otherwise than standardising the mean to 0 and the SD to 1 and assists in interpreting the results.

The p-value of the significant association of periconceptional folic acid use and *IGF2* DMR methylation was additionally adjusted for maternal education. The p-value for the significant association between maternal SAM and *IGF2* DMR methylation was also adjusted for maternal education and the SAM concentration of the child. The p-value for the significant association between *IGF2* DMR methylation and birth weight was additionally adjusted for periconceptional folic acid use and gestational age at delivery. All p-vales were two-sided and all statistical analyses were performed using SPSS 16.0.

Results

The quantitative traits, including birth weight and the biochemical markers of global DNA methylation and folate, were similar according to periconceptional folic acid use for both mothers and children (table 1). The relative methylation of the *IGF2* DMR was 4.5% higher in folic acid exposed children as compared with non-exposed children (absolute methylation 0.495 (SE 0.004) vs. 0.474 (0.007); p = 0.014, table 2). In the linear mixed model analysis additionally adjustment for maternal education level revealed an adjusted p-value of 0.009, table 3. Higher levels of methylation were also observed for individual CpG dinucleotides comprising

Table 1 Quantitative traits according to maternal periconceptional folic acid use

	Mothers			Children		
	No Folic Acid (n = 34)	Yes Folic Acid (n = 86)	p value	No Folic Acid (n = 34)	Yes Folic Acid (n = 86)	p value
Male, n (%)	-	-	-	20 (59%)	50 (58%)	0.945
Age, years	32.6 (0.8)	32.2 (0.4)	0.624	-	-	-
Age, months	-	-	-	17.1 (0.5)	17.3 (0.2)	0.709
Birth weight, g	-	-	-	3363 (98)	3490 (64)	0.287
Gestational age	-	-	-	39.4 (0.3)	39.7 (0.2)	0.496
Biochemistry						
SAM, µmol/L	79.9 (2.5)	80.2 (1.3)	0.897	102.7 (3.3)	106.4 (2.1)	0.363
SAH, µmol/L	15.0 (0.6)	14.5 (0.3)	0.438	18.5 (1.0)	17.3 (0.5)	0.278
SAM/SAH	5.5 (0.2)	5.7 (0.1)	0.357	6.1 (0.4)	6.6 (0.2)	0.267
Folate, Serum nmol/L	15.3 (0.9)	17.8 (1.2)	0.438	31.5 (2.5)	32.1 (1.6)	0.748
Folate, Red blood cell	687 (70)	720 (30)	0.357	973 (72)	1064 (41)	0.245

Data are median (range)
$^{*}p < 0.05$ vs. controls, $^{\dagger}p < 0.01$ vs. controls; Multivariable logistic regression analysis with outcome (CHD/control) as dependant variable was used to denote statistical significance for differences in biomarker concentrations between groups adjusted for age, CHD family history, medication and vitamin use at the study moment.

Table 2 ***IGF2*** **DMR methylation in the child according to maternal periconceptional folic acid use**

	Children		
	No Folic Acid (n = 34)	**Yes Folic Acid (n = 86)**	**p value**
IGF2 methylation			
Complete DMR	0.474 (0.007)	0.495 (0.004)	0.014
CpG #1	0.473 (0.009)	0.484 (0.005)	0.292
CpG #2&3	0.334 (0.006)	0.348 (0.004)	0.059
CpG #4	0.590 (0.016)	0.632 (0.010)	0.023
CpG #5	0.511 (0.011)	0.516 (0.08)	0.602

the *IGF2* DMR, particularly for CpG #4, although not always significantly. Next, we tested the association of other variables in mother and child with *IGF2* DMR methylation in children (table 3). In addition to periconceptional maternal folic acid use, a higher maternal SAM concentration, but not that of the child, was associated with a higher *IGF2* methylation in the child (p = 0.037). This association remained significant after additional adjustment for maternal education and the SAM concentration of the child (p adjusted = 0.047). To test for a possible phenotypic consequence of changes in *IGF2* methylation, we analyzed the relationship with birth weight. A 1.7% higher *IGF2* methylation in the child was associated with one SD decrease in birth weight of 584 grams (p = 0.034), which was independent of periconceptional exposure to folic acid and gestational age at delivery (p adjusted = 0.041).

Table 3 ***IGF2*** **DMR methylation in the child and parameters of mother and child**

	Mothers		Children	
	Relative methylation change (SE)	**p value**	**Relative methylation change (SE)**	**p value**
Folic acid use	+4.5% (1.8)	0.014	-	-
Female sex	-	-	+2.0% (1.6)	0.232
Age	-0.4% (0.8)	0.585	-0.7% (1.0)	0.478
Birth weight	-	-	-1.7% (0.8)	0.034
Gestational age	-	-	-0.9% (0.8)	0.276
Biochemistry				
SAM, µmol/L	+1.7% (0.8)	0.037	+1.2% (0.8)	0.129
SAH, µmol/L	+0.8% (0.8)	0.331	+0.1% (0.8)	0.882
SAM/SAH	+0.0% (0.8)	0.985	+0.3% (0.8)	0.717

Linear Mixed Model analysis. Data are presented in percentage (standard error) of mean change in relative methylation. For independent quantitative parameters the change in relative methylation is given per SD-change in that parameter. The p-value of the significant association of periconceptional folic acid use and *IGF2* DMR methylation was additionally adjusted for maternal education. The p-value for the significant association between maternal SAM and *IGF2* DMR methylation was also adjusted for maternal education and the SAM concentration of the child. The p-value for the significant association between *IGF2* DMR methylation and birth weight was additionally adjusted for periconceptional folic acid use and gestational age at delivery.

Discussion

The key finding of our study is that periconceptional folic acid use of the mother is related to an increased methylation of the *IGF2* DMR of the child. The reported stability of *IGF2* DMR methylation up to middle age supports the interpretation that the *IGF2* methylation changes we observed are explained by periconceptional folic acid exposure. [18, 22] The difference in DNA methylation associated with folic acid exposure is remarkably similar to our previous observation of a 5.2% reduced *IGF2* methylation after periconceptional exposure to famine.[16] The opposite direction of the associations suggests that the availability of methyl donors during the periconceptional period may affect *IGF2* DMR methylation. We further hypothesized that changes in *IGF2* DMR methylation would influence intrauterine growth. Our study indeed indicated an association between *IGF2* DMR methylation and birth weight as surrogate for intrauterine growth, but not between periconceptional folic acid use and birth weight.

Compared to our findings in humans, the size of the effects on DNA methylation of prenatal exposures have found to be comparable in sheep but are larger in rodents.[23-25] In this comparison we have to emphasize that human populations are inevitably more heterogeneous than the inbred rodents kept at the same, well-controlled environmental conditions. The different effects in animal studies can also be due to the common use of a combined intervention consisting of multiple methyl donors and/ or protein deficiency instead of folic acid only. Lastly, the larger effects in rodents are shown in other tissues than in blood, which are not readily accessible from human study subjects.

In addition, our study indicated an association between higher *IGF2* methylation and lower birth weight. This inverse association is compatible with a relative intrauterine silencing of the embryonic growth factor *IGF2* resulting in reduced growth.[26, 27] This links our data to the finding that *IGF2* loss of imprinting leads to somatic overgrowth (Beckwith-Wiedemann syndrome) and possibly colorectal cancer although we cannot exclude the explanation that the change in *IGF2* methylation marks possible greater changes elsewhere in the genome that underlie the association observed.[18, 19] Periconceptional folic acid use may contribute to the restoration of a loss of imprinting. It remains to be established whether DNA methylation changes contribute to the adverse effects reported for periconceptional folic acid use. [7-9, 28] Studies are required to establish optimal timing, dose and type (natural folate or synthetic folic acid) to prevent birth defects and at the same time minimize adverse effects later in life.

From this study reveals that periconceptional folic acid use is associated with epigenetic changes in *IGF2*. However, in contrast with large mother-child cohorts, we did not find a positive association between periconceptional folic acid use and higher birth weight.[29, 30] It is known that women using periconceptional folic acid supplements are generally more health conscious and higher educated. Furthermore, the women exposed to the Dutch famine were not only deprived of folate, but also of other essential macro- and micronutrients that serve as methyldonors, e.g., methionine. Thus, it should be emphasized that many factors together

including other genes besides periconceptional folic acid contribute to birth weight. This may explain the absent association between periconceptional folic acid use and birth weight in our study.

Periconceptional use of folic acid did not affect average levels of the biomarkers SAM, SAH or SAM/SAH measured at 17 months in the mother and in the child. However, we found a significant correlation between the maternal SAM concentration at the study moment and *IGF2* methylation of the child. The developmental hypothesis of health and disease states that periconceptional exposures may affect metabolic imprinting of the child. This is in line with our finding that periconceptional folic acid use can affect the metabolic imprinting of the methylation pathway of the child. Of note, this association was not influenced by fortification of food with folic acid which is absent in The Netherlands, UK and other European countries, which may have strengthened the observed associations.

Although we did not measure levels of biomarkers of methylation in the periconception period, their levels will have been comparable to those we measured 17 months after delivery. This is substantiated by Nurk et al. showing that the biomarkers of methylation show a limited variability in the periconception period and over a subsequent period of 1–2 years.[31] Furthermore, there are no substantial differences in preconceptional maternal dietary habits and lifestyles and those 1 to 1.5 year post partum which affect these biomarkers.[32]

A limitation of both our study and others is that they relied on genomic DNA extracted from whole blood so that heterogeneity in cell populations may have contributed to the outcomes.[16] Our study likely is less sensitive to such heterogeneity because the epigenetic state of imprinted loci is less dependent on cell differentiation and, importantly, a previous study showed that when demethylation of *IGF2* DMR was observed in peripheral blood lymphocytes of an individual, this was also found in colon tissue, which has a distinct embryologic origin (endoderm and mesoderm, respectively).[18] The finding that the common epigenetic variation in *IGF2* DMR might influence birth weight is intriguing but should be interpreted with care because the sample size was relatively small and other (non)genetic factors are also involved. It is currently unknown whether modestly increased *IGF2* DMR methylation upregulates *IGF2* expression. Furthermore, it has not been established whether such quantitative differences measured in lymphocytes mark a soma-wide phenomenon as was suggested whether such quantitative differences measured in lymphocytes mark a soma-wide phenomenon as was suggested for loss-of-imprinting of the *IGF2* DMR.[18] Therefore, in future human studies the sampling of different tissues should be performed.

Our study provides the first evidence that periconceptional folic acid use may be related to DNA methylation in the child. Moreover, such DNA methylation changes may have phenotypic consequences as illustrated by the association between higher *IGF2* methylation and decreased birth weight. A simple preventive strategy as periconceptional folic acid use may affect epigenetic control and as such may link the prevention of intrauterine development, i.e., birth defects such as neural tube defects, and growth due to a loss of imprinting

with the risk of chronic diseases in these children throughout life. It has to be established how folic acid intake affects the epigenetic regulation of sets of relevant genes and whether adverse effects are to be expected from an altered methylation at such loci. Given the ongoing exposure it is timely to monitor the (long term) effect on DNA methylation also of other lifestyles and environmental influences, such as overnutrition, fortification of food, smoking, stress and the use of assisted reproductive techniques.

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Chapter 7

Periconception maternal smoking and low education are associated with methylation of *INSIGF* in children at the age of 17 months

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Abstract

Maternal smoking and a low socioeconomic status are associated with adverse birth outcomes, such as fetal growth restriction and low birth weight. Of major importance in both prenatal and postnatal growth is the insulin growth factor signaling system (*IGF*). It comprises products of the maternally imprinted insulin growth factor 2 receptor (*IGF2R*) which generally inhibits growth, and the paternally imprinted insulin growth factor 2 (*IGF2*),which enhances growth. SNPs in *IGF2R* and *IGF2* DMR are associated with birth weight, and *INSIGF*, i.e., the overlapping region of *IGF2* and insulin (*INS*), has been associated with small for gestational age (SGA). From this background the objective of this study was to investigate associations between maternal smoking and other harmful exposures related to a low socioeconomic status (SES) and DNA methylation of *IGF2* DMR, *IGF2R* and *INSIGF*.

In 120 children at 17 months of age, methylation of DNA derived from white blood cells was measured. Periconception smoking and low education were independently associated with *INSIGF* methylation and showed a relative increase in methylation of +1.3% ($p = 0.043$) and +1.6% ($p = 0.021$). Smoking and low education showed an additive effect on *INSIGF* methylation (+2.8%; $p = 0.011$). There were no associations with *IGF2* DMR and *IGF2R* methylation. Our data suggest that periconception maternal smoking and low education are associated with epigenetic marks on *INSIGF* in the very young child, this warrants further study in additional populations.

Introduction

The periconception, prenatal and early postnatal period are of significant importance in the development and growth of the embryo, foetus and child. Epidemiologic studies have shown numerous associations between adverse periconception exposures, such as smoking and foetal growth restriction.[1] Next to these evident short term consequences there have been reports on long term health consequences of prenatal exposure to smoking for the offspring including but not limited to respiratory illness, type 2 diabetes and cardiovascular disease.[2]

Epigenetics is suggested to be an important mechanism linking preconceptional, prenatal and postnatal exposures in association with health and disease risks in later life. It is described as a biological mechanism to explain gene-environment interactions, which allows (heritable) changes in gene expression to occur without changing the DNA sequence.[3, 4] DNA methylation is one of the best-understood epigenetic marks, in which the differentiation of cells and tissue is programmed. Furthermore, it is hypothesized that changes induced on this mark by past intrauterine exposures may be a mechanism to adapt a priori to future environment exposures.[5]

Of major importance in both prenatal and postnatal growth is the insulin pathway in which the insulin growth factor signalling system plays an important role. It comprises products of the maternally imprinted insulin growth factor 2 receptor (*IGF2R*), which in general inhibits growth, and the paternally imprinted insulin growth factor 2 (*IGF2*) which enhances growth.[6] Polymorphisms in *IGF2R* and *IGF2* DMR are associated with birth weight.[7] Moreover, *INSIGF*, the overlapping region of *IGF2* and insulin (*INS*), has been associated with small-size-for-gestational-age at birth (SGA).[8] Of interest is that the methylation of *IGF2* DMR and *INSIGF* have been shown to be related to exposure to famine in utero.[9] Although prenatal famine shares similarities with small-size-for gestational-age at birth regarding health problems in later life, this similarity was not observed on the level of DNA methylation of *IGF2* DMR and *INSIGF*.[10-12]

Evidence is increasing that maternal smoking during pregnancy is associated with differences in DNA methylation both globally and gene-specific, both increases and decreases of DNA methylation have been reported.[13-16] A recent genome wide methylation analysis has shown significantly differential methylation among the placental genome of smokers, and a significant reduction in the child's birth weight.[17] None of the before mentioned studies examine dose-response relationships or looked specifically at periconception smoking.

From this background, we studied associations between periconception maternal smoking and DNA methylation of *IGF2* DMR, *IGF2R* and *INSIGF* genes in children at the age of 17 months. Secondly, socio-economic status, (SES), as measured by its proxy education, may be seen as an additional and cumulative determinant of harmful exposures and may therefore add additional insight. In our study we used low education defined by a maximum of 12 years of on-going education from the age of 4, as a proxy for low socioeconomic status

(SES) and considered it as a cumulative determinant and the strongest marker of SES.[18] Recently an exploratory paper showed widespread differences in DNA methylation on a genome wide level associated with SES.[19] Therefore; we investigated and included additional analyses on the influence of SES on these loci and the relation between DNA methylation and smoking.

Material and Methods

Study sample

We examined 120 children (boys n = 70, girls n = 50) at a mean age of 17 months (SD 2.5) and their mothers. These subjects served as controls in the HAVEN-study, they were previously described in detail.[20] Mothers and their children were recruited from the public child health care centres of 'Thuiszorg Nieuwe Waterweg Noord' currently known as 'Careyn' in the Rotterdam (Netherlands) area. Public child health care centres are part of the Dutch Health Care system where physicians specialized in child health care regularly check all new-borns at standardized moments on health, growth and development. Children were eligible as controls if they did not have a major congenital malformation or chromosomal abnormality according to the medical records from the regular check up at the child health centre up to the study moment. The total number of responding mothers and their children to serve as controls in the HAVEN study between October 2003 and December 2009 and willing to be contacted was 794 of which 490 (61.7%) were included in the study. Reasons for exclusion were: mother pregnant or lactating; the mother not being the birth mother; the child having a congenital defect or chromosomal abnormality; not willing to have blood drawn.

At the standardized study moment of 17 months of age, we retrospectively collected periconception, prenatal and postnatal data of the children and their mothers by self-administered questionnaires sent before the hospital visit, which were checked by the researcher for completeness and consistency at the hospital. We extracted the following data: maternal education level, periconception risk factors, e.g., folic acid supplement use, smoking, the number of cigarettes smoked, child's birth weight and health status. The latter was divided in the following categories: renal disease; liver disease; disease of the gastro-intestinal tract; thyroid disease; cardiovascular disease; epilepsy; thrombosis; malignancy; disease of the urinary tract; malaria; dermatologic disease; allergy; haematological disease; disease of the respiratory tract; or other.

Gestational age was based on the first day of the last menstrual period or calculated from the first trimester foetal ultrasound scan. Because periconception folic acid use was previously associated with *IGF2* DMR methylation, we include folic acid use in the analysis as potential effect modifier.[20] We included intake of folic acid according to the Dutch recommendation of a daily intake of a folic acid containing preparation of 400 μg from at least 4 weeks before until 8 weeks after conception. We used educational level as a proxy for socioeconomic status (SES) and considered it as a cumulative determinant and the strongest marker of SES,

dichotomized in two categories: low education defined by a maximum of 12 years of on-going education from the age of four (primary/lower vocational/intermediate secondary education), and other (higher secondary/intermediate vocational education or higher vocational/university education).[18] We additionally derived information on weight and length from the child's record at the public child health care centres of Careyn. We calculated BMI by weight in kilograms divided by squared height in centimetres. Of every child with available growth data from birth onwards, we calculated the growth rate for which the length was plotted against the square of the root of the age. The slope of each individual regression line (β) was used as variable of the postnatal growth rate.

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 on behalf of their participating child.

DNA methylation measurements

The selection of 120 mother-child pairs is described in our previous study.[20] In the present study we explored DNA methylation of *IGF2* DMR, *IGF2R* and *INSIGF* measured in 120 children. Genomic DNA was isolated from whole blood using the salting out method. One half microgram of genomic DNA was bisulfite-treated using the EZ 96-DNA methylation kit (Zymo Research) on one of two 96-well plates. Bisulfite-converted DNA-specific PCR primers were used to amplify the investigated region. DNA methylation of the CpG dinucleotides was measured in triplicate using by a mass spectrometry-based method (Epityper, Sequenom). The quantitative nature, accuracy and reproducibility of this method has been shown extensively.[11, 21] The measurements of *IGF2* DMR, *IGF2R* and *INSIGF* are part of an on-going study of a total of 8 loci including *IL10*, *TNF*, *LEP*, *KCNQ1OT1* and *FTO*. Details of the measured amplicons, including details of functional relevance and PCR primers were published before.[9, 22] In short, the region analysed for the *IGF2* DMR includes five CpG dinucleotides (chr11:2126035-2126372, in NCBI build 36.1) separated in four CpG units, e.g. fragments of DNA because of two adjacent CpG's that could not be resolved individually. The region for *IGF2R* includes ten CpG dinucleotides (chr6:160346346-160346595) resulting in four CpG units measured, because of adjacent CpG sites that could not be resolved individually and CpG's containing fragments of too little or high mass for the mass spectrometer to resolve. The region for *INSIGF*, located in the promoter of the imprinted *INSIGF* transcript originating from the *INS* promoter, includes six CpG sites in total, of which four could be resolved and all individually (chr11:2138912-2139216).

Statistical analysis

We applied linear mixed models on the raw data without imputation of missing values to calculate exposure-specific differences and associations.[9] All the analyses accounted for bisulfite plate and

the correlation between the CpG dinucleotides. The CpG sites of the studied locus were simultaneously entered with variables under study as fixed effects, with overall methylation as outcome. The linear mixed model was chosen over a standard paired t-test, because it allows for the analysis of all individual CpG dinucleotides of the locus in one test, accounts for the correlation between adjacent CpG dinucleotides, includes relevant adjustments within the model on the raw data and uses all available data. Absolute changes in DNA methylation are presented as regression coefficient with SE. Relative changes in percentage of DNA methylation were calculated by dividing the mean methylation with risk factor by mean methylation without risk factor. The association between the CpG sites and variables under study were studied separately with t-tests. Before testing the independent association of birth weight with methylation, Z-scores were calculated so that the resulting estimated effect size indicates the methylation change per standard deviation (SD) change in birth weight. Z-score does not affect a variable otherwise than standardizing the mean to zero and the SD to one and assists in interpreting the results. All p-values reported are two-sided. IBM SPSS Statistics 19.0 software was used for all analyses.

Results

Characteristics of the mother and child

In this study, we included 120 mother and child pairs (Table 1). Thirty-two mothers (28.3%) smoked during the periconception period and 31 (25.8%) had a low education. Education level was not significantly different between periconception smokers and non-smokers (p = 0.814). Maternal smoking was independently and inversely associated with birth weight (-231 gram; p = 0.021). Of the periconception smokers 19 (59.3%) continued smoking during pregnancy and 21 (65.6%) were smokers at the study moment. In the children disease of the respiratory tract was present in 10 (8.3%) who all used inhalation medication for asthma, dermatologic disease was present in three (2.5%), allergies in eight (6.7%) and ear infection was present in one child (0.8%).

DNA methylation

The average DNA methylation percentages were 48.9, 55.3 and 89.4% for *IGF2* DMR, *IGF2R* and *INSIGF*, respectively. Methylation of the separate CpG sites is listed in Table 1.

DNA methylation, periconception exposures and child characteristics

We tested for an association in all measured individuals between DNA methylation, maternal low education, smoking, no use of a folic acid supplement, gender, birth weight, growth rate, BMI and having a disease of the respiratory tract such as asthma (Table 2). Periconception low education and smoking were independently associated with a higher *INSIGF* methylation of +1.6%

Table 1 Characteristics of mothers and children

Mother	**(n = 120)**	**Missing, n (%)**
Age at birth, years	31.2 (27.6-33.6)	-
Low education	31 (25.8)	-
Folic acid supplement use, periconception	34 (28.3)	-
Smoking, periconception	32 (26.7)	-
1-10 cigarettes	22 (18.3)	-
11-25 cigarettes	10 (8.3)	-
Child	**(*n*=120)**	
Birth weight, g	3,455 (3,115-3,818)	-
Age, months	17.0 (15.1-18.6)	-
Growth rate, beta	0.995 (0.989-0.998)	21 (17.5)
BMI, kg/m^2	16.6 (15.6-17.6)	21 (17.5)
Methylation at the study moment, mean % (sd)		
IGF2 DMR		
CpG #1	48.1 (4.4)	23 (19.2)
CpG #2&3	34.4 (3.5)	10 (8.3)
CpG #4	62.1 (9.0)	5 (4.2)
CpG #5	51.4 (5.2)	18 (15)
IGF2R		
CpG #4&5	55.3 (9.0)	13 (10.8)
CpG #8-10	63.4 (8.0)	10 (8.3)
CpG #11-13	45.7 (9.9)	11 (9.2)
CpG #20&21	85.7 (4.7)	2 (1.7)
INSIGF		
CpG #2	87.3 (4.3)	2 (1.7)
CpG #4	86.6 (5.5)	9 (7.5)
CpG #5	90.2 (2.4)	1 (0.8)
CpG #6	93.2 (2.0)	1 (0.8)

Data are presented in median (p25-p75) or number (percentage).

(p = 0.021) and +1.3% (p = 0.043), respectively. Both factors showed an additive effect of +2.8% (p = 0.011) on DNA methylation. All four CpG sites of *INSIGF* showed higher methylation, tested individually for educational level this was significant for CpG #2 (p = 0.034), #5 (p = 0.042), #6 (p = 0.003) and for smoking for CpG #5 (p = 0.012). The number of cigarettes smoked was associated with an increase (p = 0.028) in overall methylation percentage of *INSIGF*. For no smoking the mean methylation percentage (se) was 89.0 (0.3), for 1 to 10 cigarettes 89.9 (0.6) and for 10 to 25 cigarettes 90.8 (0.9).

Maternal smoking and low education were not associated with overall methylation of *IGF2* DMR and *IGF2R*, this was similar for the individual CpG sites of *IGF2* DMR and *IGF2R*, except for low education and *IGF2R* CpG #20&21 (p = 0.031) and borderline significant for *IGF2R* CpG #8-10 (p = 0.050).

Table 2 Relation between DNA methylation, periconception risk factors and child characteristic

	IGF2 DMR			*IGF2R*			*INSIGF*		
	β[1]	δ%[2]	p[3]	β[1]	δ%[2]	p[3]	β[1]	δ%[2]	p[3]
Low education	-0.3 (0.9)	-0.6 (1.8)	0.706	+2.4 (1.5)	+3.9 (2.4)	0.114	+1.4 (0.6)	+1.6 (0.7)	0.021[4]
Smoking	-0.8 (0.9)	-1.8 (2.0)	0.347	-1.8 (1.5)	-2.9 (2.4)	0.232	+1.2 (0.6)	+1.3 (0.7)	0.043[5]
No folic acid supplement use	-2.1 (0.8)	-4.3 (1.6)	0.014[6]	-1.8 (1.5)	-2.9 (2.4)	0.224	+0.3 (0.6)	+0.3 (0.6)	0.549
Boys vs. girls[7]	-1.0 (0.8)	-2.0 (1.6)	0.232	-1.2 (1.4)	-1.9 (2.2)	0.396	-0.3 (0.5)	-0.3 (0.5)	0.569
Birth weight[8]	-0.9 (0.5)	-1.7 (0.8)	0.034[9]	-0.7 (0.8)	-1.1 (1.3)	0.420	-0.1 (0.3)	-0.1 (0.3)	0.838
Growth rate, beta	+1.2 (1.7)	+2.2 (3.4)	0.514	+3.3 (2.6)	+5.2 (4.1)	0.212	+0.1 (.0.1)	+0.1 (0.1)	0.941
BMI, kg/m²	-0.3 (0.3)	-0,6 (0.3)	0.408	-0.2 (0.6)	-0.3 (0.9)	0.727	-0.1 (0.2)	-0.1 (0.2)	0.781
Disease of the respiratory tract: asthma	-0.7 (1.4)	+1.4 (2.8)	0.611	+0.2 (2.4)	+0.3 (3.8)	0.425	+1.0 (0.9)	+1.1 (1.0)	0.280

[1]The regression coefficient (beta (se)) from a linear mixed model adjusted for the correlation between individual CpG dinucleotides and bisulfite batch. The investigated variable was entered as a fixed effect with overall methylation as outcome.
[2]The percentage relative change (se) in methylation of the locus.
[3]A two-sided P-value resulting from the linear mixed model.
[4]Additionally adjusted for smoking p = 0.023.
[5]Additionally adjusted for low education p = 0.047.
[6]Adjusted for birth weight p = 0.007.
[7]Gender was entered as a fixed effect.
[8]One SD increase in birth weight (584 gram), adjusted for gestational age.
[9]Adjusted for periconception folic acid use.
[10]The slope (β) of each individual regression line of length plotted against the square of the root of the age was use.
[11]One SD increase in BMI (1.3 point).

We previously reported in the same children the association between periconception folic acid use and birth weight with *IGF2* DMR methylation, and repeated here this analysis for *IGF2R* and *INSIGF*.[20] There was no association with overall methylation. Only methylation of *INSIGF* CpG #5 was inversely associated with birth weight adjusted for GA ($p = 0.035$). For none of the loci there was an association with gender, or significant additive or interaction effect of gender and smoking on DNA methylation. Growth, BMI and asthmatic condition of the child revealed no association with DNA methylation of the three loci.

Discussion

In this study, we show associations of DNA methylation of the *INSIGF* gene in very young children with exposure to periconception maternal smoking and low education. Both exposures were associated with a higher methylation of *INSIGF*. Methylation of *INSIGF* CpG #5 was positively associated with periconception smoking and inversely with birth weight. Based on our findings we hypothesize that adverse effects of the periconception environment, i.e., factors related to low education, and smoking on birth weight could be related in part to silencing of the imprinted *INSIGF* gene in the child by slightly higher DNA methylation. Smoking is considered one of the major mediators in the association between low SES and adverse pregnancy outcome, and is also in our study highly associated with a decreased birth weight.[1] However, it remains important when studying outcomes related to low education and low SES, to analyse the relationship with smoking separately, since low education and smoking both had an independent association with increased methylation of *INSIGF*. This may suggest that there are other factors that affect the methylation of *INSIGF*, that are related to low education such as bad nutrition and stress. Our finding that low SES is significantly associated with higher methylation of *INSIGF* may be a signature of the early life environment, similar to a recent finding in adult males that revealed a distinct genomic methylation pattern related to their childhood SES.[19]

A recent study examined the association between maternal smoking and *IGF2* DMR methylation in cord blood after birth, and reported a significantly higher methylation in children born to mothers who smoked throughout pregnancy compared to quitters or never smokers, which was most pronounced and only significant in males.[23] A finding which we do not replicate. In the same study, there was no association between methylation and smoking in early pregnancy compared to non-smoking, which is in line with our results in *IGF2* DMR. In one of our own studies we did not find a significant association between maternal smoking throughout the pregnancy and methylation of several loci including *IGF2* DMR and *INSIGF* at the age of 19 years.[12] Important to note is that there are several differences between our studies, such as the younger age of the children in the present study. In our previous study, we studied children born before 32 weeks of gestation with and without SGA, sometimes accompanied with multiple pregnancy complications. Furthermore, in the present study we used periconception

smoking and not smoking throughout pregnancy. Timing may be essential for *INSIGF*, as we previously reported an association with a decrease in *INSIGF* methylation and exposure to famine periconceptionally and not exposure later in gestation.[9] Supportive of the validity of our results is the relationship observed between the number of cigarettes and the increase in methylation of *INSIGF*, which may suggest a dose-response relationship. Further research on this locus and on other loci, would be interesting, especially since transgenerational effects of smoking of the mother but also of the grandmother has been reported to modify the risk for asthma in children, in which epigenetics is suggested to be the link.[24] Our group may have been too small to show an association of methylation with asthma.

Strength of our study is the fixed study moment relatively short after pregnancy in order to minimize recall bias regarding periconception exposures. Our overall power to detect statistically significant associations was limited by a relatively small sample of study subjects. In addition, many of the mothers who smoked in the periconception period were still smoking at the study moment. Current exposure may influence DNA methylation and we do not know to what extent their child was postnatally exposed to tobacco smoke.[25]

We measured DNA methylation of *IGF2* DMR, *IGF2R* and *INSIGF* in whole blood; it would be very interesting to measure DNA methylation profiles in other tissues as well, such as subcutaneous fat or muscle tissue. While the children were at a young age at the study moment, we are not able to assess diabetes, obesity or cardiovascular diseases in these children. Replication of our findings in larger groups and measuring DNA methylation of *INSIGF* in different tissues should be subject of future studies. This will be feasible because of new techniques where genome-wide methylation profiling on CpG sites and promoter regions is possible by high throughput techniques. This will help us also to gain more insight into the intra- and interindividual distribution of methylation patterns throughout the genome in different tissues in different age categories.

In conclusion, the present study has shown that periconception maternal smoking and low education, as proxy for SES, are associated with methylation of the *INSIGF* gene in very young children. These effects may modify gene expression and future health and disease risks, but this has to be confirmed in larger birth cohorts.

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Chapter 8

Duration of breastfeeding and gender are associated with methylation of the *LEPTIN* gene in very young children

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Abstract

Perinatal environmental factors have been associated with metabolic programming of the child with consequences for disease risk in later life. Epigenetic modifications leading to altered gene expression may be involved. Here, we study early life environmental and constitutional factors in association with DNA methylation of *LEP*, a non-imprinted gene implicated in appetite regulation and fat metabolism.

We investigated maternal education, breastfeeding, and constitutional factors of the child at 17 months of age. We measured DNA methylation of *LEP* in whole blood and the serum leptin concentration. Duration of breastfeeding was negatively associated with *LEP* methylation. Low education was associated with higher *LEP* methylation. Boys had higher birth weight and lower *LEP* methylation than girls. An inverse association was established between birth weight per SD increase (+584g) and *LEP* methylation. High BMI and leptin concentration were associated with lower methylation of *LEP*.

In conclusion, the early life environment and constitutional factors of the child are associated with epigenetic variations in *LEP*. Future studies must reveal whether breastfeeding and the associated decrease in *LEP* methylation is an epigenetic mechanism contributing to the protective effect of breastfeeding against obesity.

Introduction

Perinatal environmental factors, such as the socioeconomic status (SES) of the parents, nutrition and lifestyle factors are important in the growth, development and health of the child up to adulthood.[1] During pregnancy and early postnatal life, an individual can be programmed for nutritional thrift to adapt and survive in an environment of limited resources and poor nutrition.[2] Especially the mismatch between prenatal and postnatal nutrition is emphasized in association with the epidemic of obesity.[3] Epigenetics may be one of the major links between the periconception and perinatal periods in association with health and disease in later life.[4] It is described as a biological mechanism to explain gene-environment interactions, in which (heritable) changes in gene expression occur without changing the DNA sequence.[5] DNA methylation is one of the best-understood epigenetic mechanisms and an important programming mechanism of the genome, in which cells and tissues can adapt to past and current environmental exposures.[6]

The gene *LEP* has been proposed as an important candidate gene for thrifty phenotypes since it displays epigenetic variation and is involved in the development of obesity and insulin resistance.[4, 7, 8] *LEP* is primarily expressed in differentiated adipocytes of white fat tissue, and its product the hormone leptin has several functions including the regulation of food intake and expression of energy regulating peptides. Defective production of leptin or its receptor are highly associated with obesity.[9] Higher leptin levels are associated with adiposity in young children and newborns.[10, 11] The relationship between methylation of the *LEP* promoter and leptin expression has been investigated in vitro during adipose conversion. In human preadipocytes, leptin is not expressed due to hypermethylation of the *LEP* promoter. When the preadipocytes mature, the gene is switched on through demethylation.[12] Differences in methylation of the *LEP* promoter influences *LEP* expression in vitro, which suggests a functional effect of methylation on leptin levels.[7]

Illustrative of epigenetic effects on *LEP* in humans are the studies performed in the population exposed to the Dutch famine. Prenatal exposure to the Dutch famine is associated with an increased methylation of *LEP*.[13] This association is gender-specific and not related to the timing of exposure in pregnancy. The early postnatal period is another critical window where programming of growth and metabolic functions takes place. The injection of leptin during suckling to rodent neonates that are exposed to prenatal under nutrition prevents them from becoming obese.[14] This might be due to decreased methylation of the pro-opiomelanocortin (POMC) gene and increased expression of the POMC-derived peptide α-MSH which reduces food intake.[15] Leptin is also associated with the maturation of the neuro-endocrine axis. Neural projection pathways, regulating food intake and energy consumption, are disrupted in leptin deficient mice. Postnatal treatment with leptin, however, restores these projections.[16] In children, neuro-endocrine appetite regulation is influenced by prenatal exposure to leptin derived from the mother, placenta and own synthesis, and postnatally by breastfeeding and own synthesis.[17, 18] Some epidemiological studies have shown that

breastfeeding is protective against childhood obesity, which seems to be established through the programming of leptin present in breast milk but not in formula.[15, 19] Reports on the influence of breastfeeding on leptin levels are inconclusive, both increases and decreases of leptin concentrations have been reported.[20]

From this background, we study the methylation of *LEP* in association with early environmental and constitutional factors in healthy very young children at the age of 17 months.

Materials and Methods

Study sample

One hundred twenty healthy children (boys n=70, girls n=50) at a mean age of 17 months (SD 2.5) and their mothers were investigated. These mother-child pairs have been previously described in detail.[21] In summary, the mothers and their child were recruited from the public child health care centers of 'Thuiszorg Nieuwe Waterweg Noord' currently known as 'Careyn' in the Rotterdam (Netherlands) area between October 2003 and January 2007. Public child health care centers are part of the Dutch Health Care system where physicians specialized in child health care regularly check all newborns at standardized moments on health, growth and development. The included children did not have a major congenital malformation or chromosomal abnormality according to the medical records from the regular check up at the child health center up to inclusion at the age of approximately 17 months.

We collected postnatal data of the children and their mothers by self-administered questionnaires sent before the hospital visit, which were checked by the researcher for completeness and consistency at the hospital visit at inclusion. We extracted and calculated the following data: maternal education, age, periconception folic acid supplement use and smoking, gestational age at birth, child's gender, birth weight, duration of breastfeeding and body mass index at 17 months of age. We used education level as proxy for socioeconomic status (SES) and considered it as a cumulative determinant and the strongest marker of SES.[22] We dichotomized this determinant in two categories: low (primary/lower vocational/intermediate secondary education) which corresponds to a maximum of twelve years of on-going education from the age of four, and other (higher secondary/intermediate vocational education or higher vocational/university education). Because periconception folic acid use was previously associated with IGF2 DMR methylation and smoking with INSIGF methylation, we investigated both in association with *LEP* methylation.[21, 23] We included intake of folic acid according to the Dutch recommendation of a daily intake of a folic acid containing preparation of 400 μg from at least 4 weeks before until 8 weeks after conception. Gestational age was based on the first day of the last menstrual period or calculated from the first trimester fetal ultrasound.

Data on breastfeeding were derived retrospectively from the mothers in a self-administered home questionnaire, to minimize recall-bias we validated this with the information on breastfeeding

from the child's record at the public child health care centers of Careyn. Next to using breastfeeding as a grouping variable we recoded the different groups into a covariate with scores ranging from 0 - 4; (none; <1, >1-3, >3-6, >6 months). From the child's record at the public child health care center we collected child's weight and length, where after BMI was calculated by dividing weight in kilograms by squared height in centimeters. Of every child with available growth data from birth onwards, we calculated the growth rate by plotting the length against the square root of the age. The slope of each individual regression line (β) was used as variable of the postnatal growth rate. At inclusion, venous blood samples were drawn from all children to measure the serum concentration of leptin. Leptin concentrations were measured in duplicate using a specific Human Leptin Radioimmunoassay kit (Millipore, St. Charles, MO). 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 on behalf of their participating child.

DNA methylation measurements

DNA methylation of *LEP* was measured in 120 children. Genomic DNA was isolated from whole blood using the salting out method.[24] One microgram of genomic DNA was bisulphite-treated using the EZ 96-DNA methylation kit (Zymo Research, Irvine, CA) on one of two 96-well plates. Bisulphite-converted DNA-specific PCR primers were used to amplify the investigated region. DNA methylation of CpG dinucleotides was measured by a mass spectrometry-based method (Epityper, Sequenom, San Diego, CA). The quantitative nature, accuracy and reproducibility of this method have been shown extensively.[25] Details of the measured amplicon, including details of functional relevance were published before.[26] In short, the amplicon covers the proximal promoter and includes several CpG sites of which the methylation status influences transcription.[7] Data quality control and filtering were done as previously described.[26] Data filtering consisted of the removal of CpG dinucleotides of which the measurement success rate was low. Common causes of a lower success rate include fragments bordering on the upper and lower limits of the mass range that can be detected and cases of fragments of which the base of the peak signal in the mass spectrum overlapped another fragment. Details about the primer, the CpG sites included and biological relevance are provided in Supplemental Table 1.

Statistical analysis

Anova t-tests and χ^2 tests were used for the analyses of the characteristics of mother and child between gender. Associations among the study variables were studied using χ^2 test, Pearson's correlation and linear regression. We applied linear mixed models on the raw data without imputation of missing values to estimate the overall mean methylation, exposure-specific differences and associations.[13] All the analyses accounted for bisulphite plate and the correlation

between the seven CpG dinucleotides; they were simultaneously entered with the variable under study as fixed effects with overall methylation as outcome.

First, we studied the associations of each of the variables separately with methylation in the linear mixed model. These variables comprised the early environmental factors: maternal low education, no use of folic acid, periconception smoking and duration of breastfeeding; the constitutional factors of the child: gender, birth weight adjusted for gestational age, growth rate and BMI; and it comprised the biomarker concentration of leptin. These analyses are referred to as Model 1. Secondly, we entered all variables with a P-value of <0.01 for the association with *LEP* methylation simultaneously in an extensive model referred to as Model 2. Linear mixed model analysis is preferred above the more standard paired t-test, because it allows analysis of all seven individual CpG dinucleotides together in one test, it accounts for the correlation between adjacent CpG dinucleotides, it includes relevant adjustments within the model on the raw data, and uses all available data. Absolute changes in DNA methylation are presented as regression coefficient with SE. Absolute changes may seem small, but could correspond to a large relative change resulting in functional consequences. Therefore, relative changes in percentage of DNA methylation were calculated by dividing the mean methylation with the risk factor by mean methylation without the risk factor. Because methylation as well as the functional relevance may vary between individual CpG sites, analyses were also performed for the individual CpG sites with standard t-tests and linear regression. These results are provided in Supplemental Table 2.

Before testing the association of birth weight, BMI, growth rate and leptin with *LEP* methylation, Z-scores have been calculated so that the resulting estimated effect size indicates the methylation change per standard deviation (SD) change in birth weight, BMI, growth rate or leptin. The Z-score does not affect a variable otherwise than standardizing the mean to zero and the SD to one and assists in interpreting the results. All P-values reported are two-sided. Since multiple statistical tests have been applied, we performed conservative Bonferroni to adjust for multiple comparisons. SPSS 16.0 software (SPSS for Windows, SPSS Inc) was used for all analyses.

Results

Characteristics of mother and child

In this study, we included 120 mother and child pairs (Table 1). Maternal smoking was inversely associated with birth weight after adjustment for gender (Beta= -231 gram; P = 0.021). Boys had a significantly higher birth weight, adjusted for gestational age, than girls. The leptin serum concentrations were slightly higher in girls, albeit not significantly. Of 99 children with available data on breastfeeding, 75 (75.8%) were breastfed of which 14 were breastfed less than 1 month. The majority of the children were breastfed for at least one month; 21 children

Table 1 Maternal and child characteristics

Mother	All children (n = 120)	Girls (n = 50)	Boys (n = 70)	p
Age, at birth, years	31.2 (27.6-33.6)	31.3 (27.1-33.7)	31.2 (27.6-33.6)	0.718
Low education	31 (25.8)	14 (28.0)	17 (24.2)	0.647
Folic acid, periconception	34 (28.3)	14 (28.0)	20 (28.5)	0.945
Smoking, periconception	32 (26.7)	15 (30.0)	17 (24.2)	0.485
Child				
Birth weight, g	3,455 (3,115-3,818)	3,348 (3,066-3,666)	3,545 (3,185-3,917)	0.044*
Age, months	17.0 (15.1-18.6)	17.0 (15.0-18.3)	17.2 (15.5-18.8)	0.409
Leptin, serum (mmol/L)	2.7 (2.4-3.0)	2.8 (2.4-3.3)	2.7 (2.4-3.0)	0.062
Breastfeeding, *n* (%)[a]	74 (74.7)	32 (84.2)	42 (68.9)	0.096
Growth rate, beta	0.995 (0.989-0.998)	0.993 (0.984-0.997)	0.996 (0.994-0.998)	0.567
BMI, kg/m^2	16.6 (15.6-17.6)	15.8 (15.3-17.2)	17.0 (16.2-17.8)	0.002

Data are presented in median (p25-p75) or number (percentage). Data are tested between gender with Anova *t*-test or χ^2 test.
*Adjusted for gestational age
[a] Defined as any breastfeeding after birth up to the study moment, data available for 99 children

Table 2 Methylation of the CpG sites of *LEP*

Locus	All children (n = 120)
#CpG 1	25.1 (4.9)
#CpG 8	15.9 (5.3)
#CpG 16.17	15.6 (3.6)
#CpG 19.20.21	13.2 (2.9)
#CpG 22	45.1 (9.2)
#CpG 25	43.8 (8.5)
#CpG 27	6.3 (2.4)
Overall mean (se)	23.6 (0.3)

Values are presented as mean (SD) per CpG site. The overall mean (se) is estimated from the Linear Mixed Model adjusted for the correlation between individual CpG dinucleotides and bisulphite batch.

for >1-3 months (22%), 21 children for >3-6 months (21%) and 18 children for more than 6 months (18%). There was no significant association between maternal education and the duration of breastfeeding ($\chi 2 = 0.89$, $p = 0.460$). Children who were breastfed for 1-3 months had a significantly higher serum concentration of leptin (2.8 vs. 2.6 mmol/L; $p = 0.025$) than formula fed children. BMI at 17 months of age was significantly correlated with the leptin concentration (Pearson's $r = 0.228$, $p = 0.040$). Low maternal education was associated with an increased postnatal growth rate of the child (Pearson's $r = 0.234$; $p = 0.029$).

Table 3 Associations between methylation of LEP and several variables

	Model 1			Model 2		
	% absolute methylation change (se)[a]	% relative methylation change (se)	p[b]	% absolute methylation change (se)[a]	% relative methylation change (se)	p[c]
Early environmental factors						
Low education	+2.1 (0.8)	+9.1 (3.5)	0.008	+1.0 (0.8)	+4.2 (3.4)	0.233
No folic acid, periconception	+0.1 (0.8)	+0.0 (0.8)	0.910			-
Smoking, periconception	+1.3 (0.8)	+5.6 (3.4)	0.094	+0.6 (0.8)	+2.5 (3.3)	0.454
Duration of breastfeeding[d]	-0.7 (0.3)	-2.9 (1.2)	0.011	-0.6 (0.3)	-2.5 (1.3)	0.040
Constitutional factors						
Gender, male	-1.8 (0.7)	-7.3 (4.1)	0.010	-2.3 (0.8)	-9.0 (3.9)	0.005
Birth weight[e]	-1.2 (0.4)	-5.0 (1.7)	0.005*	-0.6 (0.5)	-2.5 (2.1)	0.159*
Growth rate[e,f]	0.0 (0.4)	0.0 (0.3)	0.985			-
BMI[e]	-0.8 (0.4)	-3.3 (1.7)	0.043	-0.3 (0.4)	-1.2 (1.6)	0.514
Biomarker concentration						
Leptin, serum[e]	-0.4 (0.4)	-1.7 (1.7)	0.035	-1.2 (0.5)	-4.9 (2.0)	0.028

*Adjusted for gestational age
[a]Values are the regression coefficients (beta (se)) from a Linear Mixed Model (LMM) adjusted for the correlation between individual CpG dinucleotides and bisulphite batch. They represent the mean change (%) in absolute methylation for the variable. The investigated variable was entered as a fixed effect, if continuous entered as covariate.
[b]A two-sided P-value from the LMM where all listed variables were entered separately.
[c]A two-sided P-value from the LMM where all variables from Model 1 with a $p < 0.1$ were entered simultaneously.
[d]The duration of breastfeeding was entered as a covariate with scores ranging from 0 – 4 (SD 0.4); (none; <1, >1-3, >3-6, >6 months).
[e]Z-scores have been calculated so that the resulting estimated effect size indicates the methylation change per standard deviation (SD) change in birth weight (SD 584 gram), growth rate (SD 0.03), BMI (SD 1.3 point) and leptin (0.195 mmol/L).
[f]The mean slope (β in cm/(√age (in months)) of all individual regression lines of length plotted against the square root of the age was used.

DNA methylation of the LEP gene and early environmental factors

We measured seven CpG sites of *LEP*, calculated the methylation per CpG site and estimated the overall mean methylation (Table 2). In Model 1 low education was associated with a higher *LEP* methylation (Table 3). Periconception smoking was not associated with overall *LEP* methylation, but a strong association revealed for the methylation of one CpG site (CpG #22 p = 0.001). Duration of breastfeeding was negatively associated with *LEP* methylation (–2.9%; p = 0.011). In Model 2 the duration of breastfeeding slightly attenuated but remained significant.

DNA methylation of the LEP gene and constitutional factors

To test for possible phenotypic associations with DNA methylation, we analyzed the relationship with gender and birth weight (Table 3). DNA methylation of *LEP* was 7.3% lower in boys than in girls (Model 1). In the total group an inverse association was shown between birth weight per SD increase (+584g) and *LEP* methylation (-5.0%, p = 0.005). BMI at 17 months of age was associated with an overall lower *LEP* methylation (-3.3%/1.3 kg/m2; p = 0.043). Postnatal growth rate was not associated with *LEP* methylation. In Model 2 the association between gender and *LEP* methylation became stronger and the associations with birth weight and BMI at 17 months of age disappeared.

DNA methylation of the LEP gene and leptin serum concentrations

Leptin concentration was significantly associated with the overall methylation of *LEP* (-1.7%; p = 0.035) (Table 3) and became stronger in Model 2.

Bonferroni correction

After Bonferroni correction only the association between birth weight and *LEP* methylation remained significant (p = 0.035).

Discussion

We studied methylation of the *LEP* gene in the child at 17 months of age in association with early life environmental and constitutional factors, since this gene is highly associated with obesity and insulin resistance and an important candidate for the thrifty phenotype.[4, 7, 8] We observed significant associations between the methylation of *LEP* and the duration of breastfeeding, birth weight, BMI, gender, leptin concentration in the child and maternal education. The strongest associations revealed with gender, leptin concentration and the duration of breastfeeding.

To our knowledge, this is the first report on the association between the duration of breastfeeding and lower *LEP* methylation in the very young child. Lower methylation of *LEP* leads to increased expression and higher concentrations of leptin, which is illustrated by our

finding of an increased leptin concentration with lower methylation of *LEP*. This is supported by the functional effect of methylation on leptin levels.[7, 12] The association between the duration of breastfeeding and *LEP* methylation is intriguing. In contrast to formula milk, breast milk contains unique growth factors, signaling molecules, but also leptin. Because of its interaction with almost all neuropeptides that are involved in the regulation of energy balance and food intake, leptin is important in the programming of metabolic pathways.[17] Therefore, we hypothesize that the breast milk content contributes to programming of the neuro-endocrine system by modulating methylation patterns of the *LEP* promoter. The decrease in *LEP* methylation could be one of the mechanisms by which breastfeeding contributes to the protection against childhood obesity. Although it is well known that breastfeeding is less common in mothers with a low education or low SES, the association between breastfeeding and *LEP* methylation was not confounded by maternal education.[27]

Prenatal exposure to the Dutch famine either in the periconception period or late in gestation is associated with an increased methylation of *LEP* in the offspring.[13] Therefore, we want to stress that the association we found may not only be related to breastfeeding, but could also be mediated through the dietary pattern of the mother during pregnancy, the early life feeding practice, and the current dietary pattern of the child. A study in rats showed that animals that were fed a high-fat diet became obese and had a higher methylation of *LEP* than rats that were fed a normal-diet.[28] This led to altered metabolic programming of the offspring resulting in increased body length, decreased insulin sensitivity and reduced levels of leptin, even in the next generation.[28, 29] In mice, under nutrition by a low protein diet during pregnancy and lactation, decreased the methylation of the offspring's *LEP* promoter and lead to a stronger induction of leptin secretion in response to a meal.[30] Others, however, have reported significant changes in *LEP* expression either in response to a high fat diet or weight loss without changes in methylation of the *LEP* promoter. [31, 32] Although studying dietary patterns in association with methylation patterns may be difficult, it will be essential to unravel such associations as those described here.

Contrary to our expectation, an increase in birth weight and BMI in early childhood was associated with lower *LEP* methylation. We expected to find higher methylation of *LEP* because of the association between obesity and hypermethylation that was found in rats.[28] Nevertheless, lower methylation of *LEP* related to an increase in birth weight, could explain the strong correlation between an increase in birth weight and leptin levels.[20] Because of practical and ethical constraints, we were not able to study the relationship between body composition, different adipose regions and methylation of *LEP*. Recent studies also reported on associations between DNA methylation patterns and constitutional factors in children. Total fat mass and central body fat in children at the age of 9 years have been associated with increased DNA methylation at birth of the RXR gene promoter, a locus involved in insulin sensitivity, adipogenesis and fat metabolism.[33] Furthermore, DNA methylation patterns in cord blood have been associated with body composition at the age of 9 years.[34] Although none of these

studies looked into *LEP*, these studies do indicate an epigenetic link between DNA methylation at an early age and phenotype in later life. Therefore, our findings may be an early mark and might precede an altered body composition at later age in childhood.

The gender association with *LEP* methylation replicates an earlier finding.[13] Tobi et al reported a significantly lower *LEP* methylation in males than in females in a population with a mean age of 57.1 years (SD 5.5). We now show that the gender difference in *LEP* methylation is already present at a very young age. Gender differences in methylation have been suggested to evolve from sex-steroid expression and X-inactivation resulting in differential methylation of DNA methyltransferases.[35] Gender differences are also established in leptin concentration. In general, the concentration is higher in women than in man, this difference is already present at birth.[11] Also in our study population, the leptin concentration is higher in girls than in boys, albeit not significant, probably because of our relative small sample size. Many underlying explanations for the gender difference in leptin concentration have been presented, such as differences in total fat mass, the distribution of fat mass, involvement of sex steroid expression and genetic differences.[36] Our results now indicate that epigenetic differences might be involved. One may consider that the higher leptin concentration in girls is in contrast to the higher methylation of *LEP*. However, our analysis revealed that both in girls and in boys an increase in leptin concentration was associated with lower *LEP* methylation.

The association between low maternal education as proxy for SES and a higher methylation of *LEP* warrants further investigation. We hypothesize that the adverse effects of the periconception, prenatal and postnatal environment, that are also related to low education such as occupation, nutrition, lifestyle, and health, have left a signature on the methylation of *LEP*. This finding is supported by our recent study showing the same association with INSIGF, which is the overlapping region of IGF2 and insulin (INS), a locus associated with small-size-for-gestational-age at birth (SGA).[23] This is in line with a recent exploratory paper showing a distinct genomic methylation pattern associated with early life SES in adult males.[37] Although the DNA methylation in their study was also associated with adult SES, there was only little overlap in the genomic region, which suggests an epigenetic variation specifically linked to early SES. Future studies need to identify the strongest factors of low education in association with methylation, and to assess if these changes in methylation have consequences for health and disease. With that knowledge, modifiable risk factors can be identified from which interventions can arise.

In our study, low maternal education was associated with an increased postnatal growth rate of the child, this is in line with findings in the Generation R study.[38] One of the explanations for increased growth-rate could be the difference in feeding-practices between socioeconomic groups. However, adjustment for breastfeeding (data not shown) on growth rate did not explain our finding, maybe because of our small sample size. The increased growth rate could be a response to other adverse intrauterine and postnatal exposures that are related to low maternal education.

We have to address the limitations and strength of our study. A limitation is that we

measured DNA methylation in genomic DNA extracted from whole blood and not from other tissues, therefore we have to be careful in extrapolating our data to tissue-specific methylation. The CpG sites studied in the *LEP* promoter show similar methylation in peripheral blood and adipocytes in vivo. Furthermore, methylation in adipocytes influences *LEP* expression in vitro.[7, 12] However, measurements of *LEP* mRNA have revealed that especially in children *LEP* is differentially expressed depending on the adipose region.[39]

We measured DNA-methylation at the age of 17 months, it is not known to what extent this reflects methylation patterns set at birth or methylation changes across early postnatal life. Inherent to the cross sectional design of the study we are not able to determine the direction of the associations, i.e., causality or effect, between exposures, phenotypes and methylation. Furthermore, our sample size together with multiple testing limits the robustness of the inferences that we can make on the associations we found. Therefore, it would be highly informative to study the variation in *LEP* methylation patterns from birth onwards in a larger group, and to examine the relationship with the development of body composition. Although it is often impossible to study the inter-individual phenotypic variation that is manifest in inaccessible tissues such as the brain, visceral fat and other internal organs and tissues, combined efforts

Supplemental Table 1 CpG sites of *LEP*

LEP		**chr7:127668290-127668646**	**Promoter region**
LEP 01	CpG 1	Included	
LEP 02	CpG 2-7	High Mass and rs791620	
LEP 03	CpG 8	Included	
LEP 04	CpG 9&10	Mass overlap with unit 9	
LEP 05	CpG 11	Mass overlap with units 12 and 6	
LEP 06	CpG 12&13	Mass overlap with units 5 and 12	
LEP 07	CpG 14&15	Mass overlap with unit 14	
LEP 08	CpG 16&17	Included	
LEP 09	CpG 18	Mass overlap with unit 4	
LEP 10	CpG 19-21	Included	
LEP 11	CpG 22	Included	C/EBPbeta,c-Myb, MIF-1
LEP 12	CpG 23&24	Mass overlap with units 5 and 6	
LEP 13	CpG 25	Included	Sp1 binding site, important for transcription
LEP 14	CpG 26	Mass overlap with unit 7	
LEP 15	CpG 27	Included, Low mass	
LEP 16	CpG 28	Low success rate due to partial overlap	
LEP 17	CpG_29	Excluded for rs2167270	
LEP 18	CpG_30-32	High Mass	

CpG sites are numbered from the sequence identical to the forward primer sequence. The PCR primers were reported earlier.[13,26]

For completeness (without Epityper 10mer and T7 tags):
LEP Forward: 5'GTTTTTGGAGGGATATTAAGGATTT
Reverse: 5'CTACCAAAAAAAACCAACAAAAAAA

Supplemental Table 2 Associations between methylation of the CpG sites of LEP and studied variables

	CpG 1[a]	p	CpG 8[a]	p	CpG 16.17[a]	p	CpG 19.20.21[a]	p	CpG 22[a]	p	CpG 25[a]	p	CpG 27[a]	p
Early environmental factors														
Low education	2.6 (1.1)	0.018	1.7 (1.3)	0.096	2.2 (0.7)	0.002	1.2 (0.6)	0.034	1.2 (2.0)	0.541	3.8 (1.8)	0.027	0.3 (0.5)	0.618
No folic acid, periconception	0.3 (1.0)	0.766	-1.3 (1.3)	0.320	0.9 (0.7)	0.224	0.3 (0.6)	0.649	2.5 (2.0)	0.236	0.01 (1.8)	0.994	-0.3 (0.5)	0.547
Smoking, periconception	0.3 (1.1)	0.756	-1.4 (1.3)	0.299	0.2 (0.8)	0.820	0.6 (0.6)	0.309	7.0 (2.0)	0.001	2.2 (1.8)	0.220	0.3 (0.5)	0.531
Duration of breastfeeding	-0.5 (0.4)	0.153	-0.8 (0.4)	0.042	-0.3 (0.3)	0.176	-0.3 (0.2)	0.102	-1.4 (0.7)	0.050	-1.4 (0.6)	0.027	-0.2 (0.2)	0.258
Constitutional factors														
Gender, male	-1.8 (1.0)	0.065	-1.1 (1.1)	0.328	-2.3 (0.6)	0.001	-1.4 (0.5)	0.008	-3.3 (1.8)	0.070	-2.6 (1.6)	0.106	-0.3 (0.5)	0.523
Birth weight[b]	-1.2 (0.6)	0.037	-0.6 (0.7)	0.367	-1.2 (0.4)	0.003	-0.7 (0.3)	0.037	-1.8 (1.1)	0.099	-2.5 (1.0)	0.014	-0.3 (0.3)	0.344
Growth rate	1.3 (0.7)	0.085	1.9 (1.5)	0.225	0.1 (0.5)	0.870	0.0 (0.4)	0.985	-1.2 (1.2)	0.307	0.0 (1.1)	0.993	0.1 (0.3)	0.850
BMI	-1.1 (0.5)	0.033	-1.1 (0.6)	0.084	-0.5 (0.3)	0.112	-0.5 (0.3)	0.067	-0.6 (1.0)	0.549	-0.6 (0.8)	0.457	-0.1 (0.2)	0.719
Biomarker concentration														
Leptin, serum	0.0 (0.5)	0.933	-0.4 (0.5)	0.515	-0.1 (0.4)	0.823	0.0 (0.3)	0.970	-1.0 (0.9)	0.267	-0.6 (0.9)	0.486	-0.4 (0.2)	0.148

[a] Values represent the mean difference (SE) in methylation with a P-value from a standard *t*-test. For continuous variables linear regression was performed with Z-scores so that the resulting estimated effect size indicates the methylation change per standard deviation (SD) change in birth weight (SD 584 gram), growth rate (SD 0.03 cm/(√age(in months)), BMI (SD 1.3 point) and leptin (0.195 mmol/L).
[b] Adjusted for gestational age.

such as those established through the NIH Roadmap Epigenomics Mapping Consortium, can greatly contribute to this research field.[40] Next to studying the overall methylation of *LEP*, the focus on single CpG sites may be of importance, which is illustrated by our finding of periconception smoking and increased methylation of CpG site #22. The methylation of this site affects the binding and activity of the transcription factor C/EBP which modulates expression of *LEP*.[12]

Strength of our study is the standardized study moment of 17 months after birth, which is relatively short after pregnancy thereby minimizing recall bias regarding periconception folic acid use, smoking and breastfeeding. In conclusion, the present study shows that variables associated with epigenetic differences in *LEP* at 17 months of age are low education, gender, duration of breastfeeding, birth weight, BMI and the leptin concentration. The strongest variables, however, are suggested to be gender, the duration of breastfeeding and the leptin concentration. Future studies will be necessary to replicate our findings and to reveal whether our findings have implications for future health and obesity risk.

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Chapter 9

General discussion

In this thesis, we aimed to increase our knowledge on the following three topics:

1) the influence of periconception maternal nutrition and lifestyle and the risk of having a child with a CHD;
2) biomarkers of lipid metabolism and global cellular methylation in the child and the association with CHD;
3) the influence of periconception nutrition, lifestyle and breastfeeding in association with the epigenetic programming of the healthy child.

All studies described in this thesis were performed in the HAVEN study, a case-control triad study performed in the Western part of the Netherlands.

It reveals from our studies that women carrying the *MDR1* C3435T polymorphism, who use medication without the periconception supplementation of folic acid, are at increased risk for having a child with CHD. This finding stresses the importance to use folic acid and to minimize medication use. This is in line with our finding that a dietary pattern rich in fish and seafood, is high in methyl donors, and can substantially lower the risk of having a child with CHD. From the studies in children can be concluded that in children with CHD, in particular isolated CHD, the triglyceride level was almost 40% higher compared with control children. This may suggest that children with CHD are more at risk for the development of cardiovascular disease in later life. Moreover, high concentrations of methylation biomarkers were associated with complex CHD. It appeared that Down syndrome and CHD may be associated with a global hypomethylation status, which has to be confirmed in tissues and global DNA methylation in future studies. From the epigenetic studies in healthy children reveals that several periconceptional maternal exposures are associated with DNA methylation of *IGF2* DMR, *INSIGF* and *LEP* in the child at 17 months of age. Indicators of adverse exposures, such as low SES, and periconception smoking, and presumed beneficial exposures such as periconception folic acid and long-term breastfeeding are associated with epigenetic programming. In addition, birth weight was associated with the methylation of *IGF2* DMR and *LEP*. These observations suggest that the origin of CHD and epigenetic programming are influenced by the same periconceptional and postnatal environmental exposures.

In this chapter, first the design and methodological considerations of our studies are discussed. Thereafter, we will elaborate on our main findings, the inferences and future research. A general conclusion will end this chapter.

Methodological considerations

Study design

A prospective preconception study would be ideal to investigate associations between risk factors and CHD. However, with a relatively low birth prevalence rate of 6-8/1000 live births the number of study participants needed would be very large. This is not practicable and not feasible. Therefore, we have chosen a case-control design, which is widely used in observational epidemiological research and accepted to study associations between exposures and a 'rare' outcome.

Study population

All studies described in this thesis are part of the HAVEN study, a case-control family study designed to investigate the role of genetic and lifestyle factors in the pathogenesis and prevention of CHD. The selection of CHD phenotypes was based on experimental and epidemiological studies that showed that hyperhomocysteinaemia and related gene-environment interactions are involved in the aetiology.[1-3] Included CHD phenotypes comprised of Tetralogy of Fallot, transposition of the great arteries, atrioventricular septal defect, peri-membraneous ventricular septal defect, coarctation of the aorta, valvular aortic stenosis, valvular pulmonary stenosis and hypoplastic left heart syndrome. CHDs can be classified anatomically, clinically, epidemiologically and developmentally, but the knowledge of the underlying mechanisms, while advancing, is far from complete. In some of the studies described in this thesis, we classified CHDs as isolated defects and non-isolated defects. The non-isolated CHDs were described as complex which consisted of non-syndromic CHD and syndromic CHD. The latter equals Down syndrome with CHD and other syndromic CHD. This subclassification aimed to increase homogeneity and the chance of detecting risk factors. Due to the size of our study population we were not able to study the CHD phenotypes separately, this would have improved comparability and interpretability of our studies.

Misclassification of cases and controls was prevented as much as possible by choosing the study moment of 16 months after delivery, as most CHD are diagnosed during the first year of life. However, for ethical reasons and feasibility, echocardiography or catheterisation was not performed in control children. Therefore, misclassification of controls cannot be excluded completely, but this would have led to an underestimation of the observed associations. The chosen study moment is also important, with respect to the study of maternal dietary patterns. It has been shown previously that the maternal nutritional status assessed more than 1 year after delivery can be used as a valid estimate of the periconception maternal nutritional status.[4]

Controls were recruited in collaboration with the child health care centres of "Careyn" in the Rotterdam area. Child health care centres are part of the Dutch Health Care system where physicians specialised in child health care regularly check all new-borns at standardized

moments on health, growth, and development. To exclude strong genetic factors, no familial relationship existed between cases and controls. Control children were eligible when they had no major congenital malformations or chromosomal abnormalities according to the medical records and regular health checks by physicians of the child health centres. Of the controls, a subgroup was selected for our epigenetic studies.

In a case-control study, selection bias is an important issue; it is a systematic error that occurs when the association of exposure is different for participants and non-participants. In the HAVEN-study, the response rate was 74.7% for the case families and 61.4% for the control families. We believe that the participation was not related to the exposures we studied, the included families were not aware of the hypotheses underlying our study. In case-control studies, questions about previous exposures are usually answered by the subjects after occurrence of the event of interest. This is similar in the HAVEN-study, the outcome CHD has already occurred by the time the patient is recruited into the study. The cases, therefore, may have spent more time thinking about past exposures and causes of their disease, whereas the controls are not motivated to do so. This difference between cases and controls in the accuracy and completeness of exposure information can thus introduce a differential recall bias, which can lead to underestimation or exaggeration of an effect. Moreover, especially in case-control studies of birth defects, the mothers of a baby with a birth defect are more likely to recall their exposure to potential risk factors. This might be indeed true for some exposures, but only when the exposure is known to be associated with the disease or is socially undesirable.[5] Even for alcohol or smoking, which are considered undesirable behaviours in pregnancy, differential misclassification has only a minor effect.[6] Comparing the general characteristics between cases and controls is a way of checking the comparability and to see whether selection bias might play a role; in our studies, we have presented the general characteristics of our study population. We reported all significant differences between cases and controls, and adjusted the associations for differences. Our study group comprised more non-European women than the Dutch reference population, which was according to the expectations since a higher percentage of non-Europeans live in the western part of the Netherlands.

Accuracy of the data

At the time of study, approximately 16 months after delivery of the index child, data were obtained by a self-administered questionnaire on sociodemographic characteristics, such as age, ethnicity, educational level, periconception smoking, medication use, folic acid use and nutritional intake. During the hospital visit, the questionnaire was checked by the researcher for completeness and consistency. Next to the data that were taken directly from the questionnaires, we measured various risk factors and characteristics. Here we will discuss the accuracy of the data; it is the degree a measurement represents the true value of the attribute being measured.

Measurement errors in the genotyping of the *MDR* polymorphisms are not likely because

we used a Taqman® allelic discrimination assay designed by Applied Biosystems' Assayby-Design service Applied Biosystems ([ABI], Nieuwerkerk a/d IJssel, The Netherlands) according to the manufacturers protocol. More than 3% of the samples were re-genotyped to check for consistency of genotype calling, resulting in a genotyping success rate of more than 96%. Moreover, all genotype frequencies in the control population were in Hardy-Weinberg equilibrium and comparable to other populations.[7] In addition, we removed inconsistent triads from the analyses.

Medication use was defined as any use in the periconception period. We obtained this information from the general questionnaire and classified this according to the Anatomical Therapeutic Chemical (ATC) classification that was controlled by the World Health Organization Collaborating Centre for Drug Statistics Methodology. Coding is based on the pharmacological and chemical properties of the drug. In our study, mothers were considered medication users when taking any medication. We were not able to check the use, dosage, and duration with the pharmacy because of ethical constraints. This could have led to recall bias of medication use. Especially differential recall bias could be of concern. However, an important study on the validity of parental reporting in case-control studies on different childhood diseases showed, however, that in case-control studies focusing on exposures in relation to disease, recall bias is mostly non-differential.[5] A limitation of the study is also that due to the small numbers we are not able to give the risks for the separate medicines. Although maternal use of antihistamines in early pregnancy has not shown to be harmful with respect to birth defects, our finding of a higher reported use in case mothers is interesting and worthwhile to study in further detail when larger numbers are included.

Dietary intake of macronutrients and micronutrients, as well as energy was estimated using a semi-quantitative food frequency questionnaire (FFQ), which was validated and modified for the estimation of B-vitamin intake.[8, 9] The validated FFQ was completed at the study moment and covered the intake of the previous 4 weeks, whereby day-to-day variability of food intake is minimised. The study moment is 2 years after conception of the index pregnancy in the same season as the periconception period. Thus, the seasonal influences on food intake are comparable between these periods. In addition, we have shown previously that the maternal nutritional status assessed more than 1 year after delivery can be used as a valid estimate of the preconception maternal nutritional status.[4] This is also supported by others, showing that every individual adheres to a habitual dietary pattern, which is only influenced by periods of illness, diet, pregnancy, breastfeeding and growth spurts.[10-12] It appears that after adjustment of the analysis for these factors, dietary patterns are very stable during life. Therefore, to maximize the correspondence between study moment and periconception period we excluded mothers that reported a dietary difference, were pregnant, or lactated. The median nutrient intakes were similar to those of the Dutch National food consumption survey (FCS),[13] indicating that the dietary intakes generally reflect the intakes of Dutch non-pregnant women aged 22-50 years. Nutritional under-reporting may induce a bias towards underestimating of

the energy intake. We tested this by estimating the mean basal metabolic rate (BMR) using the new Oxford equation for women aged 30–60 years: BMR (MJ/day) = 0.0407 · weight (kg) + 2.90. The physical activity level (PAL) was used to evaluate under-reporting with a cut-off value of 1.35. The PAL was calculated by dividing the mean reported energy intake (EI) by the mean BMR.[14] Both case and control mothers had a higher PAL: 1.39 and 1.44, which was not suggestive of underreporting.

The blood sampling and determination of SAM, SAH, tHcy, vitamin B12, in mothers and children, and HDL-cholesterol, cholesterol and triglycerides in the children were done in a standardized fashion in the same clinical chemistry laboratory at the Erasmus MC. Immediately after blood sampling, an EDTA-tube was put on ice and a serum separator tube was kept at room temperature. Both tubes were centrifuged at 4,000-x g for ten minutes at 4 °C and separated within one hour. All samples were stored at -80 °C and analysed in batches within five months after collection. As the samples were analysed in batches possible measurement errors were random. An important form of measurement error is the inter-assay coefficient of variation (CV); all CV's were below 10% indicating acceptable reliability.

DNA methylation of the CpG dinucleotides of *IGF2* DMR, *IGF2R*, *INSIGF* and *LEP* was measured in triplicate using a mass spectrometry-based method (Epityper, Sequenom) in a laboratory with extensive experience. The laboratory of the Department of Molecular Epidemiology of the LUMC adheres to the state-of-the-art in the field. CpG sites containing fragments of too little or high mass for the mass spectrometer to resolve were excluded from the analysis.

In our epigenetic studies we aimed to show an effect of periconceptional folic acid use, in particular an extreme effect, therefore we excluded mothers who used partially folic acid during this period. The use of folic acid in the periconception period was defined as the daily use of at least 400 µg of folic acid, either in a multivitamin preparation or as a single tablet during the complete period.

Socio-economic status refers to the "social and economic factors that influence what positions individuals or groups hold within the structure of society".[15] We used the maternal education level mentioned in the questionnaire as a proxy for socio-economic status (SES). In epidemiological studies, it is widely accepted to use this marker as a cumulative determinant of harmful exposures such as socioeconomic and health-related risk factors.[16] Low education defined by a maximum of 12 years of on-going education from the age of 4, equal to primary/lower vocational or intermediate secondary education, was used as a proxy for low SES.[17]

We used periconception smoking from the questionnaire as an exposure variable. Many of the mothers who smoked in the periconception period were still smoking at the study moment. We were not able to study current smoking in more detail, as we do not know to what extent their child was postnatally exposed to tobacco smoke. Mothers might have underreported smoking behaviour and the numbers of cigarettes they smoked. This may have led to an overestimation of the association we found.

Data on breastfeeding was derived retrospectively from the mothers in a self-administered home questionnaire. Mothers might overestimate the months that they breastfed. To minimize recall-bias and information bias we validated this with the information on breastfeeding from the child's record at the public child health care centres of Careyn, the latter was considered as the truth. From the same record child's weight and length was collected and BMI was calculated.

Power

We have used a population CHD risk of 0.008 and a type I error of 0.05 in all power calculations. Calculations revealed that we have identified high adherence to a one-carbon rich dietary pattern as a protective factor for CHD with a power of 100% (prevalence in controls 36.8%, cases n=179, OR 0.32). The increased risk for carrying the *MDR1* T-allele we found has a power of 79% (cases n = 283, *MDR1* 3435 T allele frequency 0.5). The power for the finding of 2.8-fold increased risk for CHD with the *MDR1*-polymorphism and medication use was 99% (prevalence in controls 15.3%, cases n = 283, OR 2.4.)

Main findings, inferences and future research

I The origin of congenital heart defects: determinants in the mother

General maternal medication use, folic acid, the MDR1 C3435T polymorphism

In this thesis, we have shown that the teratogenicity of the use of medication is enhanced in children of mothers carrying the *MDR1* 3435 C>T polymorphism, and do not take periconception folic acid **(Chapter 2)**. The *MDR1* 3435 TT genotype results in decreased expression of the efflux pump, thereby decreasing an adequate efflux of medication and other toxins from the circulation.[18] A previous study has shown that the *MDR1* 3435C>T polymorphism combined with medication use was associated with the risk of cleft lip/palate in the offspring.[19] In both our studies, periconception folic acid use seems to modulate the detrimental effect of medication use in the risky genotypes.

We hypothesize that when a mother carries the 3435CT/TT genotypes and takes medication, she provides an environment in which the developing embryo is exposed to higher levels of toxins, which may result in different CHD phenotypes dependent on the exposure window. This effect is counteracted by the use of folic acid. In addition, these data are in line with our previous finding that children carrying the nicotinamide N-methyltransferase A allele face additional CHD risk in combination with periconception exposure to medicines and a low dietary intake of vitamin B3.[20] It was hypothesized that the polymorphism in this gene together with decreased availability of its cofactor B3 leads to a decreased detoxification of medication undergoing methylation. The role of folic acid in our study is therefore of great

interest. We believe that the intake of folic acid in the periconception period contributed to a folate-rich environment and thereby increased the efflux of *MDR1* substrates from the cell.[21] Next to this, folic acid is an important methyl donor and provides methyl groups via SAM to aid in the detoxification of medication. The general advice to use folic acid seems enough to reduce the risk for a child with CHD when carrying the *MDR1* polymorphism. Although in general the advice is to limit medication use during pregnancy, the use has risen over the last 30 years. Almost 80% of women take at least one medicine, either prescribed or non-prescribed.[22] Therefore, prior to conception individual counselling is important to balance the risk and benefits of medication use. Sometimes medication is necessary to stable maternal conditions that are also known to be involved in adverse pregnancy outcomes. Systematically surveying birth defects and maternal medication use, as done by the birth defect registries, such as the EUROCAT registry in the Northern provinces, and the ICBDSR in Europe, are of great value to continue the increase of knowledge. It would be helpful if the Eurocats registry in the Netherlands would not only include the Northern part but could extent to all the Dutch provinces.

Dietary pattern

In this thesis, we identified dietary patterns related to the bioavailability of the SAM and SAH and the risk for CHD **(Chapter 3)**. Maternal nutrient deficiencies especially in the periconception period and early gestation, can lead to developmental abnormalities.[23] The role of folate has been widely studied and food fortification with synthetic folic acid has been associated with a decrease in the prevalence of CHD.[24] The human diet is the major source for folate, methionine and choline that serve together as substrates in the one-carbon pathway. The methyl groups provided by this pathway are essential for the transmethylation of SAM into SAH, after which homocysteine is formed. The concentrations of SAM and SAH in blood plasma and their ratio are frequently used as markers of global DNA methylation potential.[25] With these two biomarkers as outcome in RRR, we identified two dietary patterns related to the one-carbon pathway. The one-carbon-rich dietary pattern, positively associated with SAM and inversely with SAH, was characterized by a high intake of fish and seafood and was associated with a 70% reduced risk of CHD in the offspring, which is larger than the effect of periconception folic acid and similar to the association between the periconception Mediterranean diet and reduced spina bifida risk.[26] The one-carbon-poor dietary pattern was strongly positively associated with SAH and marginally with SAM; this diet contained, in particular, a high intake of snacks and sugar-rich products and beverages, which were not significantly correlated with nutrient intake or other biomarkers.

We do not know what the underlying mechanisms are by which derangements in the one-carbon pathway, induced by compromised dietary intakes of its substrates and cofactors, contribute to the development of CHD and other birth defects. In animal studies, the periconception dietary availability of methyl groups has an effect on gene methylation and phenotype.[27]

Thus, our finding may be a first link between periconception maternal nutrition and epigenetic programming of cardiac genes in embryonic development. We hypothesize that periconceptional adherence to the one-carbon-rich dietary pattern affects the methylation of genes in the offspring, with consequences for health and disease in later life. Future studies must reveal if the offspring's genome is affected and in which genes methylation differences are seen. Studying the effect of dietary interventions on gene methylation in a prospective randomized intervention trial would be ideal. Although it may be difficult to study in the population of (pre)pregnant women, studies have shown to be successful.[28, 29]

II The origin of congenital heart defects: determinants in the child

Triglyceride levels

We have demonstrated that children with CHD have higher triglyceride levels, in absence of differences in total cholesterol, HDL- and LDL-cholesterol levels compared with control children **(Chapter 4)**. Earlier we showed in the HAVEN-study that a mild maternal dyslipidaemia, i.e., elevated levels of total cholesterol, LDL-cholesterol and apolipoprotein-B, is associated with a 2-fold increased risk of CHD in the offspring.[30]

We hypothesize that next to tissue-specific epigenetic reprogramming that led to the CHD, this maternal environment may have led to a derangement in the intra-uterine programming of the lipid pathway in the child. Many studies are in support of this hypothesis. Especially nutrition during pregnancy, both under and overnutrition are of great importance for future cardiovascular health risk in the offspring.[31] There is increasing awareness that permanent epigenetic changes, possibly induced by nutritional deficiencies or overloads, by either DNA methylation or chromatin modification may be responsible for an increased risk for adult atherosclerosis.[32] An example of this is the formation of early atherosclerotic lesions that seem to persist into childhood and adulthood, when exposed in utero to maternal hypercholesterolemia.[33] This finding of our study can be of great importance for the future health of the surviving children with isolated CHD. Higher levels of triglycerides at young age are independently associated with an increased risk for cardiovascular disease risk in later life.[34] It is interesting to look into shared causalities that underlie the risk for a child with CHD and cardiovascular disease risk. Shared causalities are hyperhomocysteinaemia, obesity and diabetes.[35]

In general, increased triglyceride levels can be the result of a combination of genetic and lifestyle factors.[36] Therapeutic measures preferably target causes with dietary interventions to improve the lipid profile. Before screening for abnormal lipid profiles in children with CHD, we suggest a prospective study in a cohort of children with CHD to replicate our findings. The American Heart Association advices an aggressive risk evaluation for cardiovascular risk factors in especially children with CHD, since the anatomical changes, especially when they have obstructive lesions of the left ventricle and aorta (CoA and AS), seem to make

them already more vulnerable for atherosclerosis.[37] Moreover, some children with (repaired) CHD have limitations in their ability to exercise, which is also an independent risk factor for cardiovascular diseases.

It would be of great value based on our finding and hypothesis to study lipid levels in these patients combined with DNA methylation of genes that are involved in cardiovascular health. This could be possible in the growing surviving population of adults with CHD. Many of these adults are not regularly seen by a physician. In the Netherlands, a nation-wide publicity campaign was started to contact and register "lost" adults with a CHD by the CONCOR (CONgenital CORvitia) project group.[38] A total of 593 patients with CHD were registered, 66% were examined within one year by a cardiologist, it appeared that additional cardiac follow-up was necessary within 1 year in 22% of these patients.

Biomarkers of methylation

We have showed that young children with complex CHD, in particular syndromic CHD, have higher concentrations of biomarkers of methylation reflected by increased SAM, SAH and folate in blood. Children with Down syndrome showed the lowest SAM:SAH ratio of all groups, although they had the highest concentrations of SAM and SAH. We hypothesize that periconception exposure to the maternal metabolic derangement of a state of hypomethylation may have led not only to abnormal chromosomal segregation, but also to altered epigenetic programming of genes leading to a CHD and altered programming of the methylation pathway in the foetus. Further research is needed to understand this state of hypomethylation and to see if this state is reflected in tissue specific DNA methylation, not only in children with a complex CHD but also isolated CHD and controls. A recent study in mothers of a child with CHD has shown genome wide gene-specific differences in methylation patterns.[39] A next step will be to study if differential methylation is also observed between infants with CHD and controls, and if these methylation patterns are associated with maternal DNA methylation patterns.

In literature, there is only one other study reporting on biomarkers of methylation in children with Down syndrome and a CHD. In contrary to our result, SAM and SAH were decreased, suggestive of a reduced methylation capacity. However, the lymphocytes in these children showed increased methylation. It was hypothesized that 1) this observed increase in DNA methylation reflects the addition of the third highly methylated chromosome 21; Or that 2) the higher DNA methylation density reflects an adaptive mechanism to down-regulate overexpressed genes on chromosome 21.[40] Although our finding is different, it remains of interest because the SAM: SAH ratio is similar to the global hypomethylation status observed in the mothers of these children with Down syndrome.

III The epigenetic programming of the healthy child

Epigenetic research has emerged over the last decade as a new field of genetic research. We contributed to this field of research with our epigenetic studies in healthy young children. We studied various environmental exposures related to nutrition and lifestyle in relation to methylation of *IGF2* DMR, *IGF2R*, *INSIGF* and *LEP*. Epigenetic effects in *IGF2* DMR, *IGF2R*, *INSIGF* and *LEP* have been studied in humans that were exposed in utero to the Dutch famine revealing that 6 decades later methylation of *IGF2* DMR, *INSIGF* and *LEP* was related to this exposure, sometimes in a gender and time-specific manner.[41]

Folic acid use

In this thesis, we demonstrate that periconception folic acid use is associated with epigenetic changes in *IGF2* DMR in the child **(Chapter 6)**. We also show that the maternal SAM concentration was associated with a higher *IGF2* DMR methylation in the child.

The difference in DNA methylation associated with folic acid exposure is remarkably similar to the previous observation of a 5.2% reduced *IGF2* DMR methylation after periconceptional exposure to the Dutch famine.[42] These DNA methylation changes may have phenotypic consequences as illustrated by the association between higher *IGF2* methylation and decreased birth weight. A simple preventive strategy as periconceptional folic acid use may affect epigenetic control also in other genes, this could be the link between periconception folic acid and increased foetal growth, and a decreased risk of a growth-restricted child.[43] We did not find an association between periconception folic acid use and the methylation of *INSIGF*, *IGF2R* and *LEP* (Chapter 7 and 8). This indicates that loci are not equally affected by the same exposures.

Smoking

We showed that periconception maternal smoking and low education, as proxy for SES, are associated with increased methylation of the *INSIGF* gene in very young children **(Chapter 7)**. The *INSIGF* locus, which is the overlapping region of *IGF2* and insulin (INS), has been associated with small-size-for-gestational-age at birth (SGA).[44] A study by colleagues however, revealed no association between SGA and DNA methylation of *INSIGF*.[45]

We hypothesize that adverse effects of the periconception environment, i.e., factors related to low education, and smoking on birth weight could be related in part to silencing of the imprinted *INSIGF* gene in the child by slightly higher DNA methylation. Supportive of the validity of our results is our finding of a dose-response relationship between the number of cigarettes smoked and the increase in methylation of *INSIGF*. In literature, there are still only inconclusive studies on the effect of periconception smoking on DNA methylation of the offspring.[45, 46] Smoking is considered one of the major mediators in the association between low SES and adverse pregnancy outcome, and associated with a decreased birth weight. We expected that

both factors interacted in their association with methylation. However, both had an additive effect on the increased methylation of *INSIGF*. This may suggest that there are other factors that affect the methylation of *INSIGF* that are related to low education such as unhealthy nutrition and stress.

Although we may think it is common knowledge that smoking during pregnancy can lead to harmful effects for the baby, there is still a large percentage of woman that continue smoking. In addition, even when woman sustain from smoking, they are still at risk for adverse pregnancy outcomes; being around second hand smoke can increase the risk for a child with a birth defect.[47] This warrants the attention of caregivers involved in preconception care and pregnancy care.

Maternal SES, breastfeeding and child's constitutional factors

The epigenetic variation of *LEP* and the involvement of this gene in unhealthy phenotypes, such as obesity and insulin resistance, made *LEP* an interesting locus to study.[48] As we mentioned earlier, increased *LEP* methylation was associated with exposure to the Dutch famine. Of interest is that this observation was gender-specific and not related to the timing of exposure in pregnancy, suggestive of a broad programming window.[41] This strengthens our finding that *LEP* methylation can also be associated with a post-pregnancy exposure such as breastfeeding **(Chapter 8)**.

The association between the duration of breastfeeding with lower *LEP* methylation is fascinating. We hypothesize that the lower methylation may lead to increased expression and higher concentrations of leptin, which is substantiated by the demonstrated association between BMI-adjusted leptin concentration and methylation of *LEP* at this age. We hypothesize that the breast milk content contributes to programming of the neuro-endocrine system by modulating methylation patterns of the *LEP* promoter. This could be one of the mechanisms by which breastfeeding contributes to the protection against childhood obesity.[49] The association between low SES as the cumulative derivate of adverse exposures, and a higher methylation of *LEP* warrants further investigation. We do not know to what extent the measured methylation patterns are adequate reflections of methylation patterns set at birth or methylation changes across early postnatal life. Future studies need to focus on the variation in *LEP* methylation patterns from birth onwards in a larger group, and to examine the relationship with the development of body composition.

General Conclusion

The studies in this thesis have identified new risk factors for having a child with a CHD. The identified risk factors can add new interventions to the already existing preventive strategies. The findings in children with CHD illustrate that next to the CHD, the maternal environment

may have led to a derangement in the intra-uterine programming of the lipid and methylation pathway in the child with consequences for future health.

The observations in our epigenetic studies are suggestive of links between the early life environment and constitutional factors by epigenetics. Future studies in large birth cohorts could help to unravel to what extent positive or adverse effects on health are to be expected from altered methylation at the studied loci. By performing studies, we can identify causal associations between early exposures, long-term changes in epigenetic regulation, and disease. Epigenetic epidemiology can be a difficult field of study, nevertheless, the findings can help in the design of specific early-life interventions to improve human health.[50, 51]

Modifiable exposures that have proven to increase health such as adhering to a healthy dietary pattern, limitation of medication use, abstaining from smoking, the use of periconception folic acid and breastfeeding can be discussed and targeted in preconception counselling and maternity check-ups, also in women with low SES this can be effective.[52]

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Chapter 10

Summary / Samenvatting

English Summary

Birth defects affect ~7% of births worldwide. Of these, congenital heart defects (CHD) are the most common congenital malformation with approximately 1 million children born each year. Years of animal and human studies made it clear that the vast majority of birth defects have multifactorial origins, with contributions from environmental and genetic factors. More than 80-90% of congenital heart defects are believed to result from complex interactions between subtle genetic variations and periconception environmental exposures. The environment comprises of not only the physical, biological, and chemical external environment surrounding the pregnant woman, but it includes the internal environment of the woman's body that interacts with the developing embryo.

Epigenetics is suggested to be an important mechanism linking periconception, prenatal and postnatal exposures in association with health and disease risks in early and later life. DNA methylation is one of the best-understood epigenetic mechanisms and an important programming mechanism of the genome. Alterations in the global methylation status in blood and other tissues during pregnancy and post-weaning can derange DNA methylation and thereby modify embryonic, foetal and metabolic development. It is hypothesized that these changes may be a mechanism to adapt a priori to future environment exposures. This is known as the Developmental origin of health and disease (DOHaD) hypothesis. Adverse exposures in utero may lead to the induction of epigenetic marks, which may have consequences for health and disease in present and later life.

This thesis displays the results from the HAVEN study, a case-control family study coordinated from June 2003 until January 2010 at the Department of Obstetrics and Gynaecology, Erasmus MC, Rotterdam, The Netherlands. The studies in part I and II are conducted as a case-control study on CHD. The studies described in part III were conducted in a subset of the control children of the same HAVEN study.

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

Part I The origin of congenital heart defects: determinants in the mother

In this part we describe maternal risk factors in association with congenital heart defects. In **Chapter 2**, we present data of the interaction between maternal medication use, folic acid, and the *MDR1* C3435T polymorphism, and the risk of a child with a congenital heart defect. We determined the *MDR1* 3435C<T genotype in 283 case triads (mother, father, child) and 308 control triads. Mothers with the *MDR1* 3435CT/TT genotype that used medication in the periconception period had a two-fold increased risk of having a child with CHD compared to mothers with the *MDR1* 3435CC genotype not using medication. This risk increased to 3-fold in mothers without folic acid use and decreased in folic acid users. The *MDR1* 3435 CT/TT genotype in fathers and children were not associated with an increased risk.

In **Chapter 3** we describe the association between a periconception maternal dietary pattern linked to biomarkers of methylation and the risk of CHD in the offspring. We performed this study in 179 mothers of children with CHD and 231 mothers of children without a congenital malformation. In the control mothers we identified two dietary patterns reflecting the bioavailability of one-carbon donors. The one-carbon-rich dietary pattern, characterised by a high intake of fish and seafood, provided, in particular, total protein, vitamin B1, B2, B3, B6 and B12, zinc, EPA and DHA. Strong adherence to this dietary pattern was reflected in higher RBC and serum folate levels, and associated with a 70% reduced risk of CHD in the offspring. This estimate is much higher than the 20% reduction after the periconception use of a low dose of synthetic folic acid. The one-carbon-poor dietary pattern, characterised by a high intake of snacks, sugar-rich foods and beverages, resembles more closely a Western dietary pattern, and did not affect the biomarker levels and CHD risk.

Part II The origin of congenital heart defects: determinants in the child

This part focuses on biomarkers of lipids and of cellular methylation in children with a congenital heart defect. In **Chapter 4**, we investigated if lipid profiles in very young children are associated with CHD. We measured serum concentrations of triglycerides, total cholesterol, and HDL-cholesterol in 141 children with CHD and 178 healthy children. Furthermore, LDL-cholesterol values were calculated using the Friedewald formula. Children with CHD had a higher triglyceride level than the healthy controls. It appeared that the triglyceride concentrations were especially highest in the isolated CHD subgroup compared to the non-isolated. Total cholesterol, HDL-cholesterol and LDL-cholesterol levels were comparable between cases and controls. The finding of hypertriglyceridemia in children with isolated CHD may suggest that children with CHD are more at risk for the development of cardiovascular disease in later life.

In **Chapter 5**, we studied biomarkers of methylation in 143 children with CHD and 186 healthy children at the age of about 17 months. We determined the concentrations of S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), total homocysteine (tHcy), the vitamins folate and B12 and the functional single nucleotide polymorphisms in the folate gene *MTHFR* 677C>T and 1298A>C. All analyses are adjusted for age, use of medication and vitamin supplements, and CHD in the family. In the overall CHD group, the median concentrations of SAM, folate in serum and RBC were higher than in the controls. This could not be explained by carriership of one of the *MTHFR* variants. The subgroup of complex syndromic CHD showed the highest concentrations of SAM, SAH and folate, compared with the control group. The subgroup of children with Down syndrome showed significantly higher SAH and significantly lower SAM:SAH ratio compared with other complex CHD, suggesting a state of global hypomethylation. Thus, high concentrations of methylation biomarkers in very young children are associated with complex CHD. Down syndrome and CHD may be associated

with a global hypomethylation status, which has to be confirmed in tissues and global DNA methylation.

Part III The epigenetic programming of the healthy child

In the final part of this thesis we studied periconception exposures and DNA methylation of several loci involved in cardiovascular and metabolic health in a subset of 120 healthy control children that were included in the HAVEN study at the age of 17 months.

In **Chapter 6**, we investigated the association between periconception folic acid use and methylation of *IGF2* DMR in 120 healthy children of whom 86 mothers had used and 34 had not used folic acid periconceptionally. Children of mothers who used folic acid had a 4.5% higher methylation of the *IGF2* DMR than children who were not exposed to folic acid. *IGF2* DMR methylation of the children was also associated with the maternal SAM blood level. Furthermore, we observed an inverse independent association between *IGF2* DMR methylation and birth weight. Our results show that periconception folic acid use is associated with epigenetic changes in *IGF2* in the child; this may be a mark of intrauterine programming of growth and development.

In **Chapter 7**, we studied associations between several maternal and child characteristics in association with methylation of the insulin pathways, genes *INSIGF*, *IGF2R* and *IGF2* DMR. Periconception smoking and low education as proxy for SES, were independently associated with *INSIGF* methylation, and showed an additive effect on *INSIGF* methylation. There were no associations with *IGF2* DMR and *IGF2R* methylation. Our data suggest that periconception maternal smoking and low education are associated with epigenetic marks on *INSIGF* in the very young child.

In **Chapter 8**, we studied associations between methylation of *LEP*, maternal education as proxy for SES, breastfeeding, constitutional factors of the child and serum leptin concentration. Duration of breastfeeding was negatively associated with *LEP* methylation. Low education was associated with higher *LEP* methylation. Boys had lower *LEP* methylation than girls. An inverse association was established between birth weight and *LEP* methylation. High BMI and leptin concentration were both associated with lower methylation of *LEP*. It appears that early life environment and constitutional factors of the child are associated with epigenetic variations in *LEP*. Future studies must reveal whether breastfeeding and the associated decrease in *LEP* methylation is an epigenetic mechanism contributing to the protective effect of breastfeeding against obesity.

In the general discussion, the main findings of this case-control study are discussed and the objectives are evaluated **(Chapter 9)**. Furthermore, we discuss the implications and recommend future research.

Nederlandse Samenvatting

Wereldwijd worden jaarlijks ongeveer 7% van de kinderen geboren met een aangeboren afwijking. De meeste aangeboren afwijkingen worden veroorzaakt door een combinatie van (on)bekende erfelijke- en omgevingsfactoren in de vroege zwangerschap. De meest voorkomende afwijking is de aangeboren hartafwijking, elk jaar worden ongeveer 1 miljoen kinderen geboren met een CHD. Het merendeel (80-90%) van de aangeboren hartafwijkingen zijn het resultaat van complexe periconceptionele interacties tussen subtiele erfelijke variaties en omgevingsinvloeden. De externe invloeden betreffen niet alleen fysieke, biologische en chemische omgevingsblootstellingen van de zwangere vrouw, maar ook constitutionele oftewel interne milieu factoren die interacteren met de ontwikkeling van de organen van het embryo.

Epigenetische mechanismen lijken in toenemende mate een verklaring te zijn voor de relaties tussen periconceptionele, prenatale en postnatale blootstellingen en het optreden van ziekte en gezondheid later. Eén van de best begrepen epigenetische mechanismen is DNA methylering. Dit is een proces waarbij een methylgroep (CH3) wordt gebonden aan een DNA-molecuul waardoor er veranderingen in de transcriptie van het DNA kunnen optreden. Veranderingen in de methyleringsstatus van het DNA tijdens de zwangerschap en de postnatale periode kunnen de methylering van het DNA beïnvloeden en daardoor ook de groei en de stofwisseling van het embryo en de foetus. Deze epigenetische programmeringsprocessen zijn vermoedelijk bedoeld om het kind tijdens de zwangerschap voor te bereiden op de omstandigheden na de zwangerschap. Epigenetische mechanismen onderbouwen de `Developmental origin of health and disease´ (DOHaD) hypothese. Hieronder wordt verstaan dat ongunstige blootstellingen tijdens de zwangerschap kunnen leiden tot veranderingen in de groei en functie van organen die consequenties kunnen hebben voor ziekte en gezondheid in het verdere leven.

In dit proefschrift worden de resultaten beschreven van de HAVEN-studie (Hart Afwijkingen, Vasculaire status, Erfelijkheid en Nutriënten). Dit is een case-controle familie studie waarvan de werving heeft plaats gevonden van juni 2003 tot en met januari 2010 vanuit de afdeling Gynaecologie en Verloskunde van het Erasmus MC, Universitair Medisch Centrum in Rotterdam. De studies beschreven in deel I en II van het proefschrift zijn uitgevoerd in de HAVEN-studie. De studies beschreven in deel III zijn uitgevoerd in een groep van 120 gezonde controle kinderen uit deze zelfde HAVEN-studie.

In **Hoofdstuk 1** wordt de achtergrond van het proefschrift in een algemene introductie beschreven.

Deel I Oorzaken voor de ontwikkeling van aangeboren hartafwijkingen bij het kind: Determinanten van de moeder

Het eerste deel van dit proefschrift beschrijft associaties tussen maternale risicofactoren in

de periconceptionele periode en het risico op het krijgen van een kind met een aangeboren hartafwijking.

In **Hoofdstuk 2** presenteren we de resultaten van het onderzoek naar de interactie tussen maternaal geneesmiddelen- en foliumzuurgebruik, functionele genetische polymorfisme (SNP) in het *MDR1*-gen, en het risico op het krijgen van een kind met een aangeboren hartafwijking. We bepaalden het *MDR1* 3435C<T genotype in 283 case gezinnen (moeder, kind en vader) en 308 controle gezinnen. Moeders met het *MDR1* 3435CT/TT genotype die daarnaast een geneesmiddel gebruikten in de periconceptionele periode hadden een twee keer verhoogd risico op krijgen van een kind met een aangeboren hartafwijking vergeleken met moeders met het *MDR1* 3435CC genotype die geen geneesmiddel gebruikten. Dit risico werd drie keer zo hoog in moeders die daarnaast ook geen extra foliumzuur gebruikten, het risico was lager in moeders die wel foliumzuur gebruikten. Het *MDR1* 3435 CT/TT genotype in de vaders en de kinderen was niet geassocieerd met een verhoogd risico.

In **Hoofdstuk 3** onderzochten we het voedingspatroon van de aanstaande moeder in de periconceptionele periode in relatie tot een aantal biomarkers van methylering en het risico op het krijgen van een kind met een aangeboren hartafwijking. We includeerden 179 moeders van een kind met een aangeboren hartafwijking en 231 moeders van gezonde controle kinderen. In de controle moeders konden we twee voedingspatronen identificeren die gecorreleerd waren aan de S-adenosylmethionine (SAM) en S-adenosylhomocysteine (SAH) gehalten en daarmee de beschikbaarheid van methylgroepen weergeven. Het methyl-rijke voedingspatroon, werd gekenmerkt door hoge innamen van vis en zeevruchten, en leverde in het bijzonder eiwit, vitamine B1, B2, B3, B6 en B12, zink, en de omega=3 vetzuren EPA en DHA. Strikt gebruik van dit methyl-rijke dieet was geassocieerd met hogere foliumzuur gehalten zowel in bloed als erythrocyten, en verlaagde het risico op een kind met een aangeboren hartafwijking met 70%. Deze risicoreductie is veel lager dan het gerapporteerde 20% lagere risico bij het gebruik van extra foliumzuur in de periconceptionele periode. Het methyl-arme voedingspatroon, gekenmerkt door hoge innamen van snacks, zoetigheden, en niet-alcoholische drankjes, was niet geassocieerd met de biomarkers van methylering en het risico op het krijgen van een kind met een aangeboren hartafwijking.

Deel II Oorzaken voor de ontwikkeling van aangeboren hartafwijkingen bij het kind: determinanten van het zeer jonge kind

In deel II van dit proefschrift worden biomarkers van de vetstofwisseling en de methylering bestudeerd in relatie tot de aangeboren hartafwijking van het kind.

In **Hoofdstuk 4** onderzochten we of lipiden profielen in 141 zeer jonge kinderen met een aangeboren hartafwijking op een leeftijd van 17 maanden anders zijn dan in een vergelijkbare gezonde controle groep van 178 kinderen. We hebben de concentraties gemeten van triglyceriden, totaal cholesterol en HDL-cholesterol en berekenden het LDL-cholesterol met behulp van

de Friedewald formule. Kinderen met een aangeboren hartafwijking hebben een significant hogere triglyceriden concentratie dan gezonde kinderen. Dit bleek vooral te gelden voor kinderen met een geïsoleerde aangeboren hartafwijking... Totaal cholesterol, HDL-cholesterol en LDL-cholesterol waren niet significant verschillend. De bevinding dat er bij kinderen met een geïsoleerde aangeboren hartafwijking sprake is van een hypertriglyceridemie kan wijzen op een verhoogd risico op hart- en vaatziekten op latere leeftijd.

In **Hoofdstuk 5** onderzochten we de biomarkers van methylering in dezelfde kinderen met een aangeboren hartafwijking en gezonde controles. We hebben de gehalten gemeten van SAM, SAH, totaal homocysteine (tHcy), foliumzuur en vitamine B12 en de functionele genetische variaties in het *MTHFR* gen, namelijk C677T en A1298C. Alle analyses zijn gecorrigeerd voor leeftijd, geneesmiddelen- en vitamine gebruik en aangeboren hartafwijkingen in de familie. In de casegroep, waren de concentraties van SAM en folaat hoger dan in de controles. Deze bevinding kon niet verklaard worden door dragerschap van één van de *MTHFR* varianten. De subgroep van complexe syndromale aangeboren hartafwijking liet de hoogste gehalten zien van SAM, SAH en foliumzuur, vergeleken met de controles. Opvallend was dat de subgroep van kinderen met Down syndroom en aangeboren hartafwijking een significante hogere concentratie SAH en een significante lagere SAM:SAH ratio hadden in vergelijking met de andere complexe aangeboren hartafwijking, dit is suggestief voor de status van globale hypomethylering. Kortom, hoge gehalten van biomarkers van methylering in jonge kinderen lijken geassocieerd te zijn met een complexe aangeboren hartafwijking. Terwijl het Down syndroom met een aangeboren hartafwijking geassocieerd lijkt te zijn met een hypomethyleringsstatus, dit dient uiteraard bevestigd te worden met weefselspecifieke methylering.

Deel III Epigenetische programmering van zeer jonge gezonde kinderen

Het laatste deel van dit proefschrift concentreert zich op het bestuderen van associaties tussen periconceptionele blootstellingen en DNA methylering van verschillende genen die een rol spelen bij de cardiovasculaire en metabole gezondheid. Dit onderzoek werd uitgevoerd in de 120 gezonde controle kinderen uit de Haven studie eveneens op de leeftijd van 17 maanden.

In **Hoofdstuk 6** bestudeerden we de associatie tussen periconceptioneel foliumzuurgebruik door de moeder en de methylering van *IGF2* DMR bij het kind. Van de 120 kinderen in dit onderzoek gebruikten 86 moeders foliumzuur in de periconceptionele periode en 34 moeders gebruikten geen foliumzuur. De kinderen van de moeders die foliumzuur gebruikten hadden een 4.5% hogere methylering van *IGF2* DMR dan die niet blootgestelde kinderen. De methylering van *IGF2* DMR in de kinderen was daarnaast ook geassocieerd met de maternale SAM concentratie. Ook zagen we een onafhankelijk omgekeerd verband tussen de mate van methylering van *IGF2* DMR en het geboortegewicht. Onze resultaten laten zien dat periconceptioneel foliumzuurgebruik is geassocieerd met epigenetische veranderingen in *IGF2* DMR, dit zou kunnen wijzen op intra-uteriene programmeringsinvloeden van foliumzuur op de groei en ontwikkeling van het kind.

In **Hoofdstuk 7** onderzochten we associaties tussen de verschillende karakteristieken van moeder en kind en de methylering van genen betrokken bij het insuline metabolisme; *INSIGF*, *IGF2R* en *IGF2* DMR. Het maternale roken in de perionceptionele periode en een laag opleidingsniveau, als proxy voor SES, waren beiden onafhankelijk geassocieerd met een geringe toename in methylering van *INSIGF*. Er werden geen significante associaties vastgesteld voor de methylering van *IGF2* DMR en *IGF2R*.

In **Hoofdstuk 8**, bestudeerden we de associatie tussen de methylering van *LEP*, het opleidingsniveau van de moeder, het krijgen van borstvoeding, constitutionele eigenschappen van het kind en het leptine gehalte in het bloed. De duur van borstvoeding was negatief geassocieerd met de methylering van *LEP*. Een laag opleidingsniveau was geassocieerd met een hogere methylering van *LEP*. Jongens hadden een lagere methylering van *LEP* in vergelijking met meisjes. Een toename in BMI en het leptine gehalte waren beiden geassocieerd met een lagere methylering van *LEP*. Deze resultaten suggereren dat vroege omgevingsinvloeden en constitutionele factoren van het kind geassocieerd zijn met epigenetische variaties in *LEP*. Toekomstige studies moeten uitwijzen of borstvoeding en de daarbij gevonden lagere methylering van *LEP*, een epigenetische verklaring is die bijdraagt aan het beschermende effect van borstvoeding bij het optreden van obesitas.

In de algemene discussie (**Hoofdstuk 9**) wordt ingegaan op de doelstellingen en belangrijkste bevindingen beschreven in dit proefschrift. Daarnaast, worden de implicaties van de studies bediscussieerd en doen wij aanbevelingen voor toekomstig onderzoek.

Addendum

List of abbreviations

ANOVA	analysis of variance
AS	valvular aortic stenosis
ATC	anatomical therapeutic chemical
AVSD	atrioventricular septal defect
β	regression coefficient
BMI	body mass index
BMR	basic metabolic rate
CANOCO	canonical community ordination
CHD	congenital heart defect
CI	confidence interval
CoA	coarctation of the aorta
CpG	cytosine-guanine
δ	difference
DHA	docosahexaenoic acid
DMR	demethylated region
DNA	deoxyribonucleic acid
EDTA	plasma
EPA	eicosapentaenoic acid
FBAT	family based association test
FFQ	food frequency questionnaire
GA	gestational age
GSSG	gluthathion disulfide
HDL	high density lipoprotein
HLHS	hypoplastic left heart syndrome
HR	hazard rate
IGF2	insulin-like growth factor 2
IGF2R	insulin-like growth factor 2 receptor
INS	insulin
INSIGF	overlapping region of IGF2 and INS
IUGR	intrauterine growth restriction
IQ	interquartile
KJ	kilojoule
LDL	low density lipoprotein
LEP	leptin
LMM	linear mixed model
MDR	multi drug resistance
MTHFR	methylenetetrahydrofolate reductase
N	number
OR	odds ratio
PAL	physical activity level
p-GP	permeability glycoprotein
CVD	cardiovascular disease
PS	valvular pulmonary stenosis
pVSD	peri-membraneousventricular septal defect
RBC	red blood cell
RR	relative risk
RRR	reduced rank regression
RRR	reduced rank regression
SAH	s-adenosylhomocysteine
SAM	s-adenosylmethionine
SD	standard deviation
SE	standard error
SES	socioeconomic status
SGA	small for gestational age
SPSS	statistical package social sciences
TGA	transposition of the great arteries
tHcy	total homocysteine
TOF	tetralogy of fallot
RNA	ribonucleic acid
Wk	week
Y	year

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Sylvia A. Obermann-Borst is geboren in Velsen op 11 mei 1978, als onverwachte tweeling, acht minuten na zus Sandra. Later kregen zij er nog broertje Jasper bij. Na haar eindexamen Gymnasium in 1996 (Gymnasium Felisenum, Velsen-Zuid) was de juiste studierichting niet direct bepaald. Via "Vroedkunde" in Antwerpen en Nederlandse Taal- en Letterkunde in Leiden, startte zij uiteindelijk in 1998 met de opleiding tot HBO-Verpleegkundige (Hogeschool van Amsterdam, Amsterdam). Een paar dagen na het behalen van haar propedeuse ontmoette ze in 1999 haar man Lambert. Na haar afstuderen in 2002, werkte zij met veel plezier als verpleegkundige op de afdeling Maag-, Darm- en Leverziekten (AMC, Amsterdam). Na een jaar, besloot ze haar droom waar te maken en te streven naar het studeren van Geneeskunde. Na het behalen van de benodigde VWO-certificaten Natuurkunde en Scheikunde, werd zij in 2004 toegelaten tot de opleiding Geneeskunde via de decentrale selectie van het Erasmus MC (EUR, Rotterdam), het doctoraal werd behaald in 2008. Tijdens de doctoraal fase, startte zij ook met de Engelstalige Research Master in Clinical Research (Netherlands Institute for Health and Sciences, Rotterdam). Als onderdeel van deze Research Master volgde zij in 2008 vier cursussen in het "Summer Program" van de Johns Hopkins School of Public Health (Baltimore, MD, USA). Het afstudeeronderzoek werd verricht op de afdeling Gynaecologie en Verloskunde (Erasmus MC) onder supervisie van Prof.dr. Steegers-Theunissen, hoogleraar Periconceptie Epidemiologie. Na het behalen van de Master of Science graad in Clinical Research in 2009, startte zij als promovendus op dezelfde afdeling. In augustus 2010 startte zij met haar co-schappen. Na een succesvol oudste co-schap op de afdeling Gynaecologie en Obstetrie van het Bronovo Ziekenhuis te Den Haag, kon zij in februari 2013 haar artsenbul in ontvangst nemen. Sylvia is getrouwd met Lambert Obermann en woont in Oegstgeest terwijl haar man tijdelijk op Malta werkt voor de Europese Commissie.

Manuscripts related to this thesis

Chapter 2
Obermann-Borst SA, Isaacs A, Younes Z, van Schaik RH, van der Heiden IP, van Duyn CM, Steegers EAP, Steegers-Theunissen RPM. General maternal medication use, folic acid, the MDR1 C3435T polymorphism, and the risk of a child with a congenital heart defect. -American Journal of Obstetrics and Gynecology 2011; 204: e1-8 American Journal of Obstetrics and Gynecology 2011; 204: 236.e1-8.

Chapter 3
Obermann-Borst SA, Vujkovic M, de Vries JH, Wildhagen MF, Looman CW, de Jonge R, Steegers EAP, Steegers-Theunissen RPM. A maternal dietary pattern characterised by fish and seafood in association with the risk of congenital heart defects in the offspring. -British Journal of Obstetrics and Gynecology 2011; 118: 1205-1215.

Chapter 4
Obermann-Borst SA, Ammerlaan DS, de Jonge R, Helbing WA, Steegers EAP, Steegers-Theunissen RPM. Congenital heart defects are associated with elevated triglyceride levels: a case-control study in very young children. -Submitted for publication.

Chapter 5
Obermann-Borst SA, van Driel LM, Helbing WA, de Jonge R, Wildhagen MF, Steegers EAP, Steegers-Theunissen RPM. Congenital heart defects and biomarkers of methylation in children: a case-control study. -European Journal of Clinical Investigation 2011; 41: 143-150.

Chapter 6
Steegers-Theunissen RPM, **Obermann-Borst SA**, Kremer D, Lindemans J, Siebel C, Steegers EAP, Slagboom PE, Heijmans BT. 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:e7845.

Chapter 7
Obermann-Borst SA, Heijmans BT, Eilers PHC, Tobi EW, Steegers EAP, Slagboom PE, RPM Steegers-Theunissen. Periconception maternal smoking and low education are associated with methylation of INSIGF in children at the age of 17 months. DOHaD Journal 2012. June 1.

Chapter 8
Obermann-Borst SA, Eilers P, Tobi E, de Jong F, Slagboom PE, Heijmans BT, RPM Steegers-Theunissen. Duration of breastfeeding and gender are associated with methylation of the LEPTIN gene in very young children. –Pediatric Research 2013. June 11.

Other Manuscripts

Wijnands KPJ, **Obermann-Borst SA**, Sijbrands EJG, Wildhagen MF, Helbing WA, Steegers-Theunissen RPM. The transgenerational impact of cardiovascular disease in (great-) grandparents on the occurrence of congenital heart disease in grandchildren. -Submitted for publication.

Snijder CA, Vlot IJ, Burgdorff A, **Obermann-Borst SA**, Wildhagen MF, Steegers EA, Steegers-Theunissen RPM. Congenital heart defects and parental occupational exposure to endocrine disruptors. Human Reproduction 2012 Feb 22.

PhD Portfolio

Name PhD student:	Sylvia A. Obermann-Borst
Erasmus MC Department:	Obstetrics and Gynaecology
Research School:	NiHeS
Promotoren:	R.P.M. Steegers-Theunissen

PhD training

	Year	Workload (Hours/ECTS)
Specific courses at Erasmus MC		
Repeated Measurements in Clinical Studies	2009	1.4
Advanced Topics in Clinical Trials	2009	1.4
Analysis of Time-varying Exposures	2009	0.7
Advanced Analysis of Prognosis Studies	2009	0.7
Case-control studies	2008	0.7
Demography of Ageing	2008	0.7
Introduction to Clinical Research	2008	0.7
Working with SPSS for Windows	2008	0.15
A first glance at SPSS for Windows	2008	0.15
Introduction to Data-Analysis	2007	0.7
Regression Analysis	2007	1.4
Topics in Meta-analysis	2007	0.7
Survival Analysis	2007	1.4
Modern Statistical Methods	2007	4.3
Advanced Topics in Medical Decision-making	2007	1.4
Prognosis Research	2007	0.7
Intervention Research	2007	0.7
Diagnostic Research	2007	0.7
Study Design	2006	4.3
Principles of Research in Medicine	2006	0.7
Methods of Clinical Research	2006	0.7
Clinical Trials	2006	0.7
Pharmaco-epidemiology	2006	0.7
Topics in Evidence-based Medicine	2006	0.7
Decision Making in Medicine	2006	0.7

PhD training

	Year	Workload (Hours/ECTS)
International training		
At Johns Hopkins School of Public Health, Baltimore, MA,USA		
Infectious Disease Epidemiology	2008	1.4
Epidemiology in Evidence-Based Policy	2008	0.7
Genetic Epidemiology in Populations	2008	0.7
Gene Expression Data Analysis	2008	0.7
Presentations		
2nd European Congress on Preconception Care and Health	2012	0.7
Developmental Origins of Health and Disease (DOHaD): New results and hypotheses'. Symposium Stichting Kind en Groei.	2012	0.7
Epigenetic Epidemiology, Rotterdam/Leiden	2011	0.7
Wladimiroff Research Award Meeting	2010	0.7
Reproduction toxicology meeting	2008	0.7
Posters		
7th World Congress on DoHAD (2), Portland, Oregon, US	2011	0.6
Gynaecongres, Breda, NL	2010	0.3
Society for Gynecologic Investigation (SGI) (3) Orlando, US	2010	0.9
Kennispoort Verloskunde, Utrecht, NL	2009	0.3
6th World Congress on DoHAD (2), Santiago, Chili	2009	0.6
Gynaecongres, Utrecht, NL	2009	0.3
Society for Gynaecologic Investigation (SGI) (2) Glasgow, UK	2009	0.6
DoHAd workshop. The Embryo and its Future. Austria	2008	0.3
(Inter)national conferences		
2nd European Congress on Preconception Care and Health	2012	0.2
Society for Gynecologic Investigation (SGI), Orlando, US	2010	1
Gynaecongres, Breda	2010	0.4
Society for Gynecologic Investigation (SGI). Glasgow, UK	2009	1
Gynaecongres, Utrecht	2009	1
Kennispoort Verloskunde, Utrecht	2009	0.2
Grootstedelijke Perinatale Gezondheid	2009	0.2
Supervising Master's theses		
- Danielle Ammerlaan, EUR, Rotterdam	2009	2
- Emma van Walsem, EUR, Rotterdam	2009	2
- Ingrid Vlot, EUR, Rotterdam	2009	1
- Kim Wijnands, Nihes, Rotterdam	2008-2010	2
Total		50,4

Afterword

Het is zover dat ik mijn dankwoord mag schrijven! Ik kan het bijna niet geloven…

Beste Prof.dr. Steegers-Teunissen, beste Régine, het was in 2006 tijdens mijn 2e studiejaar Geneeskunde dat ik door gynaecoloog Helen Brandenburg bij jou geïntroduceerd werd. Nog steeds ben ik daar dankbaar voor. Voorzichtig raakte ik betrokken bij wetenschappelijk onderzoek en ik was heel blij dat je vervolgens mijn tutor werd voor het Research onderdeel van de Master. Veel dank voor het bieden van de mogelijkheid tot promotieonderzoek! Ik heb zó véél geleerd, zowel professioneel als persoonlijk. Onze overleggen gaven me vaak inspiratie en hernieuwd enthousiasme. Je passie voor de wetenschap, je kennis, creativiteit en energie zijn inspirerend!

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