Early Growth, Cardiovascular and Renal Development

The Generation R Study

Hendrik Robert Taal

Acknowledgments

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The Generation R Study

Vroege groei, de ontwikkeling van het hart, de bloedvaten en de nieren

Het Generation R Onderzoek

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof. Dr. H.G. Schmidt

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Door

Hendrik Robert Taal
Geboren te Den Haag

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Paranimfen	MSc. L.L. de Jonge BSc. A. Mulder

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Manuscripts based on this thesis

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Chapter 3.2

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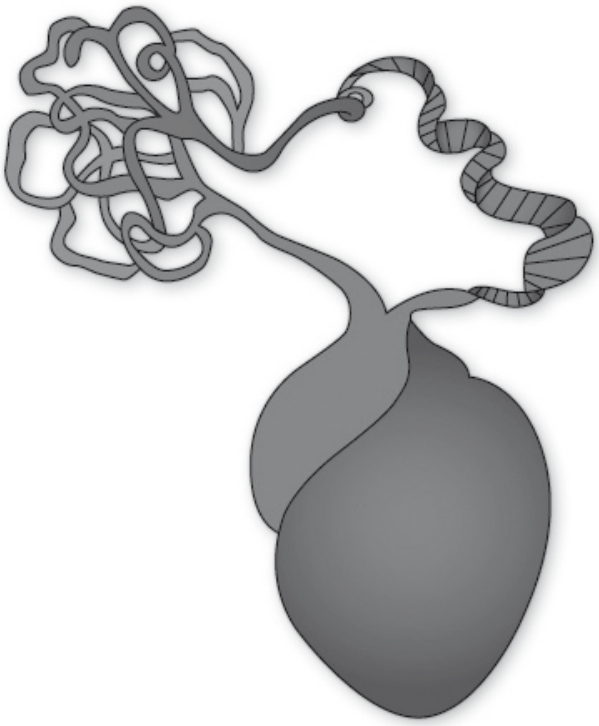
* These authors contributed equally

Chapter 1

Introduction



Introduction

Background

Cardiovascular disease is a major health problem in the adult general population. Epidemiological studies strongly suggest that early life events have an important role for the susceptibility to develop cardiovascular disease in later life. In the 1980's, Barker and Osmond showed that areas of Britain with the highest neonatal mortality rates early in the 20th century also had the highest incidence of cardiovascular disease in adults, many decades later[1-2]. Birth weight is an important predictor of neonatal mortality. After these observations, many epidemiological studies consistently showed that low birth weight is associated with adult health outcomes such as cardiovascular disease[3], type 2 diabetes[4] and kidney disease[5]. It was also noted that the risk of cardiovascular disease was highest in subjects who show a postnatal catch-up growth after being born with a low birth weight[6-7]. These observations resulted in the "fetal origins of adult disease" hypothesis, also currently known as the "Developmental Origins of Health and Disease " Hypothesis (DOHaD-hypothesis). This hypothesis states that a suboptimal fetal environment leads to developmental adaptations that permanently alter growth, physiology and metabolism, with long-term consequences for adult health. More recently, this hypothesis has been adapted to a more general "developmental plasticity hypothesis", which proposes that an organism may develop in different ways, depending on the environment it is exposed to[8]. Investigating specific adverse fetal exposures and early growth may provide new insights in mechanisms underlying the associations of low birth weight with adult disease. Different aspects of early development might be important in determining future risk of adult cardiovascular and renal diseases.

Early growth

Early postnatal growth, especially growth acceleration in infancy, has been suggested to partly explain the increased risk for adult diseases in later life among children with a low birth weight[9]. Children tend to grow according to their genetically determined growth potential. If fetal growth is restricted relative to the fetal growth potential, infant growth acceleration is likely to occur[9]. Many epidemiological studies have indeed shown that growth acceleration in the first months of life is associated with adverse outcomes in adolescence and adulthood, such as obesity and cardiovascular disease[10-12]. Other studies have shown that accelerated growth later in childhood also is important in determining the risk for coronary events, impaired glucose tolerance and type 2 diabetes in adulthood[13-14]. These different findings could be explained by differences between the studies. However, it might also indicate that growth in different periods of life, can affect the risk of adult disease.

Genetics of early growth

Genetic variants associated with early growth might partly explain the association of early growth with adult diseases[15-17]. The 'fetal insulin hypothesis' states that common genetic variants related to type 2 diabetes might partly explain the associations of low birth weight with metabolic diseases in adulthood[15]. Large scale genome-wide association studies have identified many genetic variants associated with adult body mass index[18], blood pressure[19], type 2 diabetes[20] and many other diseases. Genome-wide association studies in children are less abundant, but provide a good opportunity for gene discovery as environmental factors have been present for a relatively short time period. Recently, two genetic loci have been identified to be associated with birth weight, of which one affects insulin secretion and was previously shown to be associated with type 2 diabetes in adults[17]. These results are consistent with the 'fetal insulin hypothesis'. Furthermore, interactions between genetic susceptibility and the (fetal) environment and epigenetic changes are likely to contribute to early origins of adult health and disease[8].

Cardiovascular developmental adaptations

Also, the development of the heart and blood vessels could be affected by adverse fetal exposures or genetic variants. Low birth weight and infant growth are associated with the risks of coronary artery disease, hypertension and other cardiovascular diseases[6-7]. Fetal growth restriction might lead to impaired elastin synthesis in the vessel walls, leading to changes in the mechanical properties of these vessels[21-22]. Also, since the number of cardiomyocytes is established largely in fetal life, early developmental adaptations might lead to remodeling of the heart. Subsequently these cardiovascular adaptations might predispose an individual to higher blood pressure, left ventricular adaptations and increased risk for cardiovascular disease.

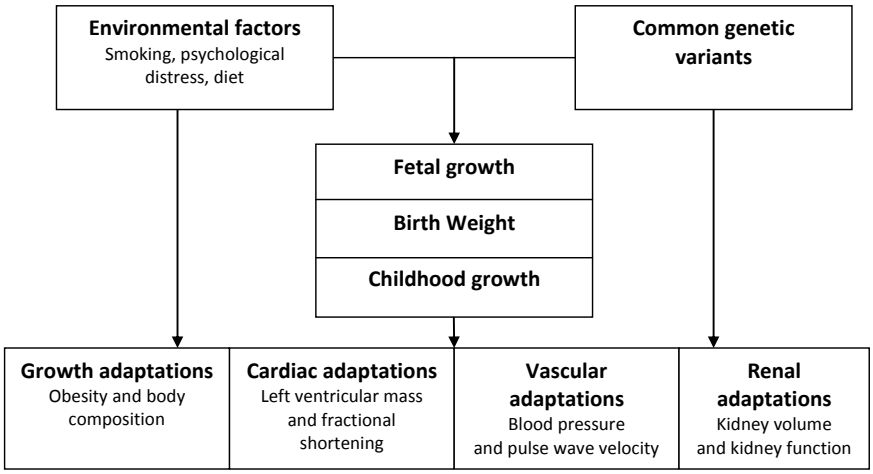
Renal developmental adaptations

Developmental adaptations in response to adverse fetal exposures may also lead to smaller kidneys with a reduced number of nephrons, which in turn leads to glomerular hyperfiltration and sclerosis, predisposing the individual to renal damage and subsequent development of higher blood pressure, impaired kidney function and end stage kidney disease in adulthood[23]. Many studies showed that low birth weight is associated with cardiovascular disease and chronic renal failure[5, 24]. Low birth weight is also associated with impaired renal growth, raised blood pressure, and impaired renal function[5, 25-28].

We designed a prospective cohort study from early fetal life until the age of 6 years to identify specific adverse fetal exposures and genetic determinants underlying the

associations of early growth with obesity, blood pressure and cardiovascular and renal structures and function (figure 1).

Figure 1. Associations studied in this thesis.



Aims

The specific aims of this thesis were:

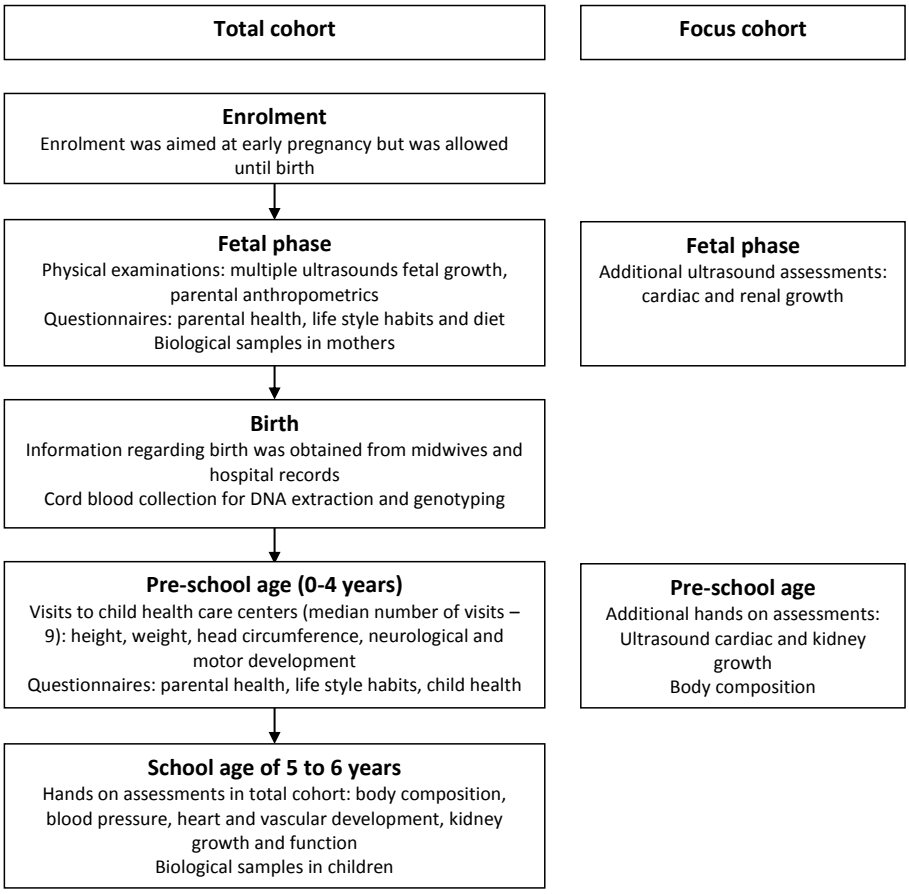
1. To examine which fetal and infant growth patterns and genetic factors are associated with childhood growth and obesity. Outcomes of interest were birth weight, infant head circumference and obesity in childhood.
2. To identify early environmental and genetic determinants related to development of the cardiovascular system in childhood. Outcomes of interest were cardiac structures and function, blood pressure, and arterial stiffness.
3. To identify early environmental and genetic determinants of kidney growth and function. Outcomes of interest were kidney volume, kidney function and blood pressure.

General design

Studies focused on environmental exposures were embedded in the Generation R Study. This is a population based prospective cohort study from fetal life until young adulthood in Rotterdam, the Netherlands[29-30]. The Generation R Study is designed to identify early environmental and genetic causes of normal and abnormal growth, development and health in fetal life, childhood and in later life. Mothers with a delivery date between April 2002 and January 2006 were eligible for enrolment in this study. Enrolment was aimed at early pregnancy, but was possible until the birth of the child. During pregnancy, assess-

ments were planned in early pregnancy (< 18 weeks of gestational age), mid-pregnancy (18-25 weeks of gestational age) and late pregnancy (>25 weeks of gestational age). In total, 9,778 mothers were enrolled in the study. Of these mothers, 91% (n=8,880) was enrolled during pregnancy. In the preschool period, from birth to 4 years of age, data collection was performed by questionnaires and visits to the routine child health care centers at the age of 2, 3, 4, 6, 11, 18, 24, 30, 36 and 48 months. At the age of 5 to 6 years, all participating children were invited to a well-equipped and dedicated research center in the Erasmus Medical Center-Sophia Children's Hospital. The measurements included were focused on several health outcomes including body composition, heart and vascular development, renal growth and obesity. Additionally, detailed assessments of fetal growth and organ development until the age of 2 years were conducted in a randomly selected subgroup of Dutch pregnancy women and their children, the Generation R

Figure 2. Design and data collection in the Generation R Study.



Focus Cohort (n=1,232). These assessments included renal and cardiac ultrasounds in fetal life and early childhood (Figure 2).

GWA studies require large sample sizes in order to increase the statistical power needed to identify common genetic variants associated with a particular trait. Therefore, we performed GWAS in collaboration with other birth cohorts, as part of the **E**arly **G**rowth **G**enetics (EGG) Consortium and **E**arly **Ge**netics and **L**ongitudinal **E**pidemiology (EAGLE) Consortium. The aim of these consortia is to identify genetic variants related to early growth, head circumference, childhood obesity and blood pressure. Furthermore, we also performed on study in the Rotterdam Study[31] and collaborated with the **C**ohorts for **H**eart and **A**geing **R**esearch in **G**enetic **E**pidemiology (CHARGE) Consortium, which aims to identify genetic variants related to adult cardiovascular disease and aging-related phenotypes[32].

Outline of thesis

We performed studies to identify early environmental and genetic determinants of childhood growth, and cardiovascular and renal development. We focused on parental exposures during pregnancy and genetic determinants of childhood growth and blood pressure.

In **chapter 2**, we focus on environmental and genetic determinants of childhood growth. We examined whether size at birth and subsequent infant growth affected the risk of adverse body composition and childhood overweight (**chapter 2.1**). Using GWAS we aimed to identify common genetic variants associated with birth weight (**chapter 2.2**) and infant head circumference (**chapter 2.3**). We also investigated whether a common variant in the nicotine receptor cluster modifies the association of maternal smoking during pregnancy and fetal growth (**chapter 2.4**). Finally, we identified genetic determinants of childhood obesity (**chapter 2.5**).

Chapter 3 focuses on parental exposures during pregnancy, genetic determinants and fetal and infant growth patterns and their association with childhood cardiovascular development. Specifically, we investigated the association of fetal and infant growth with childhood cardiovascular development (**chapter 3.1**). Furthermore, we investigated the effect of parental smoking during pregnancy (**chapter 3.2**), parental distress during pregnancy (**chapter 3.3**) and maternal diet in first trimester in pregnancy (**chapter 3.4**) on childhood blood pressure, carotid-femoral pulse wave velocity and cardiac structures and function. In **chapter 3.5** we investigated whether genetic risk scores incorporating many common genetic variants can better explain variance in blood pressure in both children and adults, as compared to the known genetic variants associated with blood pressure.

Chapter 4.1 presents a study in which we describe normal kidney growth from fetal life until the age of two years. Furthermore, we assessed whether maternal smoking

during pregnancy is associated with fetal and infant kidney volume (**chapter 4.2**). We aimed to identify early life environmental factors associated with kidney volume, kidney function and blood pressure at age of 6 years (**chapter 4.3**). Also, we examined whether common genetic variants previously associated with adult blood pressure and kidney function might affect kidney volume in fetal life (**chapter 4.4**).

Finally, **chapter 5** provides a general discussion of the finding presented in this thesis. We will discuss previous literature, possible underlying mechanisms, new insights from our studies, directions for future studies and possible implications for clinical practice.

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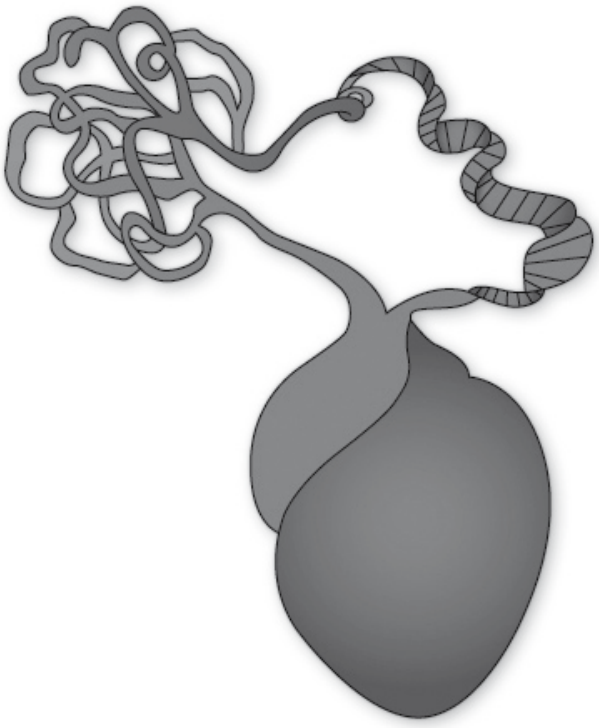
Chapter 2

Early growth and childhood overweight



Chapter 2.1

Early growth in children born small and large size for gestational age at birth and the risk of childhood overweight

H Rob Taal
Albert J van der Heijden
Eric AP Steegers
Albert Hofman
Vincent WV Jaddoe

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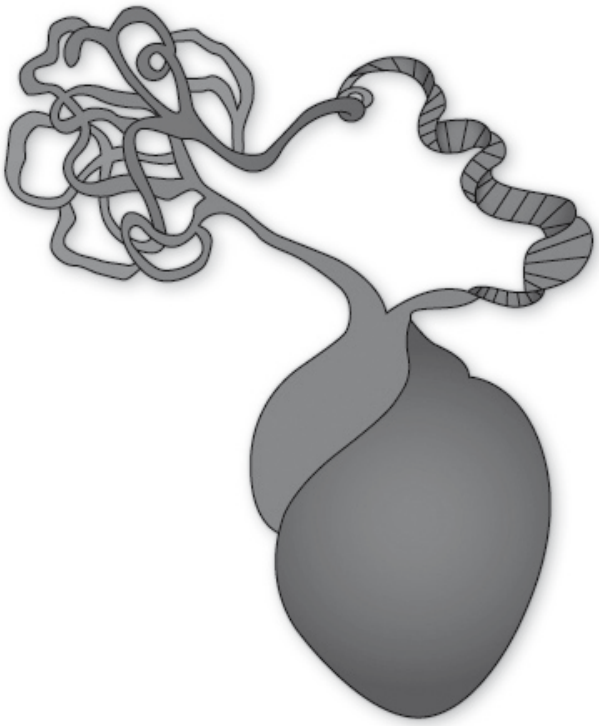


Abstract

Aims: We examined the associations of size at birth, with infant growth of head circumference, length and weight, and fat mass and body mass index in preschool children.
Methods: In a population-based prospective cohort study among 3,941 children, we repeatedly measured head circumference, length and weight until the age of 4 years. Catch-up and catch-down growth were defined as a change in standard deviation scores of >0.67 from birth to two years of age.
Results: Although most children born small and large size for gestational age showed infant catch-up and catch-down growth, respectively, their mean head circumference, length and weight remained smaller and larger respectively, until the age of 4 years. Catch-up growth in children with a small and appropriate weight for gestational age and lack of catch-down growth in children born with a large weight for gestational age were associated with higher body mass index in preschool children. Children born with an appropriate weight for gestational age with catch-up growth and children born with a large weight for gestational without catch-down growth had increased risks of childhood overweight (odds ratios: 3.11 (95% CI: 2.37, 4.08) and 12.46 (95% CI: 6.07, 25.58) respectively).
Conclusions: Children born small, appropriate and large size for gestational age have different growth patterns in early childhood and persistent differences in their head circumference, length and weight until the age of 4 years. Children born with an appropriate weight for gestational age with catch-up growth and large weight for gestational children without catch-down growth have an increased risk of overweight.

Introduction

Both a small and large size for gestational age at birth, referred to as SGA and LGA respectively, are associated with increased risks of neonatal morbidity and mortality[1-2]. In infancy, children born SGA and LGA, show growth realignment to their genetic growth potential. Approximately 80% of all SGA children show catch-up growth during the first 2 years of life[3-5]. The proportion and timing of catch-down growth in children born LGA has been studied less extensively. Also, not much is known about specific growth patterns and the time windows for growth realignment in infants born SGA and LGA for different growth parameters such as head circumference, length and weight. It has been suggested that both SGA and LGA are associated with the risk of obesity in later life[6-9]. This association may partly depend on higher growth rates in early childhood[10-15]. The interaction between size at birth and infant catch-up or catch-down growth in relation to body composition and the risk of overweight in early life is not well known.

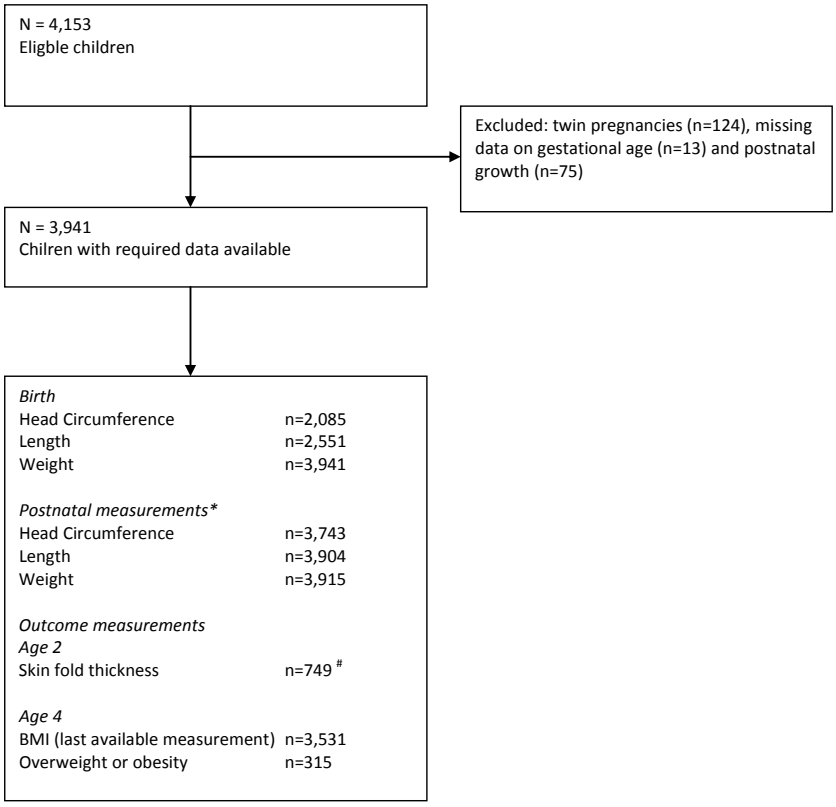
We examined in a population-based prospective cohort study among 3,941 Duch children of whom 191 were SGA and 199 were LGA for birth weight, growth of the head circumference, length and weight during the preschool period. We also examined whether infant catch-up and catch-down growth in these children are associated with body fat mass and the risk of overweight.

Methods

Study population and cohort

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood in Rotterdam, The Netherlands[16]. Enrolment in the study was aimed at early pregnancy but was possible until birth of the child. All children were born between April 2002 and January 2006[16]. Written informed consent was obtained from all participants. The Medical Ethics Committee of the Erasmus Medical Center Rotterdam has approved the study. The analyses were restricted to 4,153 Dutch children (Figure 1), defined as having both parents born in the Netherlands. Twins (n=124) were excluded from the analysis. Of the remaining 4,029 children, data on gestational age was available in 4,016 (99.7%). Data on weight, head circumference and length at birth was available in 4,016 (100%), 2,115 (52.7%) and 2,590 (64.5%) of these children, respectively. Data on postnatal growth (0-4 years) was available in 3,941 children (98.2%). In total, 1,171 of the 4,016 children participated in a sub cohort study for additional assessments of body composition[16]. At two years of age, 835 (71.3%) of these children and their parents visited our research center for additional measurements.

Figure 1. Study population – Flowchart



* At least one measurement available until the four years of age

Skin fold thickness was measured in a subgroup of children at 24 months of age

Birth characteristics and definition of small and large size for gestational age

Information about sex, gestational age, head circumference, length and weight at birth was available from midwife and obstetrics registries. We created sex-and gestational age-adjusted birth head circumference, length and weight standard deviation scores (SD scores) within our study population using Growth Analyser 3.5 (http://www.growth-analyser.org; Dutch Growth Research Foundation, Rotterdam, the Netherlands). The reference was a North European cohort[17]. We defined SGA as being <5th sex specific percentile and LGA >95th sex specific percentile for head circumference, length and weight at birth.

Growth characteristics 0-4 years

Anthropometrics were measured by well-trained staff in community health centers using standardized procedures at the ages of 2, 3, 4, 6, 11, 14, 18, 24, 30, 36 and 48 months[16]. The median number of visits to the health care centers was 9 (90% range: 3 – 12). Head circumference was measured to the nearest millimeter with a standardized tape (SECA, Hamburg, Germany) until the age of 12 months. Length was measured in a supine position to the nearest millimeter until the age of 12 months with a neonatometer, after which height was measured in standing position with a Harpenden stadiometer (Holtain Ltd, Dyfed, United Kingdom). Weight was measured with a mechanical personal scale and body mass index (BMI) was calculated (kg/m^2). Sex- and age-adjusted SD scores were constructed using Growth Analyser 3.5. We used the reference curve for height, weight, head circumference and BMI in the Netherlands to establish SD scores[18]. Catch-up and catch-down growth for weight, were defined as an increase or decrease of >0.67 SD of weight from birth to two years of age, respectively[9]. If weight at 24 months of age was not available (18.9% of study population), we used weight at 11 months, and if also not available (7.6%), weight at 6 months to assess catch-up or catch-down growth. This change represents the width of each percentile band on standard growth charts.

Subcutaneous fat mass

Subcutaneous fat mass was measured as skin fold thicknesses (SFT), at the age of 24 months on the left side of the body at four different sites (biceps, triceps, suprailiacal and subscapular) according to standard procedures by using a skinfold caliper (Slim Guide, Creative Health Products). Measurements of skin fold thickness were available in 749 (89.7%) children participating in the subgroup study. Missing skin fold thickness measurements were mostly due to crying and oppositional behaviour. Four well-trained medical assistants performed all measurements and measured SFT to the closest 0.5 mm[19]. Total subcutaneous fat mass was calculated from the sum of biceps SFT + triceps SFT + suprailliacal SFT + subscapular SFT. Central subcutaneous fat mass was calculated from the sum of suprailliacal SFT + subscapular SFT. Peripheral subcutaneous fat mass was calculated from the sum of triceps SFT + biceps SFT.

Overweight and obesity

We calculated the BMI using weight and length measured at the last visit available after the age of two years (median age 45.3 (90% range: 25.3-47.7) months). We constructed sex and age specific SD scores of the BMI measurements using Growth Analyser 3.5. Based on these SD scores prevalence of overweight and obesity was determined. Overweight and obesity were defined as described by Cole et al. as recommended by The International Obesity Taskforce[20], which created centile curves from different popula-

tions to create age and sex specific cut off points from 2 to 18 years, which correspond to overweight (> 25kg/m^2) and obesity (>30 kg/m^2) at 18 years of age.

Covariates

Maternal height was measured at enrolment. Information on parity, maternal pre-pregnancy weight, educational level and smoking during pregnancy (non/stopped when pregnancy was known/continued during pregnancy) was assessed using self-reported questionnaires. Child age at the visits to the child health care centers was collected from the staff of these centers.

Data analysis

Differences in maternal and offspring characteristics between SGA, appropriate for gestational age (AGA) and LGA groups were assessed using t-tests and χ^2-tests for independent samples. We used linear regression analysis to assess the associations of being SGA or LGA for birth weight with growth realignment (change in weight SD scores in specific time windows). AGA individuals were used as reference. The models were adjusted for the individual age period between the measurements. Similar models were used for assessing the associations of SGA and LGA for length or head circumference with subsequent change in length or head circumference SD scores per time window. The associations of being SGA, AGA and LGA for birth weight, stratified for infant catch-up or catch-down growth, with subcutaneous fat mass at the age of 2 years, BMI at the age of 4 years and the risk of overweight and/or obesity were assessed using linear and logistic regression analyses. In these analyses, we used children born AGA without catch-up or catch-down growth as reference category. Analyses concerning body composition were additionally adjusted for age and height at measurement. All models were adjusted for child sex and maternal age, height, pre-pregnancy weight, parity, educational level and smoking during pregnancy. Missing values in covariates (ranging from 0 to 22%), were multiple-imputed, to reduce potential bias associated with missing data [21]. We created five imputed datasets and each dataset was analyzed separately to obtain the effect sizes and standard errors. The results of all five imputed analyses were pooled and are presented in this paper. Because the main results did not differ materially between analyses on complete cases and imputed analyses, we only present data on imputed analyses. We performed sensitivity analyses excluding preterm born children (gestational age at birth <37 weeks). Furthermore, we performed sensitivity analyses excluding children without weight measurement available at the age of 24 months to establish the postnatal growth pattern. All measures of association are presented with their 95% confidence intervals (95% CI). We considered a P-value lower than 0.05 as statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics are shown in Table 1. The percentage of boys was 50.4%. Mothers of children born SGA group were smaller, less heavy and younger than mothers from children born AGA or LGA. They also were lower educated, smoked more often during pregnancy and were less frequently multiparous.

Table 1. Subject characteristics

	Total group (n =3,941)	**Children born small size for gestational age (weight) (n=191)**	**Children born appropriate size for gestational age (weight) (n=3,551)**	**Children born large size for gestational age (weight) (n=199)**
Maternal characteristics				
Age (yrs)	31.8 (22.7-38.5)	30.6 (21.2-38.6) *	31.8 (22.6-38.5)	33.0 (26.1-40.3) **
Height (cm)	170.5 (6.5)	167.1 (6.3) **	170.5 (6.4)	174.2 (6.3) **
Pre-pregnancy weight (kg)	67.3 (11.8)	62.8 (11.9) **	67.1 (11.4)	75.3 (14.7) **
Parity ≥ 1 (%)	40.6	23.7 **	40.2	64.6 **
Highest education (%)				
Primary/Secondary school	40.0	51.1 *	40.1	29.6 *
Higher education	60.0	48.9 *	59.9	70.4 *
Smoking during pregnancy (%)	14.4	34.5 **	13.7	4.5 *
Child characteristics at birth				
Gestational age	39.9 (37.0-42.1)	39.7 (36.9-42.0)	39.9 (37.1-42.1)	40.0 (37.0-42.0)
Male (%)	50.4	49.7	50.4	50.8
Weight (gr)	3497 (551)	2559 (365) **	3491 (467)	4501 (382) **
Length (cm)	50.5 (2.4)	47.3 (2.3) **	50.5 (2.2)	53.4 (2.1) **
Head circumference (cm)	34.0 (1.6)	32.3 (1.3) **	34.0 (1.6)	35.8 (1.4) **
Preterm birth (%)	4.4	4.7	4.4	4.0

* = p<0.05

** = p<0.001

P-values are obtained from t-tests and χ^2-tests for independent samples using the appropriate for gestational age children as reference group. Values are means (SD) or medians (95% range)

Size at birth and infant growth

Table 2 shows the change in SD scores for head circumference, length and weight in different time periods for children born SGA, AGA, and LGA children. Children who were born SGA showed substantial growth realignment for all growth characteristics, as compared to AGA children (p-value <0.001). For head circumference and length, the realignment largely occurred during the first 3 months of life, while for weight realignment was present until the age of 36 months. We observed similar patterns for children born LGA.

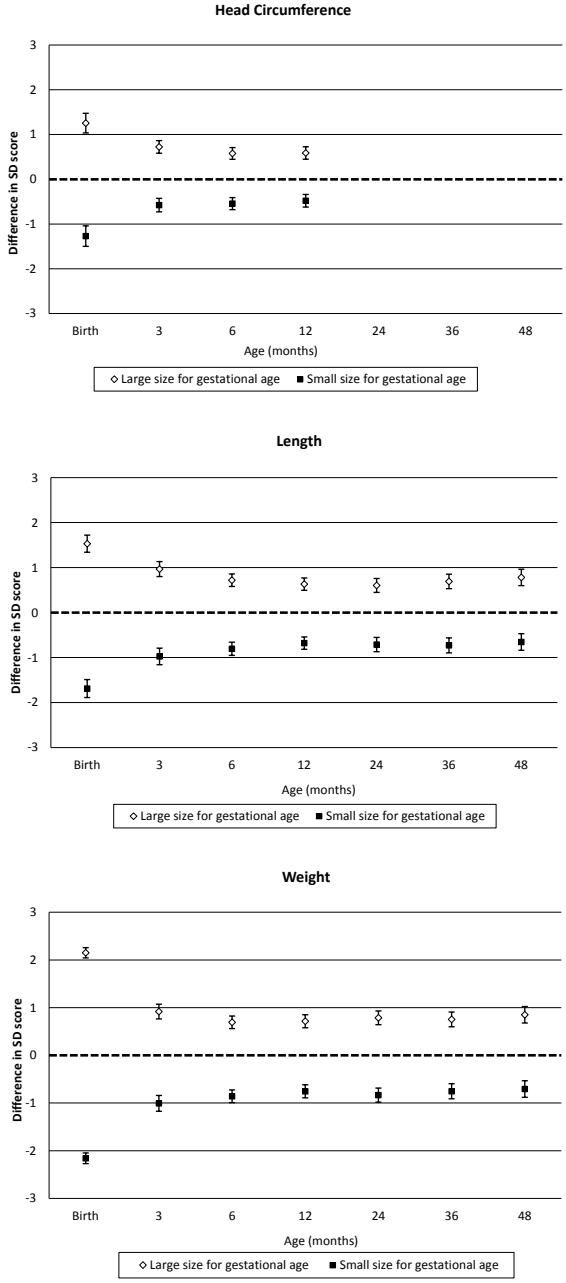
Table 2. Size at birth and growth realignment in childhood

	Age window					
	0-3 months	**3-6 months**	**6-12 months**	**12-24 months**	**24-36 months**	**36-48 months**
Head circumference at birth	**Change in standard deviation score of head circumference per age window**					
Small size for gestational age (n=105)	1.86 (1.63 , 2.09)**	0.09 (-0.04 , 0.20)	-0.11 (-0.23 , 0.01)	*NA*	*NA*	*NA*
Appropriate size for gestational age (n=1,905)	Reference	Reference	Reference	Reference	Reference	Reference
Large for gestational age (n=105)	-1.62 (-1.87 , -1.37)**	-0.02 (-0.15 , 0.12)	-0.08 (-0.20 , 0.04)	*NA*	*NA*	*NA*
Length at birth	**Change in standard deviation score in length per age window**					
Small size for gestational age (n=127)	1.75 (1.53 , 1.97)**	-0.03 (-0.18 , 0.11)	0.19 (0.07 , 0.31)*	0.04 (-0.11 , 0.18)	-0.03 (-0.14 , 0.08)	0.05 (-0.04 , 0.14)
Appropriate size for gestational age (n=2,331)	Reference	Reference	Reference	Reference	Reference	Reference
Large size for gestational age (n=132)	-1.65 (-1.88 , -1.43)**	-0.11 (-0.26 , 0.04)	-0.06 (-0.18 , 0.06)	0.04 (-0.10 , 0.18)	-0.04 (-0.15 , 0.07)	-0.01 (-0.10 , 0.08)
Weight at birth	**Change in standard deviation score in weight per age window**					
Small size for gestational age (n=191)	1.12 (0.96 , 1.29)**	0.24 (0.14 , 0.33)**	0.15 (0.07 , 0.24)**	-0.09 (-0.19, 0.01)	0.08 (0.01 , 0.16)*	-0.01 (-0.08 , 0.07)
Appropriate size for gestational age (n=3,551)	Reference	Reference	Reference	Reference	Reference	Reference
Large size for gestational age (n=199)	-1.25 (-1.41 , -1.10)**	-0.18 (-0.27 , -0.09)**	0.04 (-0.05 , 0.12)	0.01 (-0.09 , 0.10)	-0.04 (-0.12 , 0.04)	0.01 (-0.07 , 0.08)

* P <0.05, ** P < 0.001. Values are regression coefficients (95% confidence interval) and reflect the difference in SDS change in specific time windows, being small or large for gestational age, compared to being appropriate for gestational age. All regression models are additionally adjusted for the exact age span between the measurements.

Figure 2a, b and c show the differences in head circumference, length and weight SD scores until the age of 4 years between children born SGA, AGA and LGA for birth weight. Head circumference showed the most rapid catch-up growth, and the difference with AGA children was the smallest, as compared to the length or weight. The difference between SGA or LGA and AGA remained significant for all growth measures until the age of 4 years (p-value <0.001).

Figure 2. Birth weight status and postnatal growth



Associations between children born small size for gestational age and large size for gestational age and repeatedly measured postnatal growth characteristics (SD scores), compared to children born appropriate size for gestational age (dotted line, reference). Values are regression coefficients (95% CI).

a Head circumference

b Length

c Weight

Weight at birth, infant growth and subcutaneous fat mass

Table 3 shows the associations of weight at birth, and subsequent catch-up or catch-down growth with subcutaneous fat mass at the age of 2 years. As compared to children born AGA without catch-up or catch-down growth, children born SGA without catch-up growth and children born LGA without catch-down growth had the lowest and highest peripheral, central and total subcutaneous fat mass (differences for total subcutaneous fat mass -4.91 mm (95% CI: -11.91, 2.09) and 8.95 mm (95% CI: 3.93 , 13.97) respectively). Among children born AGA, infant catch-up or catch-down growth was associated with an increased or decreased total subcutaneous fat mass respectively (differences for total subcutaneous fat mass -2.84 mm (95% CI: -4.15, 1.53) and 3.50 mm (95% CI: 2.02 , 4.97) respectively) . Results of sensitivity analyses excluding preterm born children (Supplementary Table 1) and excluding children without weight measured at 24 months of age (Supplementary Table 2) showed similar associations.

Table 3. Weight at birth, infant growth and subcutaneous fats mass at the age of 2 years

Birth Weight	Peripheral fat mass (mm) (Beta (95%CI))	Central fat mass (mm) (Beta (95%CI))	Total fat mass (mm) (Beta (95%CI))
Small for gestational age			
No catch-up (n=4)	-2.35 (-6.98 , 2.27)	-2.58 (-5.91 , 0.76)	-4.91 (-11.91 , 2.09)
With catch-up (n=26)	-0.15 (-2.11 , 1.80)	-0.11 (-1.52 , 1.30)	-0.26 (-3.22 , 2.70)
P-value for difference within stratum [a]	*0.227*	*0.054*	*0.084*
Appropriate for gestational age			
With catch-down (n=174)	-1.51 (-2.37 , -0.65) **	-1.33 (-1.95 , -0.71) **	-2.84 (-4.15 , -1.53) **
No catch-up/down (n=367)	Reference	Reference	Reference
With catch-up (n=133)	1.47 (0.51 , 2.44) *	1.95 (1.25 , 2.76) **	3.50 (2.02 , 4.97) **
Large for gestational age			
With catch-down (n=22)	1.21 (-0.87 , 3.31)	1.03 (-0.51 , 2.57)	2.05 (-1.18 , 5.28)
No catch-down (n=8)	4.55 (1.24 , 7.87) *	4.42 (2.02 , 6.81) **	8.95 (3.93 , 13.97) **
P-value for difference within stratum [a]	*0.560*	*0.021*	*0.086*

* P <0.05, ** P < 0.001. Models were adjusted for child sex, age and height at measurement, maternal age, height, pre-pregnancy weight, parity, educational level and smoking during pregnancy. Values are regression coefficients (95% CI) and reflect the difference in skin fold thickness (millimeters) at the age of two years for different birth weight strata and postnatal catch-up or catch-down growth. AGA children without catch-up or catch-down growth were used as reference group. Catch-up or catch-down growth was defined as have a change in SDSweight of > 0.67 or < -0.67 from birth to two years of age respectively.

[a] The p-value for difference was obtained by conducting a linear regression analysis within each stratum assessing the difference between the growth patterns within the stratum.

Weight at birth, infant growth and risk of overweight

Table 4 shows that as compared to children born AGA without catch-up or down growth, BMI was lowest among children born SGA without catch-up growth (difference -0.95 SD (95% CI: -1.21, -0.70)) and highest among children born LGA without catch-down growth (difference 1.06 SD (95% CI: 0.77, 1.36)). Within each stratum of birth weight, infant catch-up or catch-down growth was strongly associated with body mass index (P-value for difference in each stratum <0.001). As compared to children born AGA without growth realignment, catch-up growth in children born AGA and lack of catch-down growth in children born LGA were associated with increased risks of childhood overweight (odds ratios: 3.11 (95% CI: 2.37, 4.08) and 12.46 (95% CI: 6.07, 25.58) respectively). Results of sensitivity analyses excluding preterm born children (Supplementary Table 3) and excluding children without weight measured at 24 months of age (Supplementary Table 4) showed similar associations.

Table 4. Weight at birth, infant growth and risk of overweight and obesity

Birth Weight	Body mass index (SD score) (Beta (95%CI))	Overweight/Obesity (Odds Ratio (95% CI))
Small for gestational age		
No catch-up (n=45)	-0.95 (-1.21 , -0.70) **	0.28 (0.04 , 2.07)
With catch-up (n=119)	-0.23 (-0.39 , -0.07) *	0.92 (0.43 , 1.96)
P-value for difference within stratum [a]	*<0.001*	*0.224*
Appropriate for gestational age		
With catch-down (n=840)	-0.42 (-0.49 , -0.34) **	0.31 (0.19 , 0.50) **
No catch-up/down (n=1,624)	Reference	Reference
With catch-up (n=697)	0.44 (0.37 , 0.52) **	3.11 (2.37 , 4.08) **
Large for gestational age		
With catch-down (n=135)	0.24 (0.09 , 0.40) *	1.39 (0.75 , 2.59)
No catch-down (n=34)	1.06 (0.77 , 1.36) **	12.46 (6.07 , 25.58) **
P-value for difference within stratum [a]	*<0.001*	*<0.001*

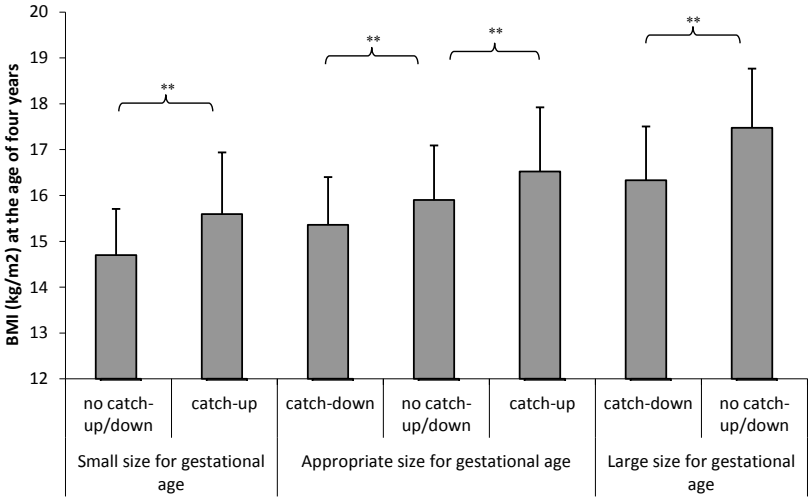
* P <0.05, ** P < 0.001

Models were adjusted for maternal age, height, pre-pregnancy weight, parity, educational level and smoking during pregnancy. Values are regression coefficients (95% CI) and reflect the difference in body mass index SD score for body mass index measured at the last visit available after the age of two years, and odds ratio's (95% CI) reflecting the difference in risk for overweight and/or obesity at the last measurement available after the age of two years, for different birth weight strata and postnatal catch-up or catch-down growth. Children with an appropriate weight for gestational age without catch-up or catch-down growth were used as reference group. Catch-up or catch-down growth was defined as have a change in weight SDS of > 0.67 or < -0.67 from birth to two years of age respectively.

[a] The p-value for difference was obtained by conducting a linear regression analysis within each stratum assessing the difference between the growth patterns within the stratum.

Figure 3 shows the BMI around the age of four years in different birth weight groups stratified by infant growth patterns. The prevalence of overweight or obesity was highest (48.5%) in LGA children who did not show catch-down growth.

Figure 3. Body mass index at the age of four years in different strata of birth weight and infant growth patterns



* P <0.05, ** P<0.001

Bars represent BMI (kg/m^2) and SD at the age of four years (median age 45.3 (90% range: 25.3-47.7) months) by birth weight status and infant growth pattern. The p-value for difference was obtained by conducting an independent samples t-test or within each stratum using the group without catch-up or catch-down growth as reference.

Discussion

Our results indicate that although children born SGA and LGA show infant catch-up and catch-down growth, respectively, their mean head circumference, length and weight are persistently different at the age of 4 years. Children born AGA with subsequent catch-up growth and children born LGA have a higher subcutaneous fat mass and body mass index in childhood.

Strength and limitations

Many studies have investigated the associations of catch-up growth and overweight in later ages in children born SGA[10, 14-15]. However, studies in children born LGA are less abundant and mostly did not study the effect of catch-down growth. Our analyses were performed in a large sample and data were prospectively collected for a large number of covariates, limiting selection and reporting bias. The median number of available

measurements per group of birth weight stratified by infant growth was largely the same and varied between 9 and 11. A limitation might be that for a small number of participants (1.8%) we did not have any postnatal growth measurements available. Birth weight of these children was lower than of children with postnatal growth measurements available (-0.33 SD (95% CI -0.57, -0.10), P=0.005). Our results would be biased if the associations of birth weight, infant growth and risk of overweight would differ between subjects included and lost to follow-up. The impact of this loss to follow-up is difficult to evaluate, but is unlikely to materially affect our results. Another limitation might be that this study was conducted within children from Dutch mothers. Therefore, generalizability to other ethnic groups might be limited. We did not exclude preterm born children, to increase generalizability to the whole range of SGA and LGA children.

Size at birth and infant growth

We observed that catch-up and catch-down growth of weight from birth to the age of 2 years occurred in 74.3% of all children born SGA and in 80.8% of all children born LGA in birth weight, respectively. Of these children, 89.9% and 86.4% showed catch-up or catch-down growth in the first six months of life respectively. The majority of the growth realignment was seen in the first three months of age for head circumference and length, while weight showed catch-up growth over a longer period of time. Our results also showed that despite catch-up or catch-down growth, children born SGA and LGA have a persistently smaller or larger head circumference, height and weight until the age of four years, compared to children born AGA. Previous studies showed similar persistent differences in length and weight after being born small or large for gestational age until the age of eight to eighteen years[3, 22-24]. Data on head circumference growth is limited[22]. The mechanism underlying these differences in growth patterns for head circumference, length and weight are not known. However, the smaller effects on head circumference at birth and faster growth realignment are in line with the relative brain sparing in children with a compromised fetal growth.

Weight at birth, infant weight growth and body composition

Previous studies have shown that catch-up growth is associated with an increased fat mass measured at different ages[5, 9, 25-26]. We explored whether these associations were present in all birth weight groups with different postnatal growth patterns. Children born LGA without catch-down growth had an increased fat mass at two years of age, while children born SGA had a lower fat mass. Hediger et al. studied 3192 children in the United States and showed that in children born LGA, the increase in fat mass was not yet observed at the age of three years, but only present at the age of six years[26]. This study did not investigate the possible modifying effect of infant growth on body composition. The majority of the children born LGA children show catch-down growth

which could be a reason that an increased fat mass is not detected when investigating LGA children irrespective of their infant growth. Our results also suggest that among all birth weight groups, infant catch-up growth is associated with higher fat mass. Longer follow-up studies are needed to evaluate whether these changes persist at later ages.

Weight at birth, infant weight growth and risks of overweight

The association of between birth weight, infant growth and obesity in childhood and adulthood is complex and has been studied extensively. Stettler et al. showed that rapid weight gain in the first four months after birth was associated with overweight at the age of seven years across the whole range of birth weight, independent of the weight attained at one year of age[27]. Several other studies have shown that catch-up growth in children born SGA is associated with a higher risk of obesity in adulthood[10-12]. In our study, this effect was not yet found in children born SGA, indicating that the increase risk of obesity develops later in life and is not yet seen at this young age. However, we did find that within children born SGA, BMI at four years of age is increased when they show catch-up growth. Catch-up and catch-down growth in children born AGA was associated with an increased or decreased risk of overweight respectively. This confirms the findings of by Ong et al. showing that children with catch-down growth in infancy have a lower BMI at the age of 5 years[9].

Children born LGA are less extensively studied. The study of Parsons et al. using data from the 1958 Birth Cohort found the association between birth weight and BMI in adulthood to be J-shaped[28]. Children in the lower ranges of birth weight in early life tended to show rapid weight gain in early life, which ultimately may lead to obesity in adulthood. Children in the upper ranges of birth weight also had an increased BMI in adulthood, showing signs of tracking[28]. Our study showed that children born LGA have an increased BMI in early childhood. Furthermore, among children born LGA, the risk of childhood overweight is modified by infant catch-down growth, with the highest risk of being overweight when not showing catch-down growth. This is in line with the study by Bueno et al. among 116 children born LGA, showing that catch-down growth was associated with a lower BMI and abdominal circumference at the age of 23-25 years[29]. Longer follow-up in this study population and other populations is needed to assess whether these associations persist and other associations develop in adolescence and adulthood.

Conclusions

In this study, we demonstrated that although children born SGA and LGA show infant catch-up and catch-down growth, respectively, their mean head circumference, length and weight are persistently different at the age of 4 years. Children born AGA with catch-up growth and children born LGA without catch-down growth have a higher subcutaneous fat mass and body mass index in childhood, and are at increased risk for childhood overweight. Results from our study suggest that these groups could be a potential target for early prevention of childhood obesity.

Note: Supplementary information is available on the Obesity website: *www.nature.com/oby*

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Chapter 2.2

A genome-wide association meta-analysis on birth weight

Momoko Hirokoshi *
Hanieh Yagootkar*
Dennis O Mook-Kanamori *
Ulla Sovio
H Rob Taal et al.
For the Early Growth Genetics (EGG) Consortium

**Authors contributed equally to this work*
For the complete list of authors please see list of publications
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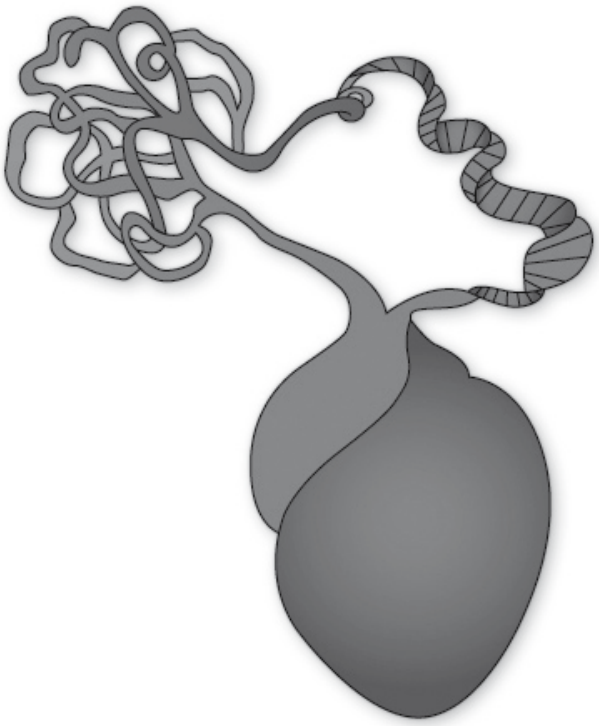


Abstract

Aims and methods: In a genome-wide association study of birth weight (up to 69,308 individuals of European descent from 43 studies), we have extended the number of genome-wide significant loci to seven, accounting for a similar proportion of variance to maternal smoking.
Results and conclusions: Five of the loci are known to be associated with other phenotypes: *ADCY5* and *CDKAL1* with type 2 diabetes; *ADRB1* with adult blood pressure; and *HMGA2* and *LCORL* with adult height. Our findings highlight genetic links between fetal growth and postnatal growth and metabolism.

Introduction

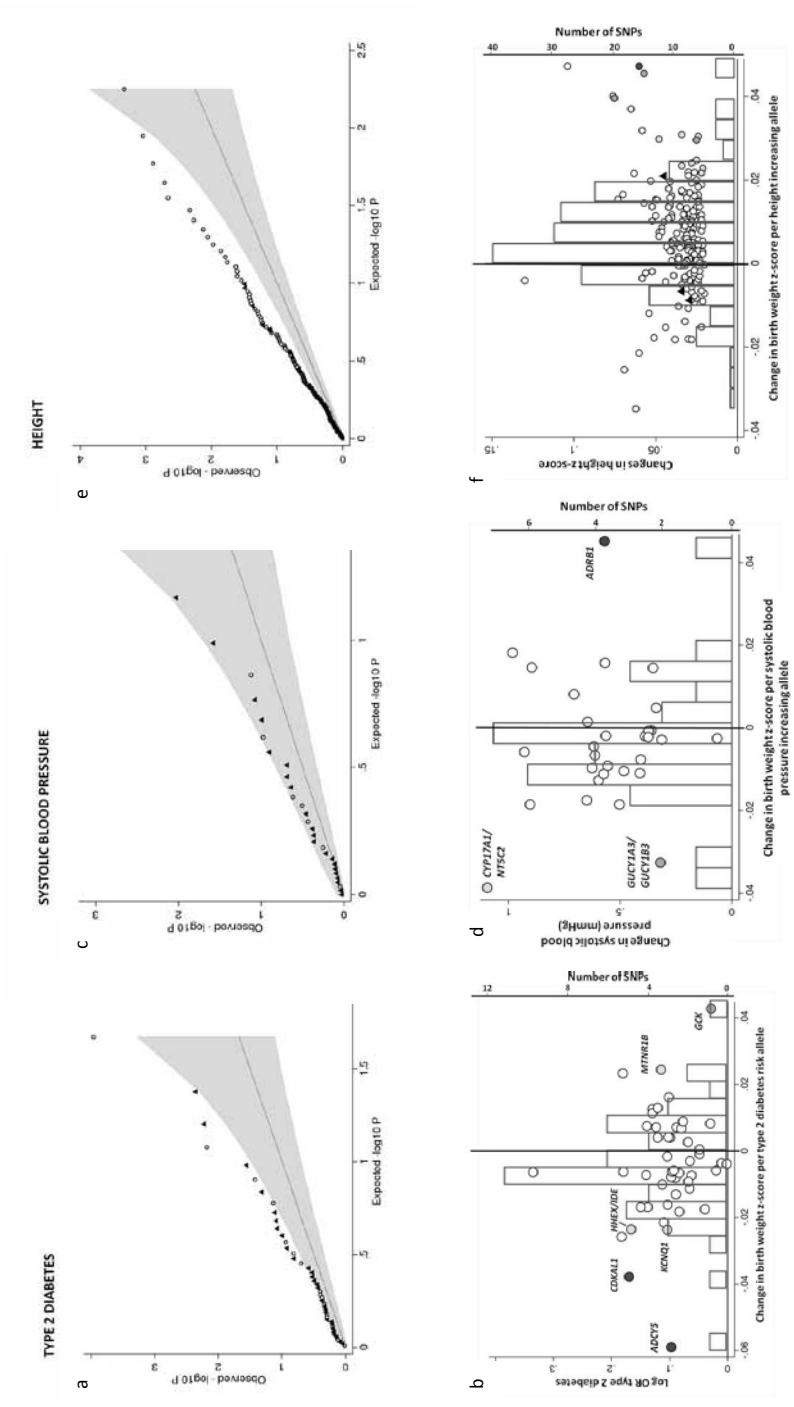
Birth weight within the normal range is associated with a variety of adult-onset diseases but the mechanisms behind these associations are poorly understood[1]. Previous genome-wide association studies identified a variant in the *ADCY5* gene associated with both birth weight and type 2 diabetes, and a second variant, near *CCNL1*, with no obvious link to adult traits[2].

Methods and results

To understand further the genetic factors involved in fetal growth and its association with adult diseases, we performed an expanded genome wide association study (GWAS) of birth weight in up to 26,836 individuals of European ancestry from 18 studies (Stage 1; Supplementary Table 1; Supplementary Figures 1 to 3; see Supplementary Online Material for a full description of the methods). After follow-up analyses of 21 of the most strongly associated independent single nucleotide polymorphisms (SNPs; $P < 1x10^{-5}$) in additional European samples (Supplementary Tables 2 and 3), we identified novel associations with birth weight at four loci ($P < 5x10^{-8}$), and confirmed three previously reported associations[2-4] (rs900400 near *CCNL1*, $P = 3.6x10^{-38}$; rs9883204 in *ADCY5*, $P = 5.5x10^{-20}$; rs6931514 in *CDKAL1*; $P = 1.5x10^{-18}$), in a joint meta-analysis of up to 69,308 individuals (Table 1; Supplementary Figures 4 and 5). The index SNPs at the four newly-associated loci were rs1042725 in *HMGA2* ($P = 1.4x10^{-19}$), rs724577 in *LCORL* ($P = 4.6x10^{-11}$), rs1801253 in *ADRB1* ($P = 3.6x10^{-9}$) and rs4432842 at chromosome 5q11.2 ($P = 4.6x10^{-8}$). The effect size estimates range from 0.034 SD to 0.072 SD per allele and equate approximately to changes in birth weight of 16 to 35g (Table 1). These estimates did not change materially in sensitivity analyses excluding studies with self- or parentally-reported birth weight data and those without a measure of gestational age (Supplementary Table 4).

Through the cellular mechanisms of gametogenesis and fertilization, fetal genotype is correlated with maternal genotype ($r \approx 0.5$). Using up to 11,307 mother-child pairs from a subset of studies, we found no evidence that the seven associations we observed at $P < 5x10^{-8}$ are driven by the maternal, rather than the fetal, genotype (likelihood-ratio test $P > 0.05$; Table 1).

Figure 1.


TYPE 2 DIABETES
HEIGHT
SYSTOLIC BLOOD PRESSURE
Observed - log10 P
Expected -log10 P
Number of SNPs
Change in birth weight z-score per height increasing allele
Change in birth weight z-score per systolic blood pressure increasing allele
Change in birth weight z-score per type 2 diabetes risk allele
Log OR type 2 diabetes
Change in systolic blood pressure (mmHg)
Change in height z-score
ADCY5
CDKAL1
HHEX/IDE
KCNJ11
MTNR1B
GCK
CYP17A1/ NT5C2
GUCY1A3/ GUCY1B3
ADRB1

Associations between birth weight and known type 2 diabetes (T2D; a and b), systolic blood pressure (SBP, c and d) or height (e and f) loci from the discovery meta-analysis of N=26,836 individuals. Plots a, c and e are quantile-quantile plots: the black triangles (associated with lower birth weight) and circles (associated with higher birth weight) represent observed *P*-values after removing the loci that achieved $P < 5x10^{-8}$ in the overall meta-analysis, and the black line represents expected *P*-values under the null. The grey area defines the approximate 95% confidence interval around the expected line. Plots b, d and f show, respectively, the T2D, SBP or height effect size (left-hand y-axis), taken from published meta-analyses[13, 19-21], against the birth weight effect size (x-axis), with a superimposed frequency histogram showing the number of SNPs in each category of birth weight effect size (right-hand y-axis). The odds ratios for type 2 diabetes are all obtained from the published DIAGRAM+ Consortium meta-analysis[21], the largest available reference sample of European descent, and while they do not necessarily reach genome-wide significance in that sample, all loci have shown associations with type 2 diabetes at $P < 5x10^{-8}$ (see Supplementary Online Material for the list of published studies). Effect sizes are aligned to the T2D risk allele or the SBP- or height-increasing allele. Colours indicate birth weight association *P*-values: P<5e-08 (red); P>=5e-08 and P<0.001 (orange); P>=0.001 and P<0.01 (yellow); P>0.01 (white). The triangles in plot f are SNPs known to be associated with age at menarche. There were more associations between height loci and higher birth weight than expected under the null, and a slight excess of associations between T2D or SBP loci and lower birth weight (binomial sign test $P = 0.02$, 0.09 and $3x10^{-10}$ for b, d and f, respectively).

Discussion

For five of the seven confirmed associations with birth weight, correspondence with GWAS findings for adult traits (type 2 diabetes, blood pressure or height) provide clues to the biological pathways involved. Two SNPs represent the same signals as known type 2 diabetes loci: *ADCY5* (previously reported[2]) and *CDKAL1* (previously examined in smaller candidate gene studies of birth weight[3-4]). We observed similar *z* score effect size estimates of the associations between each of these loci and ponderal index (calculated as weight/length^3 to indicate neonatal leanness), birth length and head circumference (Table 1), suggesting a general effect on fetal growth. At both loci, the birth weight-lowering allele is associated with greater type 2 diabetes risk[2-4]. This observation is consistent with the fetal insulin hypothesis[5], which proposes that common genetic variation influencing insulin secretion or action, both in prenatal development and adult life, could partly explain epidemiological correlations between lower birth weight and type 2 diabetes. The type 2 diabetes risk allele at *ADCY5* is associated with a number of features suggesting impaired insulin secretion: higher glucose levels after fasting and 2 hours after an oral glucose challenge[6-7]; lower 2-hour insulin levels, adjusted for 2-hour glucose levels[7]; higher fasting proinsulin (relative to mature insulin) levels[8]; and lower Homeostatic Model Assessment (HOMA)-derived index of beta-cell function HOMA-B[6] (Supplementary Table 5). The risk allele at *CDKAL1* is strongly associated with reduced insulin secretion in studies of adults[9]. Given the key role of fetal insulin in prenatal growth, we hypothesize that the *ADCY5* and *CDKAL1* risk alleles reduce fetal insulin levels, which mediate the associations with birth weight.

To investigate whether type 2 diabetes susceptibility loci other than *ADCY5* and *CDKAL1* influence fetal growth, we tested the associations between 47 additional, published type 2 diabetes loci and birth weight in our Stage 1 meta-analysis. We observed more

Table 1. Associations between seven loci associated with birth weight and various anthropometric measures taken at birth (from joint meta-analysis of up to 69,308 individuals).

Locus (Index SNP, Effect allele/Other allele)		Birth weight (combined meta-analysis of European Discovery and Follow-up studies) [in grams]	Birth weight, adjusted for maternal genotype	Birth weight, adjusted for birth length	Birth length	Birth head circumference	Ponderal Index (weight/length^3)
	N	61142	11130	36209	35953	23000	35708
		-0.072 (0.006)					
	Beta (SE)	[-35]	-0.108 (0.014)	-0.067 (0.005)	-0.025 (0.007)	-0.033 (0.009)	-0.090(0.008)
	P-value	3.6E-38	7.5E-14	1.2E-35	6.7E-04	2.3E-04	9.5E-28
	Unadj beta (SE)*	-	-0.109 (0.013)	-0.085 (0.008)	-	-	-
CCNL1 (rs900400, C/T)	Unadj P-value*	-	7.5E-18	8.81E-29	-	-	-
	N	61509	11307	36015	36084	23184	35836
		-0.059 (0.006)					
	Beta (SE)	[-29]	-0.077 (0.016)	-0.032 (0.006)	-0.035 (0.009)	-0.031 (0.010)	-0.034 (0.010)
	P-value	5.5E-20	1.5E-06	5.8E-07	5.0E-05	0.0027	2.9E-04
	Unadj beta (SE)*	-	-0.064 (0.014)	-0.058 (0.009)	-	-	-
ADCY5 (rs9883204, C/T)	Unadj P-value*	-	5.7E-06	7.4E-11	-	-	-
	N	68655	9649	35961	36030	23277	35781
		-0.047 (0.005)					
	Beta (SE)	[-23]	-0.025 (0.015)	-0.018 (0.005)	-0.046 (0.007)	-0.039 (0.009)	-0.016 (0.008)
	P-value	1.4E-19	0.096	5.5E-04	1.7E-10	5.4E-06	0.049
	Unadj beta (SE)*	-	-0.029 (0.013)	-0.053 (0.007)	-	-	-
HMGA2 (rs1042725, T/C)	Unadj P-value*	-	0.027	1.2E-12	-	-	-
	N	68822	9415	35789	35861	22894	35614
		-0.050 (0.006)					
	Beta (SE)	[-24]	-0.056 (0.017)	-0.026 (0.006)	-0.035 (0.008)	-0.019 (0.010)	-0.034 (0.009)
	P-value	1.5E-18	0.001	9.4E-06	1.7E-05	0.042	8.6E-05
	Unadj beta (SE)*	-	-0.045 (0.015)	-0.051 (0.008)	-	-	-
CDKAL1 (rs6931514, G/A)	Unadj P-value*	-	0.003	6.7E-10	-	-	-

Table 1. Associations between seven loci associated with birth weight and various anthropometric measures taken at birth (from joint meta-analysis of up to 69,308 individuals). (continued)

Locus (Index SNP, Effect allele/Other allele)		Birth weight (combined meta-analysis of European Discovery and Follow-up studies) [in grams]	Birth weight, adjusted for maternal genotype	Birth weight, adjusted for birth length	Birth length	Birth head circumference	Ponderal Index (weight/length^3)
	N	53619	6136	28465	28532	20222	28290
		-0.034 (0.006)					
	Beta (SE)	[-16]	-0.040 (0.021)	-0.018 (0.006)	-0.023 (0.008)	-0.030 (0.010)	-0.023 (0.009)
	P-value	4.6E-08	0.056	0.003	0.006	0.003	0.010
	Unadj beta (SE)*	-	-0.043 (0.018)	-0.034 (0.008)	-	-	-
5q11.2 (rs4432842, C/T)	Unadj P-value*	-	0.018	4.6E-05	-	-	-
	N	55877	8733	29956	30027	21065	29781
		-0.042 (0.006)					
	Beta (SE)	[-20]	-0.078 (0.018)	-0.010 (0.006)	-0.047 (0.009)	-0.027 (0.010)	-0.011 (0.010)
	P-value	4.6E-11	2.0E-05	0.13	8.3E-08	0.008	0.258
	Unadj beta (SE)*	-	-0.071 (0.016)	-0.042 (0.009)	-	-	-
LCORL (rs724577, C/A)	Unadj P-value*	-	8.4E-06	3.8E-06	-	-	-
	N	49660	6231	29695	29762	17833	29519
		-0.041 (0.007)					
	Beta (SE)	[-20]	-0.029 (0.023)	-0.021 (0.006)	-0.027 (0.009)	-0.033 (0.011)	-0.035 (0.009)
	P-value	3.6E-09	0.18	0.001	0.002	0.004	2.3E-04
	Unadj beta (SE)*	-	-0.036 (0.019)	-0.045 (0.009)	-	-	-
ADRB1 (rs1801253, G/C)	Unadj P-value*	-	0.058	4.3E-07	-	-	-

Results are from inverse variance, fixed-effects meta-analysis of all available study samples of European ancestry. The effect allele for each SNP is labelled on the positive strand according to HapMap. The beta value is the change in trait *z* score per birth weight-lowering allele from linear regression, adjusted for sex and gestational age (where available), assuming an additive genetic model. To obtain the equivalent birth weight effect in grams, we multiplied by 484g, the median birth weight standard deviation of European studies in [2]. There was little detectable heterogeneity between studies (all $P > 0.01$).

*Results are unadjusted for maternal genotype or birth length, but only in samples where maternal genotype or birth length is available (for direct comparison with the model that is adjusted for maternal genotype or birth length, respectively.

associations with birth weight than expected by chance (Figure 1a), with 7 associations at $P < 0.05$, of which 4 achieved $P < 0.01$ (*MTNR1B*-rs1387153, *KCNQ1*-rs231362, *HHEX-IDE*-rs5015480 and *GCK*-rs4607517), including *GCK* at $P=1x10^{-4}$. Meta-analysis of the *HHEX-IDE* result with previously published data (total $n = 51,583$) strengthened the evidence of association ($P = 6.9x10^{-7}$; Supplementary Table 6). The type 2 diabetes risk alleles at *HHEX-IDE* and *KCNQ1* follow *ADCY5* and *CDKAL1* in being associated with lower birth weight, providing additional support for the fetal insulin hypothesis, although the associations can only explain a small fraction of the epidemiological association.

In contrast, the type 2 diabetes risk alleles at *GCK* and *MTNR1B* were associated with higher birth weight (Figure 1b). Higher maternal glucose levels are associated with higher offspring birth weight[10], and both the *GCK* and *MTNR1B* loci influence fasting glucose levels throughout the normal physiological range[6]. Consistent with this, and with previous studies of the *GCK* variant[11], the effect size estimates we observed for *GCK* and *MTNR1B* were lower after adjustment for maternal genotype (Supplementary Figure 6). Well-powered studies of mothers and offspring will be required to test formally the association between maternal genotype and birth weight at these loci. The lack of a fetal association at *GCK*-rs4607517 contrasts with the strong birth weight-lowering effects of rare, heterozygous fetal *GCK* mutations[12], and suggests that the common *GCK* variant does not influence insulin secretion until postnatal life.

The association with birth weight at *ADRB1* rs1801253 (Arg389Gly) links prenatal growth with blood pressure in adulthood since the same SNP is strongly associated with both systolic and diastolic blood pressure ($P<5x10^{-8}$)[13]. Epidemiological associations between birth weight and systolic blood pressure (SBP) constitute some of the strongest evidence supporting the fetal origins of adult disease[14]. Most studies report a linear inverse association throughout the birth weight distribution, whereby lower birth weight is associated with higher adult SBP. There is also evidence that birth weights at the high end of the distribution are associated with higher SBP[15]. Based on the majority of studies, we might therefore expect a fetal SBP-raising allele to be associated with lower birth weight. However, the birth weight-lowering allele at rs1801253 (Gly389) is associated with lower blood pressure in later life. We observed similar effect size estimates for associations between *ADRB1* and various birth measures (Table 1), suggesting a general effect on fetal growth. We tested for associations between birth weight and 29 additional blood pressure loci in our Stage 1 meta-analysis. While we did not observe strong evidence of deviation from the null (Figure 1c), associations between the SBP-raising allele and lower birth weight achieved $P < 0.01$ at *GUCY1A3/GUCY1B3*-rs13139571 (P=0.0008) and *CYP17A1/NT5C2*-rs11191548 (P=0.009). These were little altered on adjustment for maternal genotype (Figure 1d; Supplementary Table 7).

The associations with birth weight at the *HMGA2* and *LCORL* loci link prenatal growth with postnatal stature. At both loci, the birth weight-lowering allele is also associated

with lower adult height and associations are consistent with a primary effect on birth length (Table 1). The *HMGA2* SNP is also strongly associated with birth head circumference and is known to associate with head circumference in infancy and intracranial volume in adulthood[16-17] suggesting a general effect on growth. Variation at *LCORL* has also been associated with peak height velocity in infancy[18], indicating an effect on growth in childhood. When testing 178 additional published height loci (see Supplementary Online Material for references to published height, blood pressure and type 2 diabetes loci), we observed more associations with birth weight than expected by chance (Figure 1e), indicating that many adult height loci influence prenatal growth. Of all 180 loci, 132 show the same direction of effect size estimate with birth weight as with height (binomial sign test $P=3x10^{-10}$), although there is no strong correlation between adult height and birth weight effect sizes (Figure 1f). We did not observe any evidence that these associations were driven by maternal genotypes (Supplementary Table 8).

The remaining two loci (near *CCNL1* and on chromosome 5q11.2) are not known to be associated with any other traits. The previously reported association near *CCNL1* represents the strongest association with birth weight, and shows a strong association with ponderal index, but relatively weak associations with birth length and head circumference (Table 1), strengthening the evidence that this locus primarily acts through non-skeletal growth. In a subset of 7 studies with available postnatal data, the association had disappeared by 3 months of age (0.001 SD [95% CI: -0.030, 0.032] per rs900400 C-allele, relative to birth weight: -0.084 SD [95%CI: -0.106, -0.062]; Supplementary Table 9; Supplementary Figure 7), suggesting that the growth effects of the *CCNL1* locus are specifically intrauterine. Little is known about the birth weight locus at chromosome 5q11.2: the nearest gene, *ACTBL2*, is approximately 400kb away and has no obvious link with fetal growth. Associations at this locus are similar across the different anthropometric birth measures (Table 1) and there are no associations with adult metabolic or anthropometric traits in published studies (Supplementary Table 5).

We were interested to explore whether the same variants have any impact on birth weight in other ethnic groups. Using data from a range of non-European studies, including those of Middle Eastern, East and Southeast Asian and African origin (total n = 11,848; Supplementary Table 10), we showed that the 7 SNPs together explained between 0.32% and 1.52% of the variance in birth weight, which was similar to that in Europeans (0.76%; Supplementary Table 11; Supplementary Figures 8 and 9).

Conclusions

To conclude, we have identified four, and confirmed three loci associated with birth weight, which explain a similar proportion of variance to maternal smoking exposure in pregnancy (Supplementary Figure 10). The associations between five of the loci and adult traits (i) highlight biological pathways of relevance to the fetal origins of type 2 diabetes, (ii) reveal complexity in that type 2 diabetes risk alleles can be associated with either higher or lower birth weight, (iii) illuminate a novel genetic link between fetal growth and adult blood pressure and (iv) demonstrate substantial overlap between the genetics of prenatal growth and adult height.

Note: Supplementary information and online methods are available on the Nature Genetics Website: www.nature.com/ng

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Chapter 2.3

Common genetic variants affect infant head circumference identified by genome-wide association meta-analysis

H. Rob Taal *
Beate St Pourcain *
Elisabeth Thiering *
Shikta Das *
Dennis O Mook-Kanamori * et al.
For the Early Growth Genetics (EGG) Consortium

**Authors contributed equally to this work*
For the complete list of authors please see list of publications
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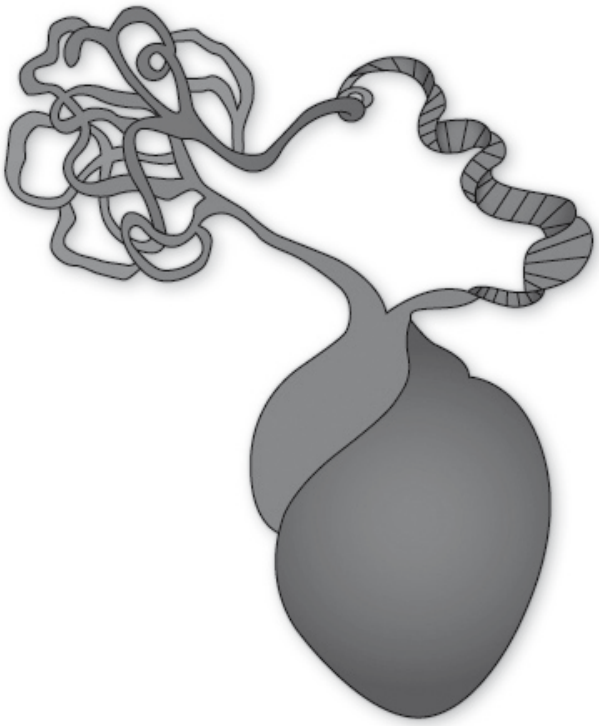


Abstract

Aims and Methods: To identify genetic variants associated with head circumference in infancy, we performed a meta-analysis of seven genome-wide association (GWA) studies (N=10,768 from European ancestry enrolled in pregnancy/birth cohorts) and followed up three lead signals in six replication studies (combined N=19,089). **Results and Conclusion:** Rs7980687 on chromosome 12q24 (P=8.1x10^{-9}), and rs1042725 on chromosome 12q15 (P=2.8x10^{-10}) were robustly associated with head circumference in infancy. Although these loci have previously been associated with adult height, their effects on infant head circumference were largely independent of height (P=3.8x10^{-7} for rs7980687, P=1.3x10^{-7} for rs1042725 after adjustment for infant height). A third signal, rs11655470 on chromosome 17q21, showed suggestive evidence of association with head circumference (P=3.9x10^{-6}). SNPs correlated to the 17q21 signal show genome-wide association with adult intra cranial volume, Parkinson's disease and other neurodegenerative diseases, indicating that a common genetic variant in this region might link early brain growth with neurological disease in later life.

Introduction

Head circumference in infancy is used as a measure for brain size and development[1-2]. Normal variation in head circumference seems to be associated with cognitive and behavioral development[3-5]. Larger head circumference in infancy is associated with higher IQ scores in childhood[5-7]. The underlying mechanisms however, are poorly understood. Head circumference is a complex trait with a high heritability of around 0.7-0.9[8]. Several rare mutations with large effects on head circumference have been identified[9-12], including those resulting in microcephaly and intellectual disability[10-12]. Common genetic variants that influence normal variation in head circumference in early life have not yet been identified.

To search for common genetic variants associated with head circumference in infancy, we performed a meta-analysis of GWA studies. We reasoned that finding such common variants might lead to enhanced understanding of molecular mechanisms important for variation in brain development.

Methods

We meta-analyzed association statistics from ~2.5 million directly-genotyped and imputed SNPs in infants of European descent from seven discovery GWA studies (N=10,768; Supplementary Table 1). In all studies head circumference in infancy (age 18 months, range 6 to 30 months) was measured from the occipital protuberance to the forehead, using a flexible, non-stretching measure tape following standardized procedures. If multiple measurements were available for one individual in this time window, only the measurement performed closest to the age of 18 months was used (Supplementary Tables 1 and 2). Since the relationship between head circumference and age during infancy is non-linear and the variance increases with age, we calculated sex- and age-adjusted SD-scores of head circumference in each study separately[13].

In the discovery phase we identified three lead signals (Manhattan plot is shown in Supplementary Fig. 1); two independent loci on chromosome 12 and one on chromosome 17, which showed suggestive evidence for association with head circumference in infancy. These three loci represent the first three independent loci of the discovery analysis and were at 12q24.31, in *SBNO1* (rs7980687, $P_{discovery}=3.3x10^{-7}$; Figure 1a), at 12q15, near *HMGA2* (rs1042725, $P_{discovery}=6.6x10^{-7}$; Figure 1b) and at 17q21.1, near *CRHR1/MAPT* (rs11655470, $P_{discovery}=1.4x10^{-6}$; Figure 1c). Other loci, suggesting an association with infant head circumference ($P<1x10^{-5}$) are described in Supplementary Table 3.

Table 1. Individual association results by study and meta-analysis

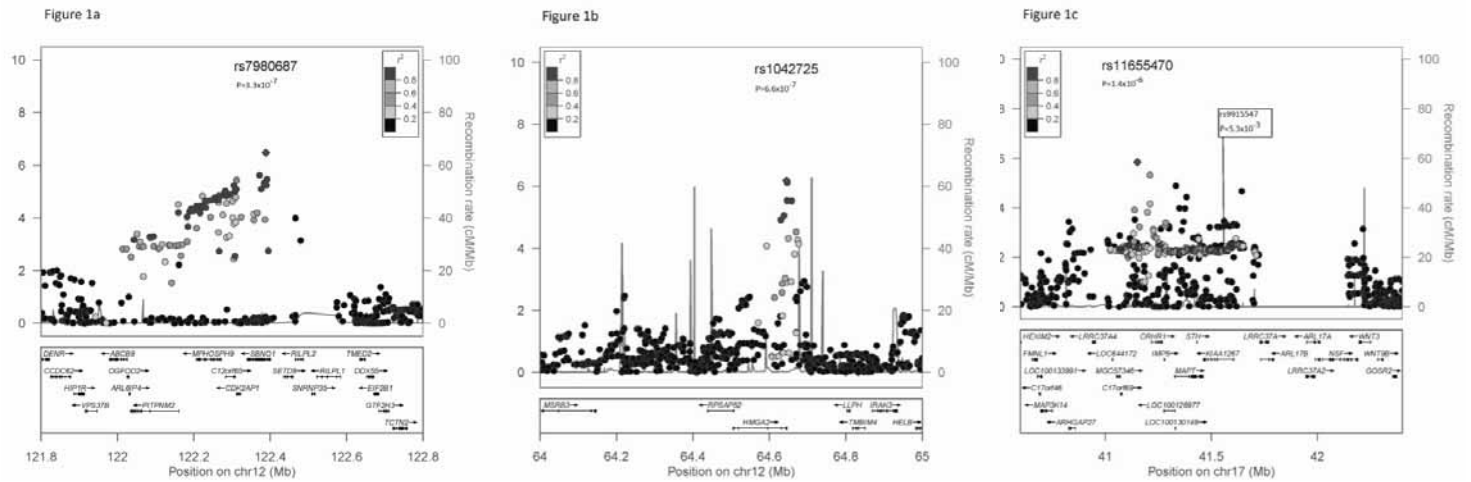
Study type	Study	Year(s) of birth	Median age (months)	Total N	% male	rs7980687_A on 12q24 SBNO1				rs1042725_T on 12q15 nearest gene HMGA2				rs11655470_T on 17q21 nearest genes CRHR1/MAPT			
						MAF	Beta	Se	P-value	MAF	Beta	Se	P-value	MAF	Beta	Se	P-value
Discovery	ALSPAC (D)	1991-2	18.9	1,748	53	0.19	0.105	0.038	$6x10^{-3}$	0.47	-0.071	0.031	0.02	0.41	0.114	0.031	$3x10^{-4}$
	CHOP	2006-10	18.5	1,008	59	0.20	0.041	0.058	0.48	0.48	-0.017	0.046	0.72	0.39	0.036	0.048	0.45
	COPSAC	1998-2001	18.1	369	49	0.19	0.083	0.086	0.33	0.47	-0.026	0.065	0.69	0.45	0.159	0.063	0.01
	Generation R	2002-6	13.1	2,240	52	0.21	0.064	0.031	0.04	0.49	-0.059	0.026	0.02	0.42	0.060	0.026	0.02
	LISA (D)	1998-9	11.8	357	56	0.21	-0.045	0.077	0.56	0.48	-0.059	0.060	0.33	0.39	0.068	0.061	0.26
	NFBC1966	1966	12.3	4,287	49	0.20	0.181	0.041	$1x10^{-5}$	0.49	-0.074	0.033	0.02	0.49	0.068	0.033	0.04
	RAINE	1989-91	13.1	759	53	0.19	0.108	0.058	0.06	0.50	-0.179	0.043	$4x10^{-5}$	0.41	-0.001	0.044	0.09
Discovery meta-analysis				10,768			0.091	0.018	$3.3x10^{-7}$		-0.072	0.014	$6.6x10^{-7}$		0.070	0.015	$1.4x10^{-6}$
Replication	ALSPAC (R)	1991-2	18.9	3,163	51	0.20	0.042	0.030	0.16	0.49	-0.088	0.024	$3x10^{-4}$	0.40	0.044	0.024	$6x10^{-4}$
	DNBC	1996-2002	12.1	531	54	0.20	0.120	0.070	0.09	0.45	-0.049	0.058	0.40	0.45	0.060	0.058	0.30
	EFSOCH	2000-4	12.1	703	52	0.20	0.054	0.061	0.37	0.50	-0.019	0.046	0.67	0.41	0.027	0.046	0.56
	INMA	2004-7	13.9	693	53	0.16	0.020	0.062	0.75	0.44	-0.029	0.045	0.52	0.36	0.022	0.046	0.64
	GINI+LISA (R)	1995-9	11.8	698	51	0.21	0.020	0.060	0.74	0.50	-0.092	0.049	0.06	0.40	-0.070	0.050	0.16
	NFBC1986	1985-6	12.0	2,533	48	0.22	0.082	0.035	0.02	0.49	-0.034	0.029	0.25	0.50	0.019	0.287	0.51
Replication meta-analysis				**8,321**			**0.055**	**0.018**	**$2.5x10^{-3}$**		**-0.058**	**0.015**	**$8.3x10^{-5}$**		**0.025**	**0.015**	**0.093**
Overall meta-analysis				**19,089**			**0.074**	**0.013**	**$8.1x10^{-9}$**		**-0.065**	**0.010**	**$2.8x10^{-10}$**		**0.048**	**0.010**	**$3.6x10^{-6}$**

MAF; Minor allele frequency, Se; standard error. Beta's reflect difference in head circumference SD score per minor allele (additive model).

P value is obtained from linear regression of the SNP against head circumference SD score (additive model). All study samples were of European descent.

Key to study names: ALSPAC (D), Avon Longitudinal Study of Parents and Children Discovery subset; CHOP, Children's Hospital Of Philadelphia; COPSAC, Copenhagen Prospective Study on Asthma in Childhood; Generation R, the Generation R Study; LISA (D), Lifestyle – Immune System – Allergy Discovery subset; NFBC1966, Northern Finland Birth Cohort 1966; RAINE, The Western Australian Pregnancy Study; ALSPAC (R), Avon Longitudinal Study of Parents and Children Replication subset; DNBC, Danish National Birth Cohort; EFSOCH, Exeter Family Study Of Childhood Health; INMA, INfancia y Medio Ambiente [Environment and Childhood] Project; GINI+LISA (R), German Infant Study on the influence of Nutrition Intervention Munich + Lifestyle – Immune System – Allergy Replication subset; NFBC1986, Northern Finland Birth Cohort 1986.

Figure 1.



Directly genotyped and imputed SNPs are plotted using filled circles with their meta-analysis P values (as –log10 values) as a function of genomic position (NCBI Build 36). In each plot, the discovery-stage SNP taken forward to replication stage is represented by a purple diamond (defining a global meta-analysis P value). Local LD structure is reflected by the plotted estimated recombination rates (taken from HapMap) in the region around the associated SNPs and their correlated proxies. The correlations of the lead SNP to other SNPs at the locus are shown on a color scale from r^2<0.2 dark blue; 0.2=<r^2<0.4 light-blue; 0.4=<r^2<0.6 green; 0.6=<r^2<0.8 orange; r^2>=0.8 red. Superimposed on the plot are the recombination rates (light blue line, second y axis). Gene annotations are shown as the dark blue arrows. The regional plots were drawn using the LocusZoom software[36].

1a Regional plot of locus 12q24.31

1b Regional plot of locus 12q15

1c Regional association plot of locus 17q21.1; downstream of the lead signal, rs9915547 is indicated (r^2 0.22 HapMap CEU with rs11655470), which showed a genome wide significant association with adult intra cranial volume ($P=1.5x10^{-12}$) as described in Ikram et al.[14]

Results

Table 1 shows the associations of these three lead SNPs in each cohort. We followed up these three associations in six independent replication samples of European descent (N=8,321; Supplementary Table 2). We genotyped the most strongly associated SNP from each locus (rs7980687 from 12q24.31; rs1042725 from 12q15; rs11655470 from 17q21.1), or a closely-correlated proxy (HapMap R^2). Consistent associations were observed for both signals on chromosome 12 in the replication samples (P=0.003 and P=8.1x10^{-5} for rs7980687 and rs1042725 respectively). Marginal evidence of association for rs11655470 was seen in the replication samples (P=0.093). Genomic control correction was applied during the discovery meta-analysis stage to adjust the statistics generated within each cohort (λ-values ranging from 1.007-1.054, Supplementary Table 1). Results from the replication cohorts were combined with the genomic control corrected discovery results to get the overall meta-analysis results. Combining discovery and replication samples (N=19,089; Table 1), each A allele of rs7980687 in *SBNO1* was robustly associated with a 0.074 SD larger head circumference (95% CI: 0.049, 0.099; P=8.1x10^{-9}, explained variance 0.24%) and each T allele of rs1042725 near *HMGA2* with a 0.065 SD smaller head circumference (95% CI: -0.085, -0.045; P=2.8x10^{-10}, explained variance 0.33%). This reflects a difference of around 1.2 and 1.0 mm in head circumference respectively. The effect of each T allele of rs11655470 near *CRHR1/MAPT* did not reach genome-wide significance in the combined analysis (effect 0.048 SD larger head circumference; 95%CI: 0.028, 0.068; P=3.8x10^{-6}, explained variance 0.21%). These three associations showed low heterogeneity (P>0.1, I^2=5-33%).

Additionally, the signals in *SBNO1* and near *HMGA2,* but not the one near *CRHR1/MAPT,* were associated with height measured at the same visit as head circumference (Supplementary Table 4). When we adjusted the model for current height, the associations of rs7980687 and rs1042725 with head circumference were slightly attenuated (effect size 0.057 SD; 95%CI: 0.035, 0.080; P=3.8x10^{-7} and -0.048 SD; 95%CI: -0.066, -0.030; P=1.3x10^{-7} for rs7980687 and rs1042725 respectively, Supplementary Table 5). The association of the third signal near *CRHR1/MAPT* was unaffected. In depth mediation analysis showed that the effects of rs7980687 and rs1042725 on head circumference were only partly (12% and 24% respectively) explained by height (Supplementary Fig. 2, Supplementary Table 6). The effect of rs11655470 was a completely direct effect of the SNP on head circumference (Supplementary Table 6). To further adjust for possible population stratification we added principal components to the model, in cohorts where these measures were available (total N = 12,763). This did not materially change the effect on head circumference, indicating that the utilized association tests are robust to population stratification (Supplementary Table 7). The three variants were not associated with other covariates such as breast feeding, socioeconomic status or educational level (data not

Table 2. Association of the three lead signals related to head circumference with additional phenotypes

Marker	Head circumference in third trimester of pregnancy (SD score)				Head circumference at birth (SD score)				Intra cranial volume (ml)				
	Total N	Beta	Se	P-value	Total N	Beta	Se	P-value	Total N	Mean age at measurement (years)	Beta	Se	P-value
rs7980687_A on 12q24	3,781	0.089	0.029	$1.9x10^{-3}$	17,330	0.050	0.012	$5.2x10^{-5}$	8,175	67.5	0.72	2.03	0.72
rs1042725_T on 12q15	3,781	-0.075	0.023	$9.9x10^{-4}$	17,074	-0.031	0.010	$1.9x10^{-3}$	8,175	67.5	-7.18	1.61	$8.8x10^{-6}$
rs11655470_T on 17q21	3,781	0.049	0.024	0.037	17,695	0.030	0.010	$2.0x10^{-3}$	8,175	67.5	3.54	1.69	0.036 #

SD; standard deviation, Se; standard error, Beta's reflect difference in head circumference SD score per minor allele, or difference in intra cranial volume (ml) per minor allele (additive model). *P* value is obtained from linear regression the SNP and sex against of head circumference SD score in fetal life (additive model); SNP, sex and gestational age at against birth head circumference SD score at birth (additive model); SNP, age and sex against Intra cranial volume (ml) (additive model)[14]. All study samples were of European descent.

A variant further downstream (rs9915547; r^2 0.22 HapMap CEU) showed a genome-wide significant association ($P=1.5x10^{-12}$) with adult intra cranial volume.

shown). We did not find evidence for an interaction of these variants with infant sex or breastfeeding after Bonferroni correction (P>0.017, Supplementary Table 8 and 9).

In order to further investigate an effect of the three lead signals on fetal head growth, we assessed the associations of the variants with head circumference using third trimester fetal ultrasound data (n=3,781) and head circumference measured at birth (n=13,775), in discovery and replication cohorts that had these data available (Supplementary Table 2). All three signals showed evidence of association with head circumference in third trimester of pregnancy and at birth (Table 2). The directions of the effects were consistent with those in infancy.

Next, we assessed the associations of the three lead signals with intra-cranial volume (ICV) in adulthood, measured by magnetic resonance imaging (MRI), in 8,175 individuals in the CHARGE-consortium[14]. There was evidence of association between the signals near *HMGA2* and *CRHR1/MAPT* and ICV (Table 2). For the signal near *CRHR1/MAPT*, a variant further downstream (rs9915547; r^2 0.22 HapMap CEU) showed a genome-wide significant association ($P<5x10^{-8}$). All directions of the effects were consistent with the observed associations for head circumference in infancy (Table 2).

We also assessed if there were possibly functional common variants in LD ($r^2 > 0.50$) with our three lead SNPs, being either non-synonymous SNPs or eQTLs. One variant, rs1060105, in high LD with our lead signal (rs7980687 with HapMap r^2 0.89), was a non-synonymous SNP located in exon 5 of SBNO1 (missense; AGT(Ser) => AAT(Asn)). The minor allele (A) of rs1060105 was associated with an increased head circumference in infancy (effect size 0.081 SD; 95%CI: 0.048, 0.115; $P=2.4x10^{-6}$ (N=10,768)). The underlying mechanism is unknown. Considering that transcription regulation is highly cell-type specific, we next evaluated whether we could find eQTLs established in brain tissue[15]. We did not find eQTLs in publicly available brain expression data[15]. Subsequently, we also explored eQTL databases from other tissues and identified three SNPs in LD with rs7980687 ($r^2 > 0.7$ HapMap CEU) associated with gene transcript expression of CDK2AP1 and MPHOSPH9 in liver tissue, monocytes and lymphoblastoid cell lines[16-18]. Little is known on these genes except that both CDK2AP1 and MPHOSPH9 are involved in cell-cycle regulation (Supplementary Table 10)[19-20].

Discussion

To our knowledge, this is the first genome-wide association study on head circumference in infancy. The top two signals (rs7980687 in *SBNO1* and rs1042725 near *HMGA2*) associated with infant head circumference have previously been associated with adult height[21]. Therefore, we also assessed the association between the 180 known height variants and head circumference during infancy[21]. A strong deviation from the null-line

was observed on the QQ-plot (Supplementary Fig. 3). Besides *SBNO1* and *HMGA2*, 23 other height variants were nominally associated with head circumference in infancy (Supplementary Table 11). After applying Bonferroni correction for multiple testing in this candidate gene analysis ($P<2.8x10^{-4}$), markers in/near *ZNFX1* ($P=6.1x10^{-6}$), *OR2J3* ($P=1.8x10^{-5}$) and *ZBTB38* ($P=1.8x10^{-4}$) remained statistically significant associated with head circumference in infancy.

The relative effect size of rs1042725 near *HMGA2* was similar for infant head circumference (0.065 SD) and adult height (0.060 SD). However, the effect size of rs7980687 in *SBNO1* on infant head circumference (0.074 SD) was considerably larger than for adult height (0.035 SD). As head size is correlated with total body size[22], it might be that the top two loci have a more general regulating role in skeletal growth and bone development. It also could be that variants in *SBNO1* affect brain growth and concurrent head circumference, or that they affect skull growth rather than skeletal growth. The *SBNO1*-gene is involved in the Notch signaling pathway[23]. In *Drosophila*, a similar gene (*sno*) is required for early embryogenesis, and absence of this gene leads to maldevelopment of the central nervous system[23]. In humans *SBNO1* has been implicated in oncogenic processes[24-25].

The variant near *HMGA2* was one of the first to be associated with adult height. Deletions and truncations in the *HMGA2*-gene in mice and humans have been associated with small and large stature[26-27]. The effect of *HMGA2* is similar for head circumference and adult height, thus it seems likely that it has a more general role in skeletal growth.

A third variant (rs11655470), in the promoter region of *CRHR1/MAPT*, was also related to head circumference, though this signal did not reach genome-wide significance. Rs11655470 lies within the 17q21 inversion, but is not strongly correlated with the inversion (r^2 0.22 HapMap CEU). This 900kb region, corresponding to the conversion, contains several genes. The SNP is closely related to the *CRHR1*-gene (r^2 0.59 HapMap CEU with rs171440). Variants in/near *CRHR1* have been associated with brain development and bone mineral density[28-29], although the underlying mechanisms are largely unknown. Another gene included in the 17q21 inversion is *MAPT* (r^2 0.22 HapMap CEU). Both common variants and mutations in *MAPT* are known to be associated with Parkinson's disease and other neurodegenerative diseases[30-34]. Other genes in this region are saitohin (*STH)* and granulin (*GRN)*. *STH* has been associated with progressive supranuclear palsy and increased risk of late-onset Alzheimer's disease[35-36]. Mutations in *GRN* have been shown to cause fronto-temporal degeneration[37]. It might be that common genetic variants in/near *CRHR1/MAPT* affect early brain development, by altering the stability and assembly of microtubules. Ikram et al. showed that a correlated SNP in the same region (rs9303525, HapMap r^2 0.22 with rs11655470) is associated with adult intra cranial volume, reaching genome-wide significance[14]. Since the LD between the variants is low, it could be that they represent separate independent effects on different pheno-

types. When we adjusted the effect of rs11655470 on infant head circumference for the CHARGE ICV signal (rs9915547), the effect was attenuated but remained significant (0.059 SD ($P=1.0x10^{-5}$) and 0.037 SD ($P=7.3x10^{-3}$) before and after adjustment for rs9915547 respectively), suggesting that these signals both tag a third marker influencing both phenotypes (Supplementary Table 12). However, although the association attenuates after conditioning on the CHARGE ICV signal, the two signals might still independently tag different causal markers in the region and the attenuation might be due to the weak LD, because of proximity, between the two signals. The marker associated with head circumference is in low LD with the chromosome 17q21 inversion, while the CHARGE ICV signal is in high LD with the inversion. Therefore, it does not seem likely that the 17q21 inversion is causally related to infant head circumference. The biological mechanisms underlying these associations are largely unknown.

Conclusions

Our study highlights early effect of variants in/near *SBNO1* and *HMGA2* on head circumference in fetal life and infancy, and shows that a variant near *CRHR1/MAPT* is marginally associated with head circumference in infancy. Our findings suggest that the genetic variants in the *CRHR1/MAPT* region might link early brain growth with neurological disease in later life. Further research is needed to elucidate whether these variants influence brain growth and neurodevelopment in early life.

Note: Supplementary information is available on the Nature Genetics website: www.nature.com/ng

Online Methods

Stage 1: GWA meta-analysis of head circumference

Discovery samples, genotyping and imputation

We selected seven population-based studies with head circumference measured in infancy (study cohort specific median age range 11-18 months) and GWA data available by the beginning of March 2010 (combined N=10,768): the Avon Longitudinal Study of Parents And Children (ALSPAC; N=1,748); The Children's Hospital of Philadelphia (CHOP; N=1,008); the Copenhagen Study on Asthma in Childhood (COPSAC; N=369); The Generation R Study (Generation R; N=2,240); the Lifestyle – Immune System – Allergy Study (LISA; N=357); the Northern Finland 1966 Birth Cohort (NFBC1966; N=4,287) and the Western Australian Pregnancy study (RAINE; N=759). Genotypes were obtained using high-density SNP arrays, and then imputed for ~2.4 million HapMap SNPs (Phase II, release 21/22, http://hapmap.ncbi.nlm.nih.gov/). The basic characteristics, exclusions (e.g. samples of non-European ancestry), genotyping, quality control and imputation methods for each discovery sample are presented in Supplementary Table 1.

Statistical analysis within discovery samples

Head circumference was measured in infancy (age window: 6-30 months). If multiple measurements were available for one individual within this age window, the measurement closest to 18 months was used. Sex- and age-adjusted standard deviation scores (SD score) were constructed using Growth Analyser 3.0 (http://www.growthanalyser.org; Dutch Growth Research Foundation, Rotterdam, the Netherlands) in each study separately[13]. The association between each SNP and head circumference was assessed in each study sample using linear regression of head circumference SD score against genotype, assuming an additive model. Imputed genotypes were only used where directly-assayed genotypes were unavailable.

Meta-analysis of discovery samples

Data exchange was facilitated by the SIMBioMS platform (simbioms.org)[38]. Prior to meta-analysis, SNPs with a minor allele frequency <1% and poorly-imputed SNPs (proper_info ≤0.4 [SNPTEST]; r^2 ≤0.3 [MACH2QTL]) were filtered. Fixed effects meta-analyses were independently conducted by two investigators (H.R.T., D.O.M-K.). Meta-analysis was performed using the software package: METAL (http://www.sph.umich.edu/csg/abecasis/metal/index.html); Genomic control[39] was applied during the meta-analysis stage to adjust the statistics generated within each cohort (see Supplementary Table 1 for individual study λ-values, discovery meta-analysis λ-value: 1.043). Meta-analysis was done using the inverse-variance method; a fixed effects model was assumed. SNPs

available in less than four discovery cohorts were excluded. Final meta-analysis results were obtained for 2,449,806 SNPs. We considered the top three lead signals (representing 3 distinct genomic regions on chromosomes 12 and 17) in the discovery analysis for further follow-up in additional samples. The two loci at chromosome 12 reached the threshold of $P<1x10^{-6}$ and were therefore selected for replication and the third locus at chromosome 17 was just above that threshold ($P=1.4x10^{-6}$) and was selected because of prior knowledge of the nearby genome wide significant hit on intra cranial volume as described by Ikram et al.[14]

Stage 2: Follow-up of three lead signals in additional samples

Follow-up samples, genotyping and analysis

We used 6 independent study samples (combined N=8,321) to follow up the three lead signals from the GWA meta-analysis (represented by index SNPs rs7980687, rs1042725 and rs11655470). Details of these study samples are presented in Supplementary Table 2. If the index SNP was unavailable, a closely correlated proxy was substituted (rs12322888 or rs12316131 for rs7980687 [HapMap $r^2=0.95$]; rs7970350 or rs1351394 for rs1042725 [HapMap $r^2=1$ and 0.91 respectively]; rs12938031 for rs11655470 [HapMap $r^2=0.58$]). In 3 of the replication studies, the index SNPs were imputed from genome-wide genotype data (see Supplementary Table 2). The head circumference analysis (as described above) was performed within each study sample.

Statistical analysis

Meta-analyses of discovery and replication samples

We performed fixed effects inverse variance meta-analyses of the head circumference association results for the three lead signals in the seven discovery samples and six replication samples combined. Fixed effects meta-analyses were conducted independently by two investigators (H.R.T., D.O.M-K.), using RMeta in R [v.2.7.0]). We used the Cochran Q test and the I^2 statistic[40] to assess evidence of between-study heterogeneity of effect sizes.

Informed consent (or parental consent, as appropriate) was obtained from all discovery and follow-up study participants and study protocols were approved by the local ethics committees.

Analyses of potential confounders

To verify that the investigated lead SNPs were not associated with other covariates which could theoretically confound the observed associations with head circumference

(including height, weight and age at measurement; breastfeeding; maternal educational level; and sex), we used linear or logistic regression models to assess the associations between each covariate and genotype, in all discovery and replication samples. For height and weight, we constructed sex- and age-adjusted SD scores using Growth Analyser 3.0 (http://www.growthanalyser.org; Dutch Growth Research Foundation, Rotterdam, the Netherlands) in each study separately, similar to the head circumference SD score. To investigate possible effects of the three lead signals on head circumference through height, we first conducted linear regression analysis with and without adjustment for height SD score. Second, we conducted a mediation analysis and assessed the direct SNP effects and indirect SNP effects (mediated through height) on head circumference for each of the signals using a seemingly unrelated regression model (STATA, StataCorp LP, College Station, TX, USA) or a simple path analysis model (MPLUS, Muthen & Muthen, Los Angeles, CA, USA), which provide identical effect estimates. To investigate whether the associations between genotypes and infant head circumference were similar in the sexes, we repeated the analyses in males and females separately. Furthermore, we evaluated possible effect modification by breastfeeding status for each of the SNPs. Where possible, we meta-analyzed results to assess overall evidence of association.

Analysis of fetal head circumference and intra cranial volume

We explored associations of rs7980687, rs1042725 and rs11655470 with third trimester fetal head circumference and head circumference at birth, assuming an additive model using linear regression. Fetal head circumference was measured by ultrasound in three studies (combined N=3,781 singleton pregnancies) in third trimester of pregnancy (gestational age window 27-36 weeks). Only one measurement per subject was included in the time window. If multiple measurements were available within the time-window, the one closest to the median of 32 weeks of the gestation was used. We calculated gestational age specific SD scores using previously published growth charts[41]. This analysis was adjusted for sex. Head circumference was measured at birth, or within the 31st day of life, in 12 studies (N=13,775; Supplementary table 2). We created SD scores for head circumference within each of the cohorts and assessed the association with each SNP, adjusted for sex and gestational age. If head circumference was measured in the first month, we used gestational age at birth + age (weeks) at measurement in the first month. Combined effect estimates were calculated using fixed effects meta-analyses.

We used the meta-analysis on intracranial volume in adults, measured by MRI, in the Cohorts for Heart and Aging Research in Genetic Epidemiology (CHARGE) consortium[42] as a third additional phenotype. Data collection methods, phenotype definition, baseline characteristics, and results of the meta-analysis are described elsewhere in this issue[14, 43].

Analysis of known adult height variants with infant head circumference

We used the discovery meta-analyses to assess the associations of the previously identified 180 known adult height loci[21] with head circumference in infancy, using the same model as described above. We also checked whether very closely related SNPs (HapMap r2 >0.95) showed higher significance levels than the originally reported SNPs. SNPs with a P-value lower than $2.8x10^{-4}$ (0.05/180) were considered significant.

Variance explained

To estimate the percentage of variation in birth weight explained by each of the associated loci, we obtained the adjusted-R^2 from univariate linear regression models of head circumference against genotype. We then calculated a mean value from all discovery and replication studies, weighted by sample size.

Non-synonymous SNPs and eQTLs

We assessed SNPs in LD with the three lead signals and checked for non-synonymous SNPs or eQTLs to identify possible functional variants explaining the associations with head circumference. First, we used the SNP Annotation and Proxy search developed by the Broad institute (http://www.broadinstitute.org/mpg/snap/) to select all SNPs in LD ($r^2 > 0.50$) with our three lead signals. We used the 1000 Genomes Pilot 1 set as SNP dataset for rs7980687 and rs1042725 and the HapMap r22 as SNP dataset for rs11655470 ($r^2 > 0.50$) since this SNP was not available on the 1000 Genomes dataset. Next, we evaluated whether these SNPs were non-synonymous using dbSNP search engine from NCBI. To evaluate whether there were cis-eQTLs in LD with our lead signals we searched publicly available eQTL databases through the NCBI GTEx (Genotype-Tissue Expression) eQTL Browser (http://www.ncbi.nlm.nih.gov/gtex/test/GTEX2/gtex.cgi) and the Generic Genome Browser (http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/). In total, these browsers search nine databases for eQTLs. Only cis-associations (defined as genes within 1Mb) that reached the P-value threshold for significance, as used in the original papers describing the gene expression datasets, were included in Supplementary Table 10. The statistics behind the eQTL analysis and calculation of the threshold for declaring significance of the associations are described in the published and validated eQTL datasets[16-18].

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Chapter 2.4

Maternal smoking during pregnancy, a common genetic variant at 15q25, and fetal growth

Elisabeth TM Leermakers
H Rob Taal
Rachel Bakker
Eric AP Steegers
Albert Hofman
Vincent WV Jaddoe

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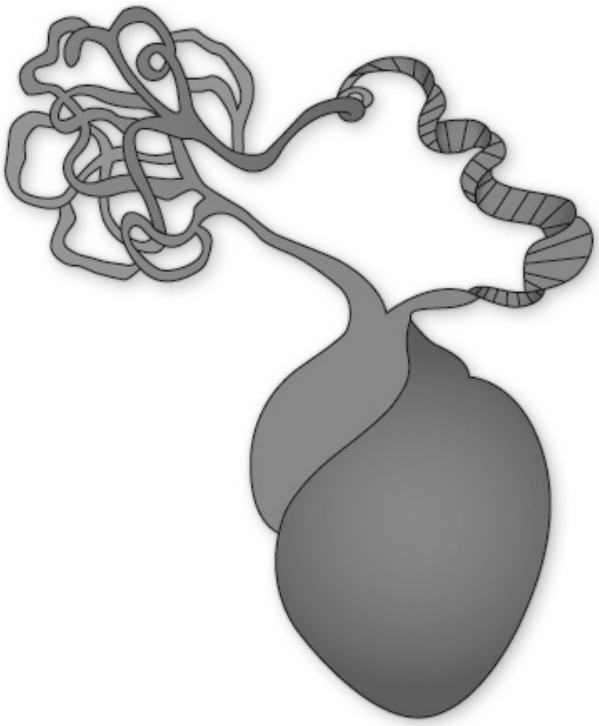


Abstract

Aims: Maternal smoking during pregnancy is associated with fetal growth retardation. We examined whether a common genetic variant at chromosome 15q25 (rs1051730), which is known to be involved in nicotine metabolism, modifies the associations of maternal smoking with fetal growth characteristics.

Methods: This study was performed in 3,563 European mothers participating in a population-based prospective cohort study from early pregnancy onwards. Smoking was assessed by postal questionnaires and fetal growth characteristics were measured by ultrasound examinations in each trimester of pregnancy.

Results: Among mothers who did not smoke during pregnancy (82.9%), maternal rs1051730 was not consistently associated with any fetal growth characteristic. Among mothers who continued smoking during pregnancy (17.1%), maternal rs1051730 was not associated with head circumference. The T-allele of maternal rs1051730 was associated with a smaller second and third trimester fetal femur length [differences -0.23 mm (95%CI -0.45 to -0.00) and -0.41 mm (95%CI -0.69 to -0.13), respectively] and a smaller birth length [difference -2.61 mm (95%CI -5.32 to 0.11)]. The maternal T-allele of rs1051730 was associated with a lower third trimester estimated fetal weight [difference -33 grams (95%CI -55 to -10)], and tended to be associated with birth weight [difference -38 grams (95%CI -89 to 13)]. This association persisted after adjustment for smoking quantity.

Conclusions: Our results suggest that maternal rs1051730 genotype modifies the associations of maternal smoking during pregnancy with impaired fetal growth in length and weight. These results should be considered as hypothesis generating and indicate the need for large-scale genome wide association studies focusing on gene – fetal smoke exposure interactions.

Introduction

Maternal smoking during pregnancy is strongly associated with increased risks of preterm birth or a small size for gestational age at birth[1]. As compared to children of mothers who did not smoke during pregnancy, those of mothers who smoked during pregnancy have a 100 to 200 grams lower birth weight[1]. It has been estimated that, in Western countries, 30 percent of children with low birth weight can be explained by exposure to tobacco smoke during pregnancy[2-3]. The effects of maternal smoking on fetal growth differ between individuals. These differences in effects might be explained by maternal genetic predisposition. A recent genome wide association study meta-analysis identified a common genetic variant, rs1051730, located within the 15q25 nicotinic acetylcholine receptor gene cluster (*CHRNA5-CHRNA3-CHRNB4*), to be associated with smoking quantity[4]. Smokers with a risk allele (T-allele) of rs1051730 seem to have higher blood levels of nicotine, compared to smokers without the T-allele[5]. Another study showed that among mothers who smoked during pregnancy, each additional copy of the maternal T-allele of rs1051730 resulted in a 28 grams lower birth weight in the offspring[6], though this effect was not significant. These findings suggest that rs1051730 might be a common genetic variant leading to an increased susceptibility of the adverse effects of maternal smoking on fetal growth and development. Therefore, we explored in a population-based prospective cohort study among 3,563 European mothers, whether maternal rs1051730 modifies the associations of maternal smoking during pregnancy with fetal growth characteristics in different trimesters.

Methods

Design

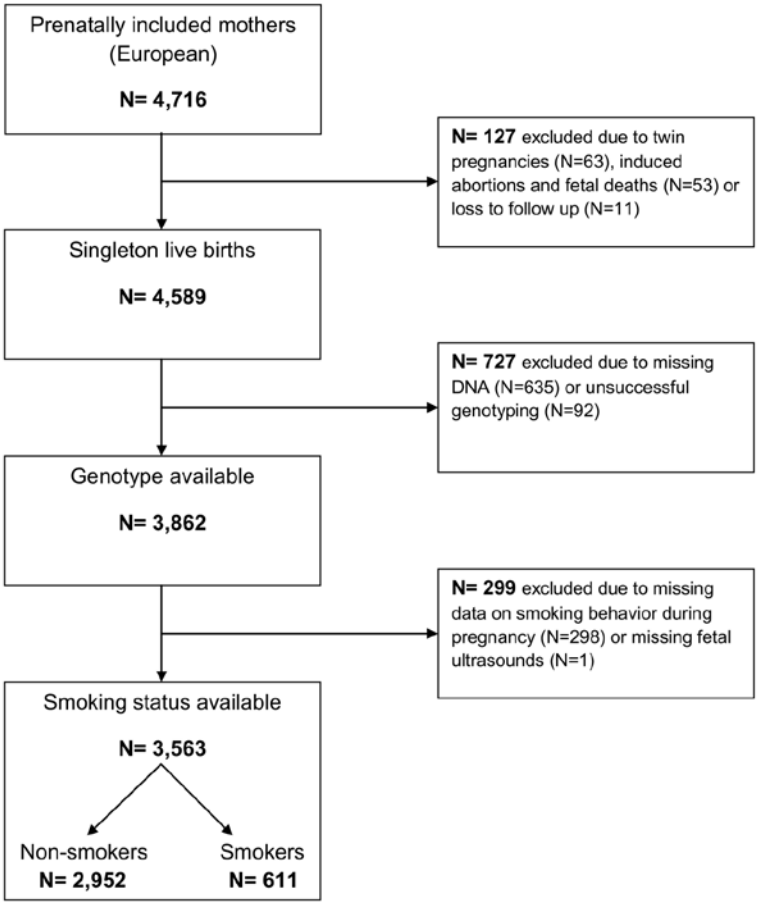
This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards in Rotterdam, the Netherlands, which has been described previously[7-8]. Enrollment was aimed at early pregnancy but was allowed until delivery. Extensive assessments focused on fetal growth and its main determinants were performed in each trimester of pregnancy. All children were born between April 2002 and January 2006. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center in Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained from all participants.

Population for analysis

In total, 4,716 mothers of European ancestry (86% Dutch, 14% other European) were included during pregnancy, of whom 3,862 mothers had a singleton live birth and had

genotype of rs1051730 available. We had smoking status during pregnancy and fetal ultrasounds available in 3,563 (92.3%) of these mothers. A participant flow chart is given in Figure 1.

Figure 1. Flow chart of participants included for analysis



Maternal smoking status

Information about maternal smoking status during pregnancy was obtained by postal questionnaires sent in first, second and third trimester. Response rates for these questionnaires were 91%, 80% and 77%, respectively[8]. Maternal smoking at enrollment was assessed in the first questionnaire by asking the mother whether she smoked during the pregnancy (no, yes, until pregnancy was known). In the second and third trimester questionnaires, the mothers were asked whether they had smoked (no, yes) during

second and third trimester, respectively. Mothers who reported to have smoked until pregnancy was known (first trimester only) were considered as non-smokers. Mothers who reported smoking in the second or third questionnaire, were considered as mothers who continued smoking during pregnancy[9]. Among mothers who continued smoking, the number of cigarettes was classified into the following categories: less than 5 cigarettes per day, 5–10 cigarettes per day and more than 10 cigarettes per day.

Genotyping

Maternal DNA was extracted from whole blood samples. Genotyping of the G>T substitution of rs1051730 in mothers was performed using TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). Child DNA was isolated from cord blood. Genotyping was carried out using the Illumina 610k Quad arrays. The frequency distribution of maternal rs1051730 genotype did not deviate from the Hardy-Weinberg equilibrium in subjects with European ancestry (GG: 45.4%, GT: 43.1%, TT: 11.6%, *P*-value= 0.78). Distribution of fetal genotype was similar (GG: 47.3%, GT: 41.8%, TT: 10.8%, *P*-value= 0.73).

Fetal growth and birth outcomes

Fetal ultrasound examinations were carried out in two dedicated research centers in each trimester of pregnancy. Reliability and reproducibility of these ultrasound examinations was tested in early pregnancy and was very good (interobserver intraclass correlation coefficients above 0.988 for all fetal characteristics and interobserver coefficient of variation all between 2.4% and 3.8%)[10]. Fetal ultrasound examinations were used for both establishing gestational age in early pregnancy and assessing second and third trimester fetal growth characteristics. These methods have previously been described in detail[8]. Establishing gestational age by the first day of the last menstrual period is not reliable for a variety of reasons, including the large number of women who do not know their exact date, have irregular menstrual cycles or amenorrhea, use oral contraceptive pills or bleed in early pregnancy[11-12]. Pregnancy dating curves were constructed for subjects with complete data on gestational age measured by ultrasonography and the last menstrual period[12]. Crown-rump length was used for pregnancy dating up to until a gestational age of 12 weeks and 5 days (crown-rump length smaller than 65 mm) and biparietal diameter thereafter (gestational age from 12 weeks and 5 days onwards, biparietal diameter larger than 23 mm). Second and third trimester growth measurements (head circumference, abdominal circumference and femur length) were measured to the nearest millimeter using standardized ultrasound procedures[13]. Estimated fetal weight was calculated using the formula by Hadlock[14]. Gestational age adjusted standard deviation scores were constructed for all fetal growth measurements based on reference growth curves from the whole study population[12]. Information on

date of birth, sex and birth anthropometrics (head circumference, length and weight) was obtained from community midwife and hospital registries. Gestational age and sex-adjusted standard deviation scores for birth anthropometrics were constructed using growth standards from Usher and McLean[15]. Because head circumference and length were not routinely measured at birth, missing birth measures were completed with data from the first month visit at the routine child health center. Of all measurements, 32% and 19% were based on the first month visit for head circumference and birth length, respectively. No differences in genotype between children with measurements at birth and those with measurements at child health center were observed (Chi-square tests: P =0.67 for head circumference and P =0.51 for birth length).

Covariates

Maternal age and information about maternal educational level and parity was obtained by a questionnaire at enrollment in the study. Maternal alcohol consumption habits were assessed by questionnaires in each trimester. Maternal anthropometrics, including height and weight, were measured without shoes and heavy clothing and body mass index (BMI) was calculated (weight/height^2 [kg/m^2]) at enrollment[16].

Statistical analysis

Differences in basic characteristics between mothers who did not smoke and who smoked during pregnancy were assessed by using a Student T-test for continuous variables and Chi-square tests for categorical variables. We performed cross-sectional analyses using linear regression models to assess the associations of the maternal risk allele (T-allele of rs1051730) with fetal growth characteristics in second and third trimester (head circumference, femur length, estimated fetal weight) and at birth (head circumference, body length, weight). Based on previous studies, we considered an additive model, but performed a sensitivity analysis using a T-dominant model. All other analyses were based on an additive model and the effect estimates reflect the effect for each additional copy of the risk allele. Analyses were performed in the total group, and in strata of mothers who did not smoke during pregnancy and mothers who smoked during pregnancy. We tested the interaction between maternal smoking and maternal genotype, to assess whether the associations of maternal smoking with fetal growth were modified by the T-allele of rs1051730. All models were adjusted for sex and gestational age at visit. Analyses in the total population were adjusted for smoking status. To assess possible confounding effects, we additionally adjusted for first trimester smoking quantity as a covariate. Next, a fully adjusted model was explored, with further adjustment for maternal age, body mass index at enrollment, parity, educational level and alcohol consumption. The regression models with neonatal head circumference and length as outcome were additionally adjusted for postconceptional age (gestational age

at birth for measurements at birth or gestational age at birth plus postnatal age for measurement from the child health centers) and for the source of the measurement (birth or child health center). We repeated the analyses for fetal genotype. The associations of smoke status, rs1051730 genotype and longitudinally measured fetal growth were analyzed using unbalanced repeated measurement regression models. The repeatedly measured outcome data for this analysis were the gender and gestational age adjusted SD scores. These models take the correlation between repeated measurements of the same participant into account and allow for incomplete outcome data[17]. We have used a fixed effects model without higher order terms. This approach uses the exogenous sampling maximum likelihood to assess the best model to calculate the effect estimates. The genotype categories of non-smokers and smokers categories were included in these models as intercept and as an interaction term with gestational age. Each individual could have a maximum of three measurements per growth characteristic available. The analyses were based on 3,563 subjects with in total 9,751, 9,843 and 10,405 measurements for head circumference, length, and weight, respectively. For all three growth characteristics, the minimum number of measurements was 1, maximum 3 and median was 3 (90% range 2 -3) for head circumference, length, and weight. The outcomes reflect the differences in growth in gender and gestational age specific standard deviation scores (SDS). The repeated measurement analysis was performed using the Statistical Analysis System version 9.2 (SAS, Cary, NC), including the Proc Mixed module for unbalanced repeated measurements. All other analyses were performed using the Statistical Package of Social Sciences for Windows (SPSS Inc, Chicago, IL, USA), version 17.0.

Results

In our population, 610 (17.1%) mothers continued smoking during pregnancy (Table 1). Mean maternal age was 31.1 years. As compared to mothers who did not smoke during pregnancy, mothers who smoked during pregnancy were shorter, had higher weight and were lower educated. Children of mothers who smoked during pregnancy had a lower birth weight than children of mothers who did not smoke. Distribution of maternal genotype of rs1051730 was different between non-smokers and continued smokers. Genotype was not associated with smoking quantity. Subject characteristics per maternal genotype group among continued smokers are given in Table S1.

In the total population, we did not observe associations of the maternal T-allele of rs1051730 with fetal growth characteristics (Table 2). Among mothers who continued smoking, we observed non-significant tendencies toward smaller (fetal) head circumference for each additional copy of the maternal T-allele of rs1051730. The T-allele of maternal rs1051730 was associated with a smaller second and third trimester fetal femur

Table 1. Subject characteristics of the mothers per smoking status (n=3,563)

	Total	**Non-smokers**	**Continued smokers**
	N= 3,563	**N= 2,953 (82.9%)**	**N= 610 (17.1%)**
Mother			
Age (years)	31.1 (4.5)	31.4 (4.2)	29.7 (5.6)**
Gestational age at enrollment[1] (weeks)	13.6 (10.1 to 23.2)	13.6 (10.2 to 22.9)	14.1 (9.5 to 29.4)**
Height (cm)	170.3 (6.5)	170.4 (6.5)	169.3 (6.5)**
Weight (kg)	70.2 (12.6)	70.0 (12.2)	71.2 (14.0)*
Body mass index (kg/m^2)	24.2 (4.1)	24.1 (4.0)	24.8 (4.5)**
Parity (% nullipara)	59.8	60.4	57.4
Highest education finished (%)			
Primary school	4.4	2.6	13.3**
Secondary school	38.5	33.7	62.5**
Higher education	57.1	63.8	24.2**
Alcohol consumption during pregnancy (% yes)	65.3	66.2	61.3*
Number of cigarettes smoked (%)			
< 5 per day	NA	NA	45.2
5-10 per day	NA	NA	32.7
>10 per day	NA	NA	22.0
Genotype (%)			
G/G	45.4	46.3	41.0*
G/T	43.1	42.2	47.4*
T/T	11.6	11.5	11.6
Child			
Gestational age at birth[1] (weeks)	40.1 (35.6 to 42.3)	40.3 (35.8 to 42.3)	40.0 (34.8 to 42.3)**
Birth weight (grams)	3476 (558)	3514 (553)	3290 (546)**
Sex (% Boys)	49.6	49.1	52.5

Values are means (SD) or percentages, [1] Median (95% range)

Differences in distributions between groups were evaluated using a Student T-test for continuous variables and Chi-square tests for categorical variables *P-value <0.05; **P-value <0.01

length [differences -0.23 mm (95%CI -0.45 to -0.00) and -0.41 mm (95%CI -0.69 to -0.13), respectively] and tended to be associated with a smaller birth length [difference -2.61 mm (95%CI -5.32 to 0.11)] (Table 2). For all fetal length measures, we found a significant interaction between maternal smoking status and maternal genotype of rs1051730 [$P_{interaction}$<0.05]. Among mothers who smoked during pregnancy, the T-allele was associated with a lower third trimester estimated fetal weight [difference -32.7 grams (95%CI -55.4 to -10.0)], and tended to be associated with lower birth weight [difference -38.1 grams (95%CI -89.2 to 12.9)]. Among the mothers who smoked during pregnancy, those who had a T/T genotype gave birth to children with a birth weight that was 117 grams lower (95% CI -229 to -4 grams), as compared to children whose mothers had a G/G genotype. We found a significant interaction between maternal smoking status and maternal genotype of rs1051730 for fetal weight at all time points [$P_{interaction}$<0.05].

Table 2. Cross-sectional associations of maternal rs1051730 genotype with fetal growth characteristics in different trimesters (n= 3,563)

	Second trimester	**Third trimester**	**Birth**
	Head circumference	**Head circumference**	**Head circumference**
	Difference (95% CI) (mm)	Difference (95% CI) (mm)	Difference (95% CI) (mm)
Total Group *N= 3,546*	-0.26 (-0.56 to 0.04)	0.11 (-0.33 to 0.55)	0.16 (-0.63 to 0.95)
Non-smokers *N= 2,940*	-0.23 (-0.55 to 0.10)	0.23 (-0.25 to 0.71)	0.31 (-0.55 to 1.17)
Smokers *N= 606*	-0.43 (-1.21 to 0.35)	-0.44 (-1.55 to 0.66)	-0.67 (-2.71 to 1.37)
Interaction[1]	*P=0.62*	*P=0.24*	*P=0.39*
	Femur length	**Femur length**	**Body length**
	Difference (95% CI) (mm)	Difference (95% CI) (mm)	Difference (95% CI) (mm)
Total Group *N= 3,549*	0.00 (-0.09 to 0.09)	-0.01 (-0.12 to 0.11)	0.33 (-0.77 to 1.42)
Non-smokers *N= 2,943*	0.04 (-0.05 to 0.14)	0.08 (-0.04 to 0.20)	0.82 (-0.38 to 2.02)
Smokers *N= 606*	-0.23 (-0.45 to -0.00)*	-0.41 (-0.69 to -0.13)**	-2.61 (-5.32 to 0.11)
Interaction[1]	*P=0.03*	*P<0.01*	*P=0.04*
	Estimated fetal weight	**Estimated fetal weight**	**Weight**
	Difference (95% CI) (g)	Difference (95% CI) (g)	Difference (95% CI) (g)
Total Group *N= 3,563*	0.28 (-1.88 to 2.43)	3.05 (-6.23 to 12.32)	15.95 (-5.59 to 37.48)
Non-smokers *N= 2,953*	1.36 (-0.99 to 3.71)	10.37 (0.22 to 20.51)*	26.81 (3.05 to 50.57)*
Smokers *N= 610*	-5.36 (-10.73 to 0.01)	-32.66 (-55.36 to -9.97)**	-38.13 (-89.16 to 12.90)
Interaction[1]	*P=0.02*	*P<0.01*	*P=0.03*

Effect estimates (with 95% confidence interval) reflect the differences in each growth characteristic for each additional copy of the T-allele of rs1051730 (assuming an additive model)

[1]Interaction term = maternal genotype x smoking status

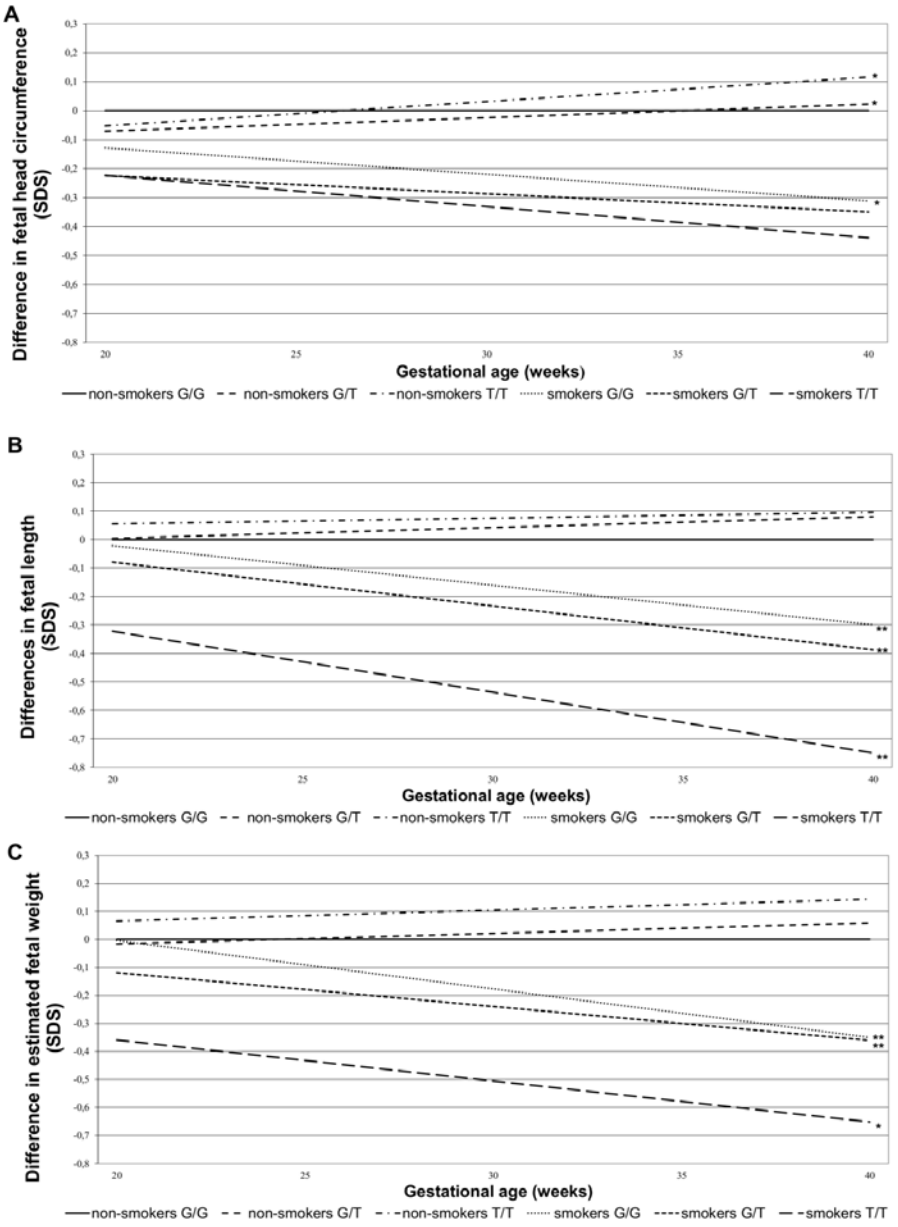
**P*-value <0.05; ***P*-value <0.01

All analyses were adjusted for gestational age at visit and sex. Analyses in the total group were additionally adjusted for smoking status (yes, no). Birth length and head circumference at birth were additionally adjusted for source of the birth measurements

In Figure 2, the results of maternal genotype of rs1051730 per smoking status with longitudinal fetal growth patterns are presented as difference in standard deviation scores, adjusted for gestational age and sex. This gives the relative effect in different periods of pregnancy. All genotype categories of mothers who smoked during pregnancy had smaller fetal head circumference, length and weight measures, as compared to the mothers who did not smoke during pregnancy. Among mothers who did not smoke during pregnancy, the maternal T-allele of rs1051730 had a positive effect on head circumference growth, but was not significantly associated with length and weight growth. Among mothers who smoked during pregnancy, the T-allele was associated with the smallest head circumference, length and weight growth. The effects seem to be larger for length and weight than for head circumference. The specific coefficients of the gestational age independent and dependent differences (interaction smoke status, rs1051730 genotype and gestational age) for these models are given in Table S2.

We performed several sensitivity analyses. First, results of the analyses with a dominant genetic model are shown in Table S3. Second, additional analyses showed that the fetal genotype of rs1051730, was not associated with any fetal growth characteristic

Figure 2. Effect of maternal genotype and smoking status on fetal growth characteristics (SD scores)



Values are based on repeated linear regression models and reflect the differences in growth in gender and gestational age specific standard deviation scores (SDS) between the number of risk alleles and smoking status compared to the reference group (genotype G/G, non-smokers). Estimates are given in the Supplementary Table S2.
**P*-value <0.05; ***P*-value <0.01
(Figure 1a= head circumference growth, Figure 1b= length growth, Figure 1c= weight growth)

after adjustment for the maternal genotype (Table S4). Furthermore, adjustment for maternal smoking quantity did not affect our results (Table S5). Additional adjustment for maternal age, body mass index at enrollment, parity, educational level and alcohol consumption slightly attenuated the effect of maternal rs1051730, although the directions of the effects remained similar (Table S6). In these fully adjusted models, the interaction of maternal smoking and genotype remained significant in third trimester of pregnancy.

Discussion

Main findings

Results from this prospective population-based cohort study suggest that maternal genotype of the common genetic variant, rs1051730, located within the 15q25 nicotinic acetylcholine receptor gene cluster (*CHRNA5-CHRNA3-CHRNB4*), influences the susceptibility of impaired fetal growth by maternal smoking. Among mothers who smoked during pregnancy, the T-allele resulted in smaller femur length and lower estimated fetal weight from second trimester onwards.

Methodological considerations

An important strength of this study was the prospective design and the large sample size of 3,563 participants being studied from early pregnancy onwards. To our knowledge, this study is the first study that examined the associations of maternal smoking during pregnancy, and rs1051730, with fetal growth characteristics in different trimesters of pregnancy. A potential limitation of our study is that information about smoking during pregnancy was collected by questionnaires. Although assessing smoking during pregnancy by questionnaire seems to be a valid method, misclassification may occur[18]. To overcome these limitations, previous studies have used biomarkers of tobacco exposure, including cotinine -a metabolite of nicotine- in maternal urine samples[19-20]. However, it has been demonstrated that use of cotinine levels is not superior to the use of self-reporting questionnaires in studying the effect of maternal smoking in pregnancy on birth weight[21]. In general, confounding is not likely to be a major issue in gene - outcome association studies. The unadjusted and adjusted models focused on the associations of rs1051730 genotype with fetal growth characteristics did not show large differences in effect estimates. However, smoking quantity might be a relevant confounder in our study, since rs1051730 is known to be associated with smoking quantity[4]. We adjusted the analyses for the number of cigarettes smoked per day reported in first trimester, which did not change the effect estimates. Furthermore, in our population of mothers who smoked during pregnancy, smoking quantity was not associated with rs1051730 genotype in neither first, second nor third trimester.

We also performed a sensitivity analysis using second and third trimester smoking quantity instead of first trimester smoking quantity in the models, which did not alter our results. Therefore it seems unlikely that smoking quantity has biased our results. Another potential limitation could be that women who quitted smoking when pregnancy was known (first trimester only) were considered as non-smokers in our analyses. This could have resulted in biased effect estimates if fetal growth would be different between quitted smokers and non-smokers. However, we have previously reported that first trimester smoking only does not affect second and third trimester fetal growth[9]. We performed a sensitivity analysis in which we excluded women who quitted smoking in first trimester, which did not change our results (data not shown).
We established gestational age by ultrasound. By using this method, growth variation of the fetal characteristics used for pregnancy dating is assumed to be zero. In our study, first trimester crown-rump length and biparietal diameter were used for pregnancy dating but not for assessing fetal growth. Since pregnancy dating characteristics and growth characteristics are correlated throughout pregnancy, growth variation in head circumference, abdominal circumference, and femur length may be reduced by dating the pregnancy on the other fetal characteristics. This may have led to underestimation of our effect estimates. However, we expect this effect to be small in our results. This underestimation will consequently be lowest in women included in the first trimester (54.7% of the population for analysis). We performed a sensitivity analysis using only these women, but results did not change (data not shown).

Maternal rs1051730 genotype, smoking during pregnancy and fetal growth
The effects of maternal smoking status during pregnancy on fetal growth have been studied previously, showing that among mothers who continued smoking during pregnancy, children had smaller femur length from second trimester onwards and smaller head circumferences from third trimester onwards, as compared to children from mothers who did not smoke during pregnancy[9]. As compared to children of mothers who did not smoke during pregnancy, those of mothers who smoked during pregnancy have a 100 to 200 grams lower birth weight[1]. This fetal growth restriction might be caused by developmental adaptations in placental vasculature and adaptations in fetal arterial resistance. A study that investigated these adaptations showed an increased umbilical resistance artery pulsatility index (PI) in pregnant women who smoked, which indicates higher feto-placental resistance. Also, a higher umbilical artery PI was associated with lower estimated fetal weight and birth weight[22].
The effects of maternal smoking on fetal growth are known to differ between individuals. We have previously demonstrated that maternal first trimester folic acid supplementation use reduces the adverse effects of maternal smoking during pregnancy on fetal growth[23]. Differences in effects of maternal smoking on fetal growth might also be

explained by maternal genetic variation. We hypothesized that maternal genotype of rs1051730 modifies the association of maternal smoking during pregnancy with fetal growth. The T-allele of rs1051730 is associated with higher smoking quantity and higher nicotine levels in adults[4-5, 24]. A previous study showed that children from mothers who smoked during pregnancy, had a tendency towards a 28 grams lower birth weight per T-allele. No association was found among mothers who did not smoke during pregnancy[6]. In line with this previous study, we observed that among mothers who smoked during pregnancy, the T-allele resulted in smaller femur length and lower estimated fetal weight from second trimester onwards. We did not observe any effect on fetal head circumference. The effects of maternal smoking in fetal head circumference seem not to be affected by rs1051730. Our results also suggest that rs1051730 does not have a direct effect on birth weight, but needs environmental factors to exert its effect. Surprisingly, among non-smokers, we found a positive effect on fetal weight with each copy of the risk allele. We can not clearly explain this finding. It might me hypothesized that specific gene-environmental interactions, such as DNA methylation, changes the effects of specific genetic variants[25]. In a recent meta-analysis, the risk allele of rs1051730 was associated with a lower body mass index among smokers, while some studies of this meta-analysis showed a higher body mass index among non-smokers with the risk allele. However, the overall effect of the meta-analysis showed no effect of genotype among non-smokers[26]. We think that further research is necessary to explore the different effects of risk alleles of rs1051730 on various health outcomes among both smokers and non-smokers. The mechanism through which rs1051730 modifies the associations between maternal smoking and fetal growth is yet unknown. Rs1051730 is located in the nicotinic acetylcholine receptor gene cluster (*CHRNA5-CHRNA3-CHRNB4*) on chromosome 15q25. This locus has been investigated previously and common genetic variants in high linkage disequilibrium with rs1051730 (HapMap $r^2 > 0.8$) were associated with higher circulating cotinine levels, but not with number of cigarettes smoked per day[24]. A previous study that assessed the association of rs1051730 with the risk of lung cancer, observed that subjects with the T-allele had higher mean nicotine equivalents. In this study, the T-allele seemed to behave as a dominant model after adjustment for smoking quantity[5]. The authors therefore concluded that T-allele carriers extract a greater amount of nicotine per cigarette and are exposed to a higher internal dose of tobacco-specific nitrosamine, as compared to subjects without the T-allele[5]. Whether this is caused by more intense smoking or by a change in the nicotine metabolism, is not clear yet. Other studies suggested an additive genetic model for the T-allele of rs1051730[6, 27], which was also most compliant with our data. Thus our results suggest that children of mothers who smoked during pregnancy are exposed to higher levels of nicotine when the mother has a T-allele of rs1051730. Therefore, these children might be more susceptible to the

adverse effects of maternal smoking during pregnancy. Further research is necessary to determine the exact mechanism and underlying genetic model.

Gene-environment interaction studies are of increasing importance in modern medicine. Although smoking cessation should be advised to all pregnant women, a genetic risk profile might help us to target more effectively those at higher risk of growth retardation and adverse pregnancy outcomes. Further research is needed to identify groups at risk and develop new preventive strategies.

Conclusions

Our results suggest that maternal genotype of the 15q25 variant rs1051730 influences the susceptibility of impaired fetal growth in length and weight by maternal smoking. This association of rs1051730 with fetal growth was present in mothers who continued smoking during pregnancy, but not in mothers who did not smoke during pregnancy. These results should be considered as hypothesis generating and indicate the need for large-scale genome wide association studies focusing on gene – fetal smoke exposure interactions.

Note: Supplementary information is available on the Plos One website: www.plosone.org

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Chapter 2.5

A genome-wide association meta-analysis on childhood obesity

Jonathan P Bradfield *
H Rob Taal * et al.
For the Early Growth Genetics (EGG) Consortium

**Authors contributed equally to this work*
For the complete list of authors please see list of publications
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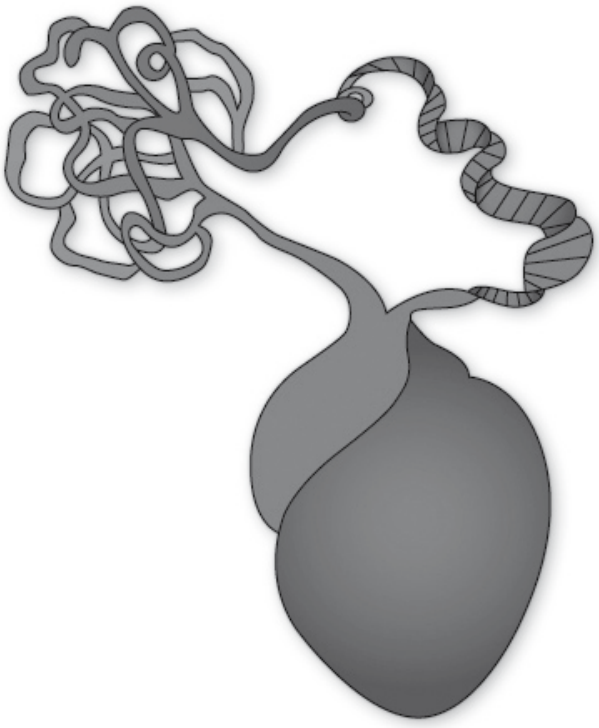

Abstract

Aims and Methods: Multiple genetic variants have been associated with adult obesity and a few with severe obesity in childhood; however, less progress has been made to establish genetic influences on common early-onset obesity. We performed a North American-Australian-European collaborative meta-analysis of fourteen studies consisting of 5,530 cases (≥95th percentile of body mass index (BMI)) and 8,318 controls (<50th percentile of BMI) of European ancestry.

Results and Conclusions: Taking forward the eight novel signals yielding association with $P < 5x10^{-6}$ in to nine independent datasets (n = 2,818 cases and 4,083 controls) we observed two loci that yielded a genome wide significant combined P-value, namely near *OLFM4* on 13q14 (rs9568856; $P=1.82x10^{-9}$; OR=1.22) and within *HOXB5* on 17q21 (rs9299; $P=3.54x10^{-9}$; OR=1.14). Both loci continued to show association when including two extreme childhood obesity cohorts (n = 2,214 cases and 2,674 controls). Finally, these two loci yielded directionally consistent associations in the GIANT meta-analysis of adult BMI.

Introduction

Obesity is the major, increasingly prevalent health problem affecting modern societies. The problem is particularly severe for children in developed countries, where the prevalence of obesity is on the increase. Obesity present in adolescence is associated with increased overall mortality in later life[1]. Whereas the change in prevalence of obesity is likely to be explained by environmental changes over the last 30 years, there is also strong evidence for a genetic component to the risk of obesity. This is reflected in familial occurrences of childhood obesity, where concordance for fat mass among monozygotic twins is reported to be higher than in dizygotic twins.

In the past four years, many genetic loci have been implicated for body mass index (BMI) / obesity from the outcomes of genome-wide association studies (GWAS), primarily in adults. The first locus reliably found to harbor variation associated with adiposity, the fat mass- and obesity-associated gene (*FTO*)[2], has been shown subsequently to be associated with obesity in all sufficiently sized study groups. Subsequent larger studies have revealed additional BMI/obesity genes. The largest meta-analysis of adult BMI to date came from the GIANT consortium, which confirmed fourteen known obesity susceptibility loci and revealed eighteen new loci associated with BMI in a study involving a total of 249,796 individuals[3]. However, these loci only account for a small fraction of the heritability that is known to contribute to obesity. There has been some work on extreme obesity in childhood (>99.5th percentile of BMI) but little progress has been made on less marked definitions of obesity more relevant to public health.

We reasoned that distillation of the genetic component in this complex phenotype should be easier in children, where environmental exposure and impact has been for a relatively short period of their lifetime. The relationship between BMI and body fat in children varies widely with age and with pubertal maturation. The Center for Disease Control and Prevention defined overweight as at or above the 95th percentile of BMI for age[4]. By late adolescence, these percentiles approach those used for adult definitions; the 95th percentile is then approximately 30 kg/m^2 [5].

Methods

In an effort to systematically search for childhood obesity susceptibility loci, we performed a large scale meta-analysis of fourteen existing GWAS datasets for childhood obesity, totaling 5,530 cases (≥95th percentile of BMI achieved before the age of 18 years old, representing 5-30% of any given cohort) and 8,318 controls (relatively conservatively defined as <50th percentile of BMI consistent throughout all measures during childhood) of European ancestry (see Supplementary Table 1 and Supplementary Note).

Results

Following the meta-analysis of 2.7 million SNPs (directly genotyped or imputed), signals at seven discrete locations reached genome-wide significance at $P < 5.0x10^{-8}$. All these loci have been previously reported within GWAS for adult BMI (*FTO, TMEM18, POMC, MC4R, FAIM2, TNNI3K and SEC16B*), and robustly reflect previous reports on individual pediatric cohorts[6-7]. *FTO* gave the strongest evidence for association while *TNNI3K* and *POMC*, which were only detected in adult studies when using hundreds of thousands of participants, were readily detected in our relatively small sample size (Figure 1 and Supplementary Tables 2 and 3). Excluding the French and German studies from the meta-analysis, we did not observe association with variants previously reported as novel in their extreme childhood obesity cohorts[8] at the loci harboring *TNKS-MSRA* (rs17150703; $P = 0.22$) and *SDCCAG8* (rs12145833; $P = 0.57$).

We took forward all eight novel signals yielding association with $P < 5.0x10^{-6}$ (Table 1 and Supplementary Table 4, the latter of which includes a heterogeneity analysis showing that the different distributions in each study did not affect our results; in addition Supplementary Table 5 shows the results after applying a second genomic control correction to the overall discovery meta-analysis results) in order to test for replication in multiple independent existing datasets, the majority of which were *in silico* analyses. In our replication effort, we initially tested these eight SNPs in nine study groups that had a

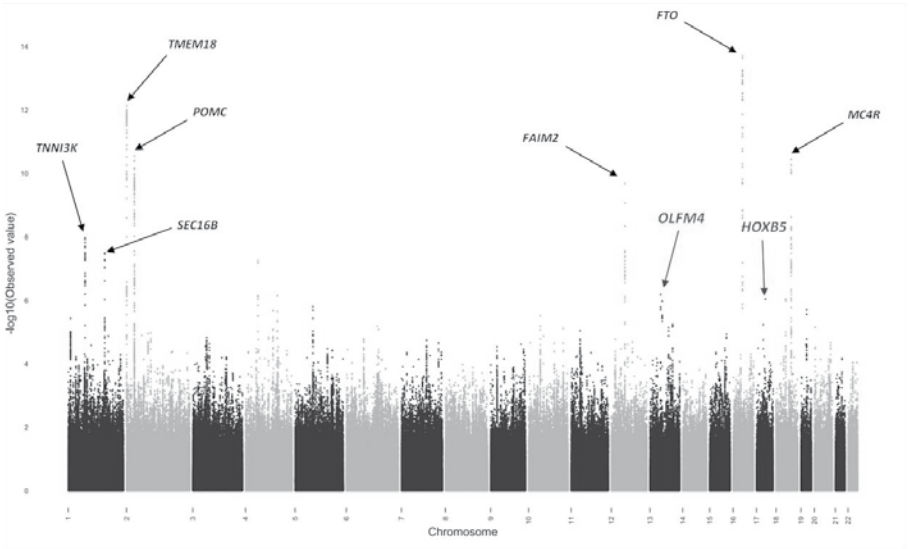

Figure 1

Manhattan Plot of the meta-analysis of childhood obesity GWAS runs in the discovery stage (5,530 cases and 8,318 controls), with each locus achieving genome wide significance ($P < 5x10^{-8}$) indicated in black text. In addition, the novel loci uncovered in this study are indicated in red text.

Table 1: The two key novel loci established to be associated with common early-onset obesity.

	Locus	SNP	Allele1/2	Nearest Gene	Direction	OR [95% C.I.]	P-value
Discovery	13q14	rs9568856	A/G	OLFM4	+++++++++++-+	1.210 [1.123, 1.305]	$6.58x10^{-7}$
	17q21	rs9299	T/C	HOXB5	++-++-++++++++	1.144 [1.084, 1.207]	$9.12x10^{-7}$
Replication	13q14	rs9568856	A/G	OLFM4	+-++++-++	1.225 [1.089, 1.378]	$7.13x10^{-4}$
	17q21	rs9299	T/C	HOXB5	++-++++++	1.145 [1.056, 1.242]	0.00104
Combined	13q14	rs9568856	A/G	OLFM4		1.215 [1.140, 1.294]	$1.82x10^{-9}$
	17q21	rs9299	T/C	HOXB5		1.144 [1.094, 1.196]	$3.54x10^{-9}$

The two loci did not reach genome wide significance but yielded $P < 5x10^{-6}$ in the discovery stage (5,530 cases and 8,318 controls). The outcome of the replication effort of these loci taken forward in to nine comparable independent cohorts (n = 2,818 cases and 4,083 controls) is also indicated. Separate discovery and replication data plus combined data are shown, with the latter indicating genome wide significance in both instances.

comparable set of affected subjects i.e. BMI distributed normally from the 95th percentile upwards (n = 2,818 cases and 4,083 controls). From this attempt we observed two loci that yielded consistent evidence of association when combined with the discovery cohort, namely near olfactomedin 4 (*OLFM4*) on 13q14 (rs9568856; $P_{combined} = 1.82x10^{-9}$; OR = 1.22) and within the gene encoding homeobox B5 (*HOXB5*) on 17q21 (rs9299; $P_{combined} = 3.54x10^{-9}$; OR = 1.14) (Table 1; see also the regional plots in Supplementary Figures 1 and 2 for the discovery meta-analysis data).

Previous GWAS reports for extreme obesity case-control samples have demonstrated both confirmation of signals seen in less extreme or population based samples, such as *FTO*, as well as novel signals that are distinct from those seen at the population level[8]. We reasoned therefore that further exploration in existing extreme datasets [two cohorts totaling 2,214 cases (exclusively individuals approximately >4 standard deviations above the mean, equating to BMI>99.5th percentile] and 2,674 controls) would offer further insight in to how these signals operate, acknowledging the phenotypic differences and limits of sample size. Indeed, both loci emerging from the main replication step continued to show association folding in this more extreme phenotype (*OLFM4*; rs4833407; $P_{overall} = 5.33x10^{-9}$; OR = 1.18 and *HOXB5*; rs9299; $P_{overall} = 1.54x10^{-8}$; OR = 1.13) (Supplementary Table 6).

As the ALSPAC cohort leveraged BMI measures made before the age of two years old as part of defining cases and controls, we ran sensitivity analyses limiting case and control definitions to children over two years of age (Supplementary Tables 7-9). In addition to no diminishment in the odds ratios for the *OLFM4* and *HOXB5* loci, we observed support for rs4864201 at *BC041448* and rs4833407 at *ALPK1* (Supplementary Tables 8 and 9).

Finally, we were interested to see whether our two main signals of interest, namely at *OLFM4* and *HOXB5*, were evident in the GIANT adult BMI meta-analysis results (n = 123,864). Indeed, both loci yielded evidence of association in this quantitative setting

(*P*-values= $7.75x10^{-5}$ and 0.015, respectively) with the same alleles in the same direction. Overall, seven of the eight signals initially taken forward in to the replication stage yielded consistent directionality, albeit not all being statistically significant, with the exception being rs1290002 (Supplementary Table 10).

Discussion

Overall, these data indicate that the genetic architecture of BMI and obesity overlap to a large extent in children as well as adults. In addition to the previously reported loci, we have uncovered at least two new loci associated with obesity in early life. The adult BMI data available from GIANT[3] reveals that the influence of these two loci is also detected in adulthood. Interestingly, in addition to *OLFM4* and *HOXB5*, GIANT also supports an association with three more of the eight loci initially taken forward in to the replication effort, namely rs4864201 at *BC041448*, rs4833407 at *ALPK1* and rs2300095 at *MTOR-ANGPTL7* loci, despite these signals not formally replicating in the main defined overall pediatric setting, suggesting that these loci should be followed up further to fully understand their role in the pathogenesis of obesity as a whole.

The gene encoding olfactomedin 4 (*OLFM4*) is the nearest gene to rs9568856 but is still approximately 500kb from the associated signal; the gene product has never been directly implicated in obesity but has been extensively studied in the context of various cancers. OLFM4 is a secreted glycoprotein that facilitates cell adhesion via lectins and cadherin on the cell surface. Although the function of OLFM4 is not well understood, there are several intriguing observations that link it to gut microflora and to a relationship between the gut microbiome and obesity risk. For example, the *OLFM4* gene product down regulates innate immunity against infection by the stomach bacterium, *Helicobacter pylori*[9], with obese subjects having a higher occurrence of *Helicobacter pylori* infection than lean counterparts[10-11]; indeed, weight-loss induced by obesity surgery eradicates *Helicobacter pylori*[12].

rs9299 is in the 3′ untranslated region of the gene encoding homeobox B5 (*HOXB5*) within a homeobox B cluster. HOXB5 is spatially and temporarily regulated during gut development[13], but its role in obesity has been suggested by a study observing up-regulation of homeobox transcription factors after fat loss[14]. Taken together, it is possible that *OLFM4* and *HOXB5* may impact BMI via different aspects of gut function.

Conclusions

In summary, as a consequence of extensive North American-Australian-European collaborative genome-wide meta-analyses on children, we have uncovered two novel obesity loci which have their strongest evidence for association with elevated adiposity in the first eighteen years of life. Further functional characterization of these signals is required to elucidate the precise mechanism behind these observations.

Note: Supplementary information is available on the Nature Genetics website: www.nature.com/ng

Online Methods

Research Subjects

The descriptions of the individual cohorts are presented as a Supplementary Note. The discovery set for the meta-analysis consisted of 14 studies with body mass index (BMI) measured in childhood (age range 2-18 years, except for ALSPAC, which also leveraged BMI data available from the first four clinical examinations prior to 2 years old) and genome-wide genotype data available by the beginning of May 2010): the Avon Longitudinal Study of Parents and Children (ALSPAC, n= 976 cases / 1,244 controls); Northern Finland 1966 Birth Cohort (NFBC1966, n= 700 cases / 521 controls); British 1958 Birth Cohort – Type 1 Diabetes Genetics Consortium subset (B58C-T1DGC, n= 192 cases / 367 controls); British 1958 Birth Cohort – Wellcome Trust Case Control Consortium Subset (B58C-WTCCC, n= 188 cases / 428 controls); French Young study (FRENCH YOUNG, 670 cases/ 349 controls); Lifestyle Immune System Allergy Study (LISA, n=27 cases / 250 controls); Western Australian Pregnancy Cohort study (RAINE, n= 232 cases / 125 controls); Children's Hospital of Philadelphia (CHOP, n= 1,445 cases / 2,802 controls); Essen Obesity Study (ESSEN, n=397 cases / 435 controls); Helsinki Birth Cohort Study (HBCS, n= 261 cases/ 403 controls); Cardiovascular Risk in Young Finns Study (YF, n= 167 cases / 537 controls); Copenhagen Study on Asthma in Childhood (COPSAC, n= 62 cases / 99 controls); CM-GOYA study (CM-GOYA; n= 21 cases / 34 controls) and Generation R Study (GENERATIONR, n= 192 cases / 724 controls).

The phenotypically comparable cohorts used for the replication effort were Healthy Lifestyle in Europe by Nutrition in Adolescence study (HELENA; n= 56 cases / 563 controls), Young Hearts studies (n= 44 cases / 450 controls), the Lifestyle – Immune System – Allergy Study plus German Infant Study on the influence of Nutrition Intervention (LISA+GINI, n= 40 cases / 457 controls), Children's Health Study (CHS; n= 311 cases / 330 controls), Avon Longitudinal Study of Parents and Children (ALSPAC; n= 1,452 cases /

1,042 controls), INfancia y Medio Ambiente [Environment and Childhood] Project (INMA; n= 55 cases / 213 controls), Project Viva (VIVA; n= 48 cases / 184 controls), Prevention and incidence of asthma and mite allergy birth cohort study (PIAMA; n= 68 cases / 85 controls) and the Northern Finland 1986 Birth Cohort (NFBC1986; n= 744 cases / 759 controls). The two extreme obesity replication cohorts consisted of 705 German trios and SCOOP-UK cohort (n= 1,509 cases / 2,674 controls). Selected signals were further investigated in the GIANT[1] cohort, using the publically available dataset at http://www.broadinstitute.org/collaboration/giant/index.php/GIANT_consortium_data_files.

All cases and controls were of European ancestry. Cases were defined as having a BMI>95th percentile at any point in childhood. Controls were defined as consistently having a BMI<50th percentile throughout childhood for all measurements available for that individual. BMI percentiles were based on national standard growth curves, except in the Helsinki Birth Cohort Study (HBCS) and the Northern Finland 1966 Birth Cohort (NFBC1966) as pediatric measurements were made two decades ago, thus contemporary curves will not be appropriate. HBCS and NFBC1966 generated their own reference curves. In addition, the density of data available longitudinally in the ALSPAC study gave rise to two differences in cases/control definition. Firstly, this collection factored in subjects from the first four clinical examinations of childhood and thus participants less than 2 years old in their consideration of trait definition (sensitivity analyses considering the use of data from participants limited to being over the age of 2 years old are included in Supplementary Tables 7-9). Secondly, owing to the regularity of measures (11 measures available), controls in the ALSPAC sample were defined as those BMI<50th percentile on at least 5 occasions. Known syndromic cases of obesity were excluded, since these individuals are likely to have a different underlying genetic architecture. Unless otherwise noted, all discovery sample analysis followed the same protocol and analysis plan.

Informed consent was obtained from all discovery study participants (or parental consent, as appropriate), and study protocols were approved by the local ethics committees.

Statistical approaches

Stage 1: GWA meta-analysis of childhood obesity

Statistical analysis within discovery samples

Genotypes were obtained using high-density SNP arrays, and then imputed for ~2.54 million HapMap CEU SNPs (Phase II, release 22, http://hapmap.ncbi.nlm.nih.gov). Prior to imputation, we excluded SNPs with a Hardy-Weinberg equilibrium *P*-value (HWEP) < $1.0x10^{-6}$, call rate < 95 percent and minor allele frequency < 1 percent. Post imputa-

tion, SNPs imputed with IMPUTE were excluded if the proper info was < 0.40 and SNPs imputed with MACH were excluded if the r2hat was < 0.30. SNPs were also excluded post imputation if the minor allele frequency was < 1 percent. The association between each SNP and case-control status was assessed in each study sample using logistic regression of case-control against genotype, assuming an additive model and taking into account genotype uncertainty. Imputed genotypes were only used where directly-assayed genotypes were unavailable. Unless otherwise stated, all discovery analysis followed the former protocol.

Meta-analysis of discovery samples

Prior to meta-analysis, SNPs with a minor allele frequency <1% and poorly-imputed SNPs (proper_info ≤0.4 [SNPTEST]; r^2 ≤0.3 [MACH2QTL]) were filtered. Fixed effects meta-analyses were conducted by two independent investigators (J.B., H.R.T.). Meta-analysis was performed using the software package: METAL (http://www.sph.umich.edu/csg/abecasis/metal/index.html); Genomic control[15] was applied to each cohort prior to meta-analysis. Meta-analysis was carried out using the inverse-variance method, fixed effects model was assumed. SNPs available for less than half of the total expected sample were excluded. We used the Cochran Q test to assess evidence of between-study heterogeneity of effect sizes.

A total of 2.7 million SNPs were analyzed in the meta-analysis unfiltered for the number of cohorts they appear in. Seven SNPs reached genome wide significance, all of which were reported previously in the adult BMI GWAS[1,16]. Those loci that reached a *P*-value threshold of $<5x10^{-6}$ in the discovery meta-analysis, and were not identified with obesity related traits before (n = 8), were considered for further follow-up in additional samples.

Stage 2: Follow-up of three lead signals in additional samples

Follow-up samples, genotyping and analysis

We used nine study samples representing a comparable dataset (combined n = 2,818 cases and 4,083 controls) and two study samples representing an extreme obesity dataset (combined n = 2,214 cases and 2,674 controls) to follow up the eight novel signals from the GWAS discovery meta-analysis (represented by index SNPs: rs2300095, rs4833407, rs4864201, rs28636, rs1290002, rs9568856, rs9299 and rs17697518). If the index SNP was unavailable, the most closely correlated proxy available was substituted. In four of the replication studies, the index SNPs were imputed from genome-wide genotype data.

Meta-analyses

We performed fixed effects inverse variance meta-analyses of the association results for the eight lead signals in the fourteen discovery samples and the nine comparable replication samples. We subsequently did the same with the fourteen discovery samples combined with the two extreme cohorts. Lastly we combined all datasets for the final overall meta-analysis. Fixed effects meta-analyses were conducted independently by two investigators (J.B, H.R.T.), again using METAL.

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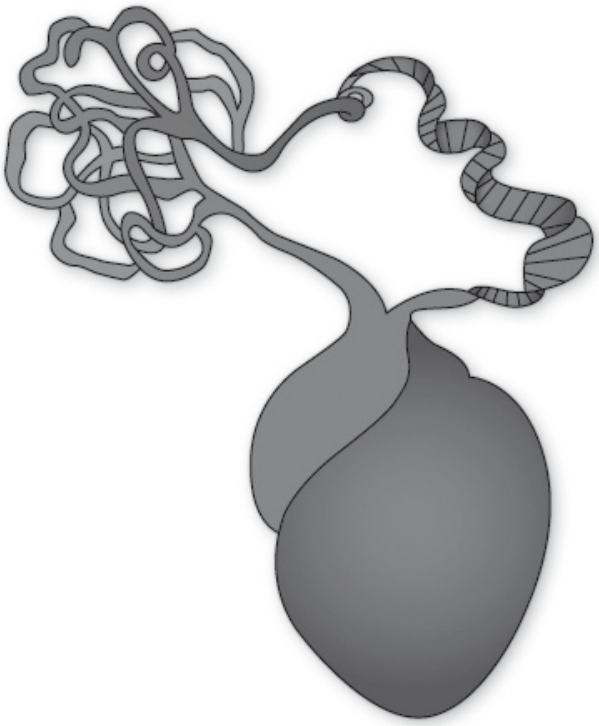
Chapter 3

Childhood cardiovascular structure and function

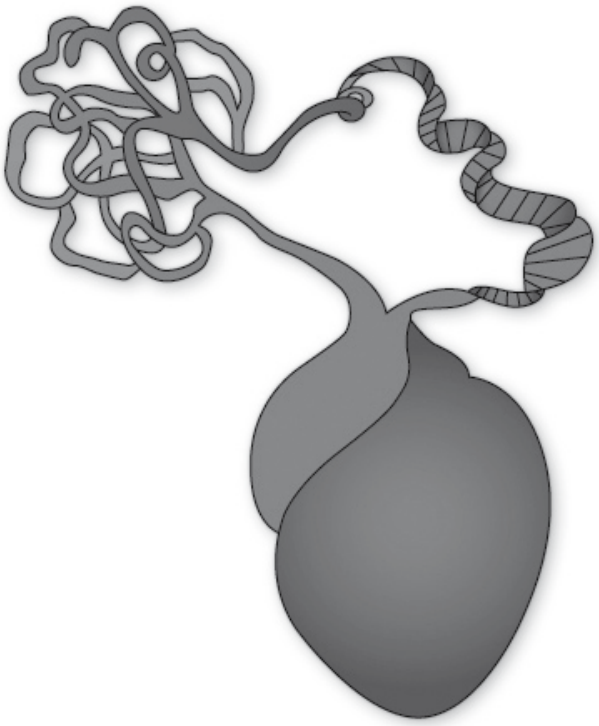


Chapter 3.1

Fetal and infant growth and childhood cardiovascular development

Layla L de Jonge
H Rob Taal
Denise HM Heppe
Lennie van Osch-Gevers
Albert Hofman
Willem A Helbing
Vincent WV Jaddoe

Submitted



Abstract

Background: Growth in early life influences the susceptibility to cardiovascular disease in adulthood. Early growth induced cardiovascular programming may partly explain this association. We aimed to evaluate the associations of different fetal and childhood growth patterns with cardiovascular development in children.

Methods: In a population-based prospective cohort study among 6,416 children, fetal femur length and estimated fetal weight were assessed by second and third trimester ultrasound examinations. Child length and weight were measured at birth and at the ages of 0.5, 2 and 6 years. Blood pressure, carotid-femoral pulse wave velocity, left atrial diameter, aortic root diameter, left ventricular mass, and fractional shortening were assessed at the age of 6 years.

Results: Decreased birth weight and birth weight for gestational age were associated with higher systolic blood pressure, and smaller left cardiac structures at the age of 6 years (all $P<0.05$). Length and weight gain between second and third trimester of pregnancy were positively associated with aortic root diameter and left ventricular mass. As compared to children with normal fetal and infant weight gain, children with low fetal and high infant weight gain had higher systolic blood pressure, whereas those with both high fetal and infant weight gain had higher left ventricular mass. No consequent effect of early growth on arterial stiffness was observed.

Conclusion: These observations suggest that fetal and child growth patterns influence cardiovascular outcomes in childhood. Early programming of cardiovascular development may partly underlie the mechanisms that link early growth and cardiovascular disease in later life.

Introduction

Epidemiological studies have provided broad evidence that early environmental exposures influence the risk of various health outcomes in adulthood. Low birth weight has been repeatedly associated with cardiovascular disease and its risk factors in later life[1-2]. Follow up studies have shown that individuals with low birth weight and high rates of childhood weight gain have increased risks of cardiovascular disease, suggesting that not birth weight per se, but both fetal and childhood growth are related to cardiovascular morbidity and mortality[3]. The exact growth patterns and underlying biological mechanisms linking early growth with cardiovascular health and disease are not known.

There is accumulating evidence that early growth induces primary cardiovascular programming, introducing a possible mechanism through which the path of early growth initiates future cardiovascular disease. Comprehensive studies in children and adults have shown that early growth variation leads to cardiovascular structural and functional adaptations in early life[4-7]. We have previously demonstrated that third trimester fetal growth variation within the normal range is associated with cardiac remodelling and cardiac output changes consistent with a gradual increase in afterload and compromised arterial compliance[8]. Higher fetal and infant length and weight gain from third trimester of pregnancy to the age of 2 years was associated with a higher systolic and diastolic blood pressure in early childhood[9]. Persisting cardiovascular adaptations might explain part of the association between early growth and cardiovascular disease in adulthood. Detailed studies focused on the effects of specific fetal, and child growth patterns on cardiovascular development in childhood might extend our knowledge on the origins of cardiovascular disease in the earliest phase of life, and may provide a window of opportunity for the early prevention of cardiovascular dysfunction.

In this population-based, prospective cohort study among 6,416 children, we investigated the associations of different fetal, infant and child growth patterns with cardiovascular structures and function at the age of 6 years.

Methods

Design and study population

The study was embedded in the Generation R Study, a population-based, prospective cohort study from fetal life onwards in Rotterdam, the Netherlands[10]. Enrolment in the study was aimed at early pregnancy, but was possible until the birth of child. Fetal and childhood growth were repeatedly assessed by ultrasounds and physical examinations. Between March 2008 and January 2012, all children and their mothers were invited to a dedicated research center to participate in detailed growth and cardiovascular follow up

measurements. Written informed consent was obtained from all parents of participants. In total, N = 9,506 singleton live births participated in the study. Information on birth weight was available in 9,425 children (99%). Of these children and their parents, 6,510 visited the research center (median 6.0 years, 95% range 5.6 – 7.9 years). Blood pressure, carotid-femoral pulse wave velocity, or cardiac ultrasound measurements were performed in 6,455 children. Children with echocardiographic evidence of congenital heart disease or kidney disease were excluded from the study (N = 40), leaving 6,416 children for the current analyses (Figure S1). The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam.

Fetal and infant growth

In a dedicated research facility, fetal ultrasound examinations were carried out in each trimester of pregnancy, using an Aloka® model SSD-1700 (Tokyo, Japan) or the ATL-Philips® Model HDI 5000 (Seattle, WA, USA). First trimester fetal measurements were not included as growth characteristics because these examinations were primarily performed to establish gestational age[11]. Second trimester (median 20.5 weeks, 95% range 18.6 – 23.4) and third trimester (median 30.4 weeks, 95% range 28.4 – 33.1) fetal head circumference (HC), abdominal circumference (AC), and femur length (FL) were measured to the nearest millimetre using standardized ultrasound procedures[11]. Estimated fetal weight (EFW) was calculated using the Hadlock formula[12]. Gestational age adjusted standard deviation scores (SDS) for all fetal growth characteristics were constructed on data from the study group[11]. At birth, information on weight was obtained from community midwife and hospital registries. Preterm birth was defined as birth before 37.0 weeks of gestation. Gestational age adjusted SDS were constructed for birth weight, using reference growth charts[13]. Fetal growth rate was defined as the change in SDS in the intervals between the second and third trimester, and between third trimester and birth. We defined small size for gestational age (SGA) as being <5th sex specific percentile and large size for gestational age (LGA) as being >95th sex specific percentile for weight at birth.

Infant growth was repeatedly measured at the Community Health Centers according to standardized procedures by a well-trained staff at the median ages of 6.2 months (95% range 5.2 – 8.3), and 24.8 months (95% range 23.4 – 28.2). Length was measured in supine position to the nearest millimetre and weight was measured using a mechanical personal scale (SECA). Sex and age adjusted SDS for infant growth characteristics were obtained using Dutch reference growth curves (Growth Analyzer 3.0, Dutch Growth Research Foundation). For each infant age interval, we defined growth as the change in SDS between the two time-points. Catch-down and catch-up growth for weight was defined as a decrease or increase of >0.67 standard deviation (SD) of weight from

birth to 24 months of age. This change represents the width of each percentile band on standard growth charts[14].

Childhood anthropometrics

At the age of 6 years, child height and weight were measured without shoes and heavy clothing, and body mass index (BMI) (kg/m^2) and body surface area (BSA) (m^2) were calculated. All anthropometric measurements were converted to SDS. Overweight and obesity were defined as recommended by The International Obesity Taskforce[15].

Child cardiovascular structures and function

We measured blood pressure with the child in supine position quietly awake. Systolic and diastolic blood pressure was measured at the right brachial artery, four times with one minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus™ (Paramus, NJ, USA).[15] A cuff was selected with a cuff width approximately 40% of the arm circumference and long enough to cover 90% of the arm circumference. More than 90% of the children who visited the research center had four successful blood pressure measurements available.

Carotid-femoral pulse wave velocity, the reference method to assess aortic stiffness[16], was assessed using the automatic Complior SP device (Complior; Artech Medical, Pantin, France) with participants in supine position. The distance between the recording sites at the carotid (proximal) and femoral (distal) artery was measured over the surface of the body to the nearest centimeter. Through piezoelectric sensors placed on the skin, the device collected signals to assess the time delay between the upstroke of carotid and femoral waveforms. Carotid-femoral pulse wave velocity was calculated as the ratio of the distance travelled by the pulse wave and the time delay between the waveforms, as expressed in meters per second[17]. To cover a complete respiratory cycle, the mean of at least 10 consecutive pressure waveforms was used in the analyses. Recently, it has been shown that pulse wave velocity can be measured reliably, with good reproducibility in a large pediatric population-based cohort[18]. Two-dimensional M-mode echocardiographic measurements were performed using the ATL-Philips Model HDI 5000 (Seattle, WA, USA) or the Logiq E9 (GE Medical Systems, Wauwatosa, WI, USA) devices. The children were examined in a quiet room with the child awake in supine position. Missing echocardiograms were mainly due to restlessness of the child or unavailability of equipment or sonographer. Left atrial diameter, interventricular end-diastolic septal thickness (IVSTD), left ventricular end-diastolic diameter (LVEDD), left ventricular end-diastolic posterior wall thickness (LVPWTD), interventricular end-systolic septal thickness, left ventricular end-systolic diameter, left ventricular end-systolic posterior wall thickness, aortic root diameter, and fractional shortening were measured using methods recommended by the American Society of Echocar-

diography[19]. Left ventricular mass (LV mass was computed using the formula derived by Devereux et al[20]:LV mass = $0.80 \times 1.04((IVSTD + LVEDD + LVPWTD)^3 - (LVEDD)^3) + 0.6$. To assess reproducibility of ultrasound measurements, the intraobserver and interobserver intraclass correlation coefficients were calculated previously in 28 subjects (median age 7.5 years, inter-quartile range 3.0 – 11.0) and varied for the main outcome measures between 0.91 to 0.99 and 0.78 to 0.96, respectively[21].

All cardiovascular outcome measurements were converted to SDS for comparison of effect estimates.

Covariates

Information on maternal age, pre-pregnancy weight, parity, educational level, and smoking status (yes/no) during pregnancy was obtained by questionnaires. Maternal education was defined as the highest followed education according to the classification of Statistics Netherlands and was categorized in primary and secondary, or higher[22]. Maternal height was measured without shoes and pre-pregnancy BMI was calculated (kg/m^2). Date of birth and infant sex were obtained from midwife and hospital registries. Infant ethnicity was classified by the countries of birth of the parents, according to the Dutch standard classification criteria of Statistics Netherlands and was categorized as European or non-European[22]. Breastfeeding (yes/no) was assessed using questionnaires.

Statistical analysis

First, we assessed the associations of fetal, infant and child characteristics with cardiovascular outcomes at the age of 6 years using linear regression models adjusted for sex and current age. Since we measured blood pressure four times, we applied linear mixed models[23], that fit the four blood pressure measurements within the same child as repeated outcome measures. One of the advantages of this approach is that subjects with the maximum number of blood pressure measurements available and the least individual variability in their blood pressure measurements are assigned the highest weight in the analysis[24]. Second, to investigate the associations of clinical cut off levels of birth characteristics with cardiovascular outcomes, we performed analyses using categories of gestational age, birth weight and gestational age adjusted birth weight. Third, we explored the associations of fetal and infant growth in different age intervals with cardiovascular outcomes. We used the following intervals: Second to third trimester; third trimester to birth; birth to 6 months; and 6 months to 24 months. Finally, we assessed the associations of infant growth until 24 months of age and BMI categories with blood pressure and left ventricular mass, known risk factors for cardiovascular disease in adulthood, within strata of gestational age adjusted birth weight (SGA, appropriate size for gestational age (AGA), and LGA). All models were adjusted for relevant confounders, including maternal pre-pregnancy BMI, parity, educational level, smoking status during

pregnancy, and child's gestational age, birth weight, sex, ethnicity, breastfeeding and current age and BMI. The percentages of missing covariate values within the population for analysis were lower than 15.0%, except for maternal pre-pregnancy BMI (25.2%) and breastfeeding (22.1%). Missing covariate data were imputed using the multiple imputations procedure (N = 5 imputations) and the imputed datasets were analyzed together. All measures of association are presented with their 95% confidence intervals (CI). The mixed-models were fitted using the Statistical Analysis System version 9.2 (SAS, Institute Inc., Gary, NC, USA). All other Statistical analyses were performed using the Statistical Package for the Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics

Table 1 provides a comparison in characteristics of the children included in the study between the original dataset and after the multiple imputation procedure. Sex-specific descriptives of all fetal and infant growth characteristics are given in the supplementary material (Table S1). Table S2 shows consequent positive associations of cross-sectional second and third trimester estimated fetal weight and birth weight with left atrial diameter, aortic root diameter and left ventricular mass at 6 years, with stronger effect estimates in third trimester. Child growth characteristics were all positively associated with blood pressure, left atrial diameter, aortic root diameter and left ventricular mass. The strongest effect estimates observed for weight at the age of 24 months.

Table 1. Characteristics of study participants (N = 6,416)

Maternal characteristics	
Age, y, median (95% range)	31.1 (19.8 – 39.9)
Height, cm, mean (SD)	167.5 (7.4)
Weight before pregnancy, kg, mean (SD)	66.5 (12.6)
Pre-pregnancy body mass index, kg/m^2, mean (SD)	23.6 (4.2)
Parity, % (n)	
0	54.6 (3,500)
≥1	42.2 (2,707)
Missing	3.3 (209)
Educational level mother, % (n)	
Primary or Secondary	48.5 (3,109)
Higher	42.5 (2,729)
Missing	9.0 (578)
Smoking during pregnancy, % (n)	
No	64.9 (4,161)
Yes	21.9 (1,408)

Table 1. Characteristics of study participants (N = 6,416) (continued)

Missing	13.2 (847)
Birth characteristics	
Male sex, % (n)	3,221 (50.2)
Gestational age at birth, wks, median (95% range)	40.1 (35.9 – 42.3)
Birth weight, g, mean (SD)	3,427 (554)
Preterm birth (<37 weeks), % (n)	5.0 (318)
Small for gestational age, % (n)	4.2 (272)
Large for gestational age, % (n)	4.9 (317)
Ethnicity, % (n)	
European	62.6 (4,019)
Non-European	34.9 (2,236)
Missing	2.5 (161)
Breastfeeding during infancy, % (n)	
Ever	72.0 (4,620)
Never	5.9 (379)
Missing	22.1 (1,416)
Child characteristics at 6 years	
Age, y, median (95% range)	6.0 (5.6 – 7.9)
Height, cm, mean (SD)	119.5 (6.1)
Weight, kg, mean (SD)	23.3 (4.3)
Body mass index, m/kg^2, mean (SD)	16.2 (1.9)
Systolic blood pressure, mmHg, mean (SD)	102.7 (8.1)
Diastolic blood pressure, mmHg, mean (SD)	60.7 (6.7)
Carotid-femoral pulse wave velocity, m/s, mean (SD)	5.5 (0.9)
Left atrial diameter, mm, mean (SD)	25.2 (2.7)
Aortic root diameter, mm, mean (SD)	19.3 (1.8)
Left ventricular mass, g, mean (SD)	53.4 (11.6)
Fractional shortening, %, mean (SD)	35.2 (4.5)

Missing values continuous variables: maternal height N = 612, pre-pregnancy weight N = 1,605, pre-pregnancy body mass index = 1,614, child gestational age at birth N = 44, current height N = 8, current weight N = 8, and current body mass index N = 8. Subject characteristics based on imputed values of covariates are given in the supplementary materials.

Fetal and childhood growth characteristics and cardiovascular outcomes

Gestational age, birth weight, and gestational age adjusted birth weight were all inversely associated with systolic blood pressure, and positively associated with cardiac structures at 6 years of age in the fully adjusted models (Table 2). Children born preterm had smaller left atrial diameter, aortic root diameter and left ventricular mass. Children born with a birth weight <2,000 grams had a higher blood pressure and decreased aortic root and left ventricular mass, compared to children born with a birth weight 3,000 - 3,499 grams. We did not observe consistent associations of birth characteristics with carotid-femoral pulse wave velocity.

Table 3 shows that fetal length growth rate was not associated with childhood blood pressure. Fetal length growth rate from second to third trimester, but not from third trimester to birth, was positively associated with aortic root diameter and left ventricular

Table 2. Birth characteristics and cardiovascular structures and function at the age of 6 years

	Difference in cardiovascular outcomes expressed as SD (95% Confidence Interval)						
Birth characteristics	**Systolic blood pressure (SD = 8.1 mmHg)**	**Diastolic blood pressure (SD = 6.7 mmHg)**	**Pulse wave velocity (SD = 0.9 m/s)**	**Left atrial diameter (SD = 2.7 mm)**	**Aortic root diameter (SD = 1.8 mm)**	**Left ventricular mass (SD = 11.6 g)**	**Fractional shortening (SD = 4.5 %)**
Gestational age N = 6,372							
<37.0 weeks	0.05	-0.01	-0.09	-0.11	-0.18	-0.10	0.02
N = 318	(-0.05, 0.14)	(-0.09, 0.07)	(-0.22, 0.04)	(-0.21, 0.00)*	(-0.28, -0.08)†	(-0.20, -0.01)*	(-0.09, 0.13)
37.0-41.9 weeks	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
N = 5,622							
>=42.0 weeks	-0.07	-0.04	-0.05	0.03	0.03	0.01	-0.09
N = 432	(-0.15, 0.02)	(-0.11, 0.03)	(-0.16, 0.06)	(-0.07, 0.12)	(-0.07, 0.12)	(-0.07, 0.10)	(-0.18, 0.01)
P for trend	<0.01	0.31	0.53	<0.01	<0.01	<0.01	0.16
Birth weight N = 6,416							
<2,000 grams	0.18	0.08	-0.28	-0.20	-0.32	-0.21	0.05
N = 78	(-0.01, 0.37)	(-0.09, 0.25)	(-0.53, -0.03)*	(-0.41, 0.01)	(-0.53, -0.12)†	(-0.39, -0.02)*	(-0.18, 0.27)
2,000-2,499 grams	-0.03	-0.03	-0.04	0.04	-0.21	-0.19	-0.03
N = 208	(-0.15, 0.09)	(-0.14, 0.08)	(-0.21, 0.12)	(-0.09, 0.17)	(-0.34, -0.08)†	(-0.31, -0.07)†	(-0.17, 0.11)
2,500-2,999 grams	0.00	0.02	-0.06	0.00	-0.16	-0.15	-0.01
N = 944	(-0.06, 0.07)	(-0.04, 0.08)	(-0.15, 0.02)	(-0.07, 0.07)	(-0.23, -0.10)†	(-0.22, -0.09)†	(-0.09, 0.06)
3,000-3,499 grams	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
N = 2,223							
3,500-3,999 grams	-0.09	-0.07	-0.01	0.09	0.19	0.11	-0.07
N = 2,063	(-0.14, -0.04)†	(-0.12, -0.02)†	(-0.08, 0.06)	(0.04, 0.15)†	(0.13, 0.24) †	(0.06, 0.16)†	(-0.13, -0.01)*
4,000-4,499 grams	-0.12	-0.08	0.02	0.18	0.26	0.25	-0.09
N = 751	(-0.19, -0.05)†	(-0.15, -0.02)*	(-0.08, 0.11)	(0.10, 0.26)†	(0.18, 0.34) †	(0.18, 0.32)†	(-0.17, 0.00)*
≥4,500 grams	-0.17	-0.09	0.02	0.15	0.46	0.41	-0.16
N = 149	(-0.31, -0.03)*	(-0.21, 0.04)	(-0.16, 0.21)	(0.00, 0.31)	(0.31, 0.61) †	(0.27, 0.55)†	(-0.32, 0.01)
P for trend	<0.01	<0.01	0.04	<0.01	<0.01	<0.01	<0.01
Birth weight for gestational age N = 6,367							
Small for gestational age	0.15	0.13	-0.11	-0.04	-0.31	-0.21	0.06
N = 272	(0.04, 0.25)†	(0.04, 0.22)†	(-0.25, 0.03)	(-0.16, 0.07)	(-0.43, -0.20)†	(-0.32, -0.11)†	(-0.06, 0.18)
Normal for gestational age	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
N = 5,778							
Large for gestational age	-0.07	-0.08	0.00	0.08	0.24	0.34	-0.07
N = 317	(-0.16, 0.03)	(-0.17, 0.00)	(-0.13, 0.13)	(-0.03, 0.19)	(0.13, 0.35)†	(0.24, 0.44)†	(-0.18, 0.05)
P for trend	<0.01	<0.01	0.06	<0.01	<0.01	<0.01	<0.01

Values are regression coefficients (95% CI) based on mixed models or linear regression models and reflect the change in SDS of each cardiovascular outcome for each birth weight or gestational age group, compared to the reference group. Models are adjusted for maternal age, pre-pregnancy body mass index, parity, educational level, and smoking status during pregnancy, and child sex, ethnicity, breastfeeding and current age and body mass index. Models with ultrasound outcomes are additionally adjusted for ultrasound device and performing sonographer. *P<0.05 †P<0.01

Table 3. Fetal and infant growth in different periods and cardiovascular structures and function at the age of 6 years

Growth characteristics	Difference in cardiovascular outcomes expressed as SD (95% Confidence Interval)						
	Systolic blood pressure (SD = 8.1 mmHg)	Diastolic blood pressure (SD = 6.7 mmHg)	Pulse wave velocity (SD = 0.9 m/s)	Left atrial diameter (SD = 2.7 mm)	Aortic root diameter (SD = 1.8 mm)	Left ventricular mass (SD = 11.6 g)	Fractional shortening (SD = 4.5 %)
Length							
Second trimester (SD = 0.97 change in SDS) N = 5,378	-0.02 (-0.04, 0.00)	-0.01 (-0.03, 0.01)	0.00 (-0.03, 0.03)	0.02 (0.00, 0.05)	0.06 (0.04, 0.09)†	0.05 (0.03, 0.07)†	0.00 (-0.03, 0.03)
Third trimester (SD = 1.25 change in SDS) N = 3,702	-0.02 (-0.04, 0.00)	-0.01 (-0.03, 0.01)	0.00 (-0.03, 0.03)	0.01 (-0.01, 0.04)	0.01 (-0.01, 0.04)	0.01 (-0.01, 0.04)	-0.01 (-0.03, 0.02)
Birth – 6 months (SD = 1.20 change in SDS) N = 2,664	0.04 (0.01, 0.06)†	0.03 (0.00, 0.05)*	0.01 (-0.03, 0.04)	0.02 (-0.01, 0.05)	0.05 (0.02, 0.08)†	0.07 (0.04, 0.09)†	0.02 (-0.02, 0.05)
6 – 24 months (SD = 0.78 change in SDS) N = 3,248	0.08 (0.05, 0.12)†	0.00 (-0.03, 0.03)	0.03 (-0.02, 0.08)	0.03 (-0.01, 0.07)	0.01 (-0.04, 0.05)	0.07 (0.03, 0.11)†	0.03 (-0.01, 0.07)
Weight							
Second trimester (SD = 0.98 change in SDS) N = 5,334	-0.03 (-0.05, -0.01)*	-0.02 (-0.04, 0.00)	0.02 (-0.01, 0.05)	0.02 (-0.01, 0.04)	0.07 (0.04, 0.09)†	0.04 (0.02, 0.07)†	-0.01 (-0.04, 0.02)
Third trimester (SD = 0.93 change in SDS) N = 5,639	-0.02 (-0.05, 0.00)	-0.01 (-0.03, 0.01)	-0.02 (-0.05, 0.01)	0.00 (-0.03, 0.02)	0.01 (-0.02, 0.04)	0.02 (-0.01, 0.04)	-0.01 (-0.04, 0.02)
Birth – 6 months (SD = 1.06 change in SDS) N = 4,677	0.06 (0.04, 0.08)†	0.06 (0.04, 0.08)†	0.00 (-0.03, 0.03)	0.00 (-0.03, 0.02)	-0.01 (-0.04, 0.02)	0.00 (-0.03, 0.02)	0.02 (-0.01, 0.05)
6 – 24 months (SD = 0.76 change in SDS) N = 3,680	0.05 (0.01, 0.09)†	-0.01 (-0.05, 0.02)	0.03 (-0.02, 0.08)	0.06 (0.02, 0.10)†	0.06 (0.02, 0.11)†	0.12 (0.08, 0.15)†	-0.03 (-0.07, 0.02)

Values are regression coefficients (95% CI) based on multiple linear regression models and reflect the change in SDS of each cardiovascular outcome per change in SDS of fetal and childhood growth. Models are adjusted for the time interval between growth measurements, maternal age, pre-pregnancy body mass index, parity, educational level, smoking status during pregnancy, and child sex, ethnicity, breastfeeding, and current age and body mass index. Models with ultrasound outcomes are additionally adjusted for ultrasound device and performing sonographer.
*P<0.05 †P<0.01

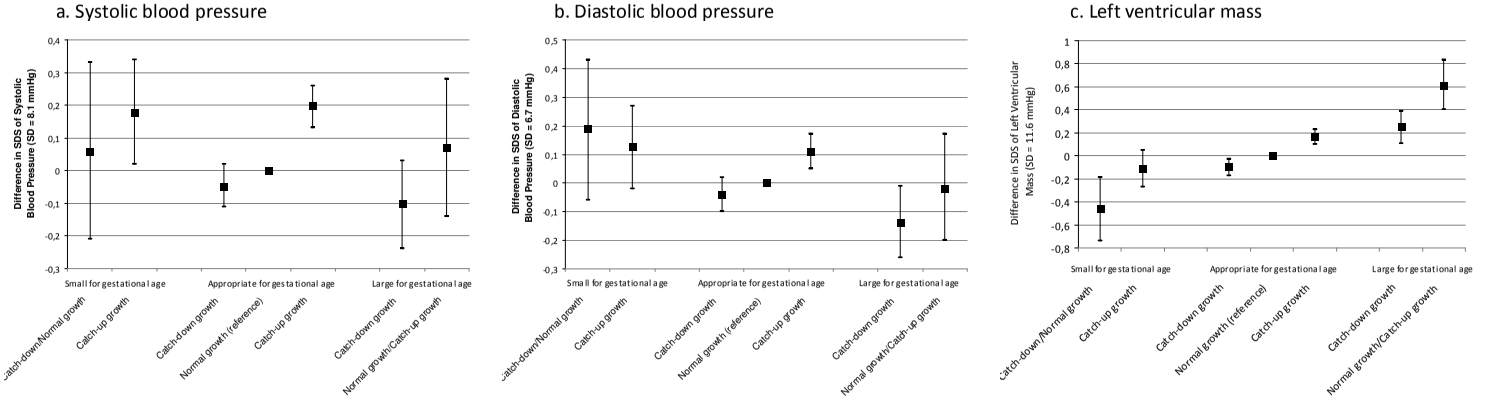
mass (0.06 SDS, 95% CI 0.04, 0.09, and 0.05 SDS, 95% CI 0.03, 0.07, respectively). Infants with increased length gain in the first 24 months of life had a higher systolic blood pressure. Although fetal weight growth rate in the period from second trimester to birth was inversely associated with systolic blood pressure, infant weight gain in the first 24 months of life was positively associated with blood pressure. Fetal weight growth rate from second to third trimester was positively associated with aortic root diameter and left ventricular mass. No consistent associations were found of fetal or infant growth rate with childhood carotid-femoral pulse wave velocity or fractional shortening.

Size at birth, infant growth and childhood obesity and cardiovascular outcomes

As compared to children born AGA with normal infant weight gain until 24 months of age, children born SGA or AGA followed by catch up weight gain had the highest systolic blood pressure (0.20 SDS, 95% CI 0.04, 0.36, and 0.20 SDS, 95% CI 0.13, 0.27, respectively), whereas children born LGA with a normal or catch up growth had the highest left ventricular mass (0.61 SDS, 95% CI 0.39, 0.82) (Figure 1). Children born SGA had a smaller left ventricular mass compared to the reference group, with the smallest left ventricular mass in children without catch up growth.

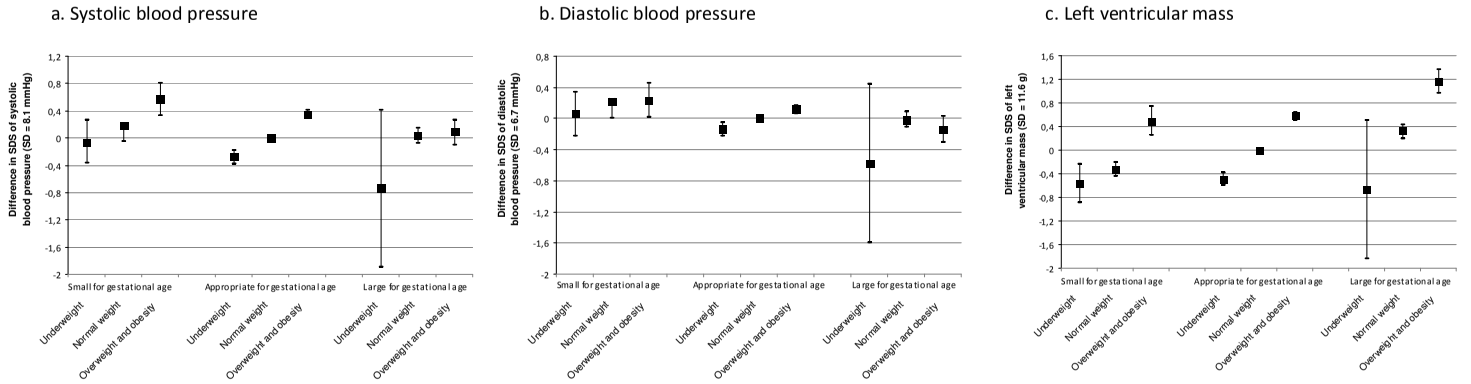
Children born SGA or AGA who became overweight or obese at the age of 6 years, had the highest systolic and diastolic blood pressure as compared to AGA children with a normal weight, as presented in Figure 2. Children born LGA who were underweight had the lowest blood pressure. Children born LGA who became overweight or obese had the highest left ventricular mass (1.17 SDS, 95% CI 0.97, 1.37).

Figure 1. Size at birth, infant growth and cardiovascular structures and function at the age of 6 years



Values are regression coefficients (95% CI) based on multiple linear regression models and reflect the change in SDS of blood pressure and left ventricular mass for the different categories of infant growth, stratified for fetal growth. Trend tests have been performed on infant growth as a continuous variable. Models are adjusted for maternal age, pre-pregnancy body mass index, parity, educational level, smoking status during pregnancy, and child sex, ethnicity, breastfeeding, and current age and body mass index. Models with ultrasound outcomes are additionally adjusted for ultrasound device and performing sonographer.

Figure 2. Size at birth, child weight status and cardiovascular structures and function at the age of 6 years



Values are regression coefficients (95% CI) based on multiple linear regression models and reflect the change in SDS of blood pressure and left ventricular mass for the different categories of child weight status, stratified for fetal growth. Models are adjusted for maternal age, pre-pregnancy body mass index, parity, educational level, smoking status during pregnancy, and child sex, ethnicity, breastfeeding, and current age. Models with ultrasound outcomes are additionally adjusted for ultrasound device and performing sonographer.

Discussion

In this large, population based prospective cohort study, we demonstrated that fetal, infant and child growth characteristics are associated with cardiovascular structural and functional development in childhood, independent of current BMI. These results suggest that both fetal and childhood growth might have persisting influence on cardiovascular growth and development.

Early critical growth periods for cardiovascular development

Suboptimal cardiovascular developmental adaptations in response to or associated with early growth variation might partly explain the previously demonstrated increased predisposition to cardiovascular disease in individuals born with low birth weight[1-2]. The rates of elastin synthesis in human blood vessels increase to a maximum in the perinatal period, followed by a rapid fall in the synthesis rates thereafter[25-26], suggesting a critical period for the development of the vascular system in the perinatal phase. It has been hypothesized that impaired fetal growth is associated with a deficient synthesis of elastin in the walls of the aorta and large arteries, and that this deficiency would lead to permanent changes in the mechanical properties of these vessels[26]. Indeed, adaptations in arterial properties have been observed in both children[21, 27-29] and adults[4, 30] born with low birth weight, although results seem inconsistent[31-32]. We observed an inverse association of birth weight and gestational age adjusted birth weight with systolic and diastolic blood pressure at the age of 6 years. Furthermore, length and weight growth rates from second to third trimester of pregnancy were consequently associated with aortic root diameter, supporting the hypothesis of the presence of a critical window of developmental programming of the vascular system[26]. However, we did not find consistent evidence of an association of fetal or infant growth with carotid-femoral pulse wave velocity at the age of 6 years. Although in contrast with previous studies[4], our results are in line with findings of Montgomery et al. in adults aged 25 years, where neither low size at birth nor the interaction between birth size and adult size were associated with pulse wave velocity[31]. The discrepancy in results between studies in older and younger individuals is suggested to be explained by the influence of atherosclerotic lesions on arterial compliance with advancing age[31].

Detailed studies in animals and humans have shown that intrauterine growth restriction can program structural and mechanical cardiac properties in the offspring[7, 33-34]. In a case-control study among young children, fetal growth restriction induced primary changes in cardiac morphology, including an increase in transversal diameters and more globular ventricles[7]. The authors postulated that this cardiac remodelling is most likely the result of the inability of the myocardium to develop adequate hypertrophic changes in response to an increased workload under conditions of sustained hypoxia and

undernutrition[7]. Fetal growth of the human heart mainly involves myocardial cell hyperplasia, while postnatal heart growth is characterized by myocardial cell hypertrophy and hyperplasia of nonmuscle cells[35]. Although cardiomyocyte proliferation is observed in the heart of adults, factors that influence cardiac histology established during fetal life through either reduced cardiomyocyte proliferation or increased apoptosis, might exert a lasting effect on cardiac development. In rats, intrauterine growth restriction led to a reduced number of cardiomyocytes per heart at birth[34]. In a post-mortem study among four human infants with severe intrauterine growth retardation, hypoplasia of myocardial fibers was found[36]. The smaller left ventricular mass we observed in children with restricted fetal growth from second to third trimester and born small for gestational age, may reflect the inability of the left ventricle to adequately adapt according to the increased workload that is imposed from birth onwards, due to a lower number of cardiomyocytes.

Longitudinal approach of fetal and child growth

It has been hypothesized that the pattern of postnatal growth may modify the association between restricted fetal growth and the risk of cardiovascular disease. An observational study from Finland with detailed growth data described that reduced birth weight followed by accelerated child growth was related to a higher risk of coronary heart disease in later life[3]. In 1,258 men participating in a follow up study in South Wales, the risk of coronary heart disease associated with low birth weight only observed among individuals with a high adult BMI[37]. We observed that children born SGA or AGA with catch up growth or overweight at the age of 6 years had a higher systolic blood pressure than children born AGA followed by normal growth. Children born LGA with catch up growth or overweight in childhood had a higher left ventricular mass. These results support a mechanistic pathway where the pattern of postnatal growth following fetal growth influences cardiovascular development.

Methodological considerations

Main strengths of this study are its population-based, prospective design starting in fetal life, and the large number of fetal, infant and child growth measurements available. The majority of studies have no access to measurements of fetal growth other than birth measures, which are crude summary indices of fetal growth. To move beyond birth measures, we investigated the influence of fetal and postnatal growth, in addition to birth size. The repeated fetal and postnatal growth measurements provided the opportunity to identify critical growth periods that might influence cardiovascular development. A limitation of the study is that of all children, 69.1% participated in the follow-up measurements and 68.5% underwent one or more cardiovascular measurements. No differences in birth weight between children with and without one of the cardiovascular

measurements were observed. Selection bias due to selective loss to follow-up is of concern if the associations of early growth characteristics with cardiovascular structures and function differ between those included and those not included in the analyses. Although this seems unlikely, it cannot be excluded. It is expected that the potential confounding effect of factors related to the risk of cardiovascular disease is confined in young children. However, the influence of residual confounding should be considered, as in all observational studies.

Conclusions

This study suggests that fetal, infant and child growth are associated with cardiovascular development in childhood and that critical periods of growth can be identified. This early cardiovascular programming may subsequently increase the susceptibility to cardiovascular disease in adulthood. However, the long-term clinical importance of the changes in cardiovascular development detected in childhood due to impaired early growth is unknown. Whether these adaptations predict a greater risk of cardiovascular disease in later life, is topic for further research.

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Supplement

Figure S1. Flow chart of participants included in the analysis

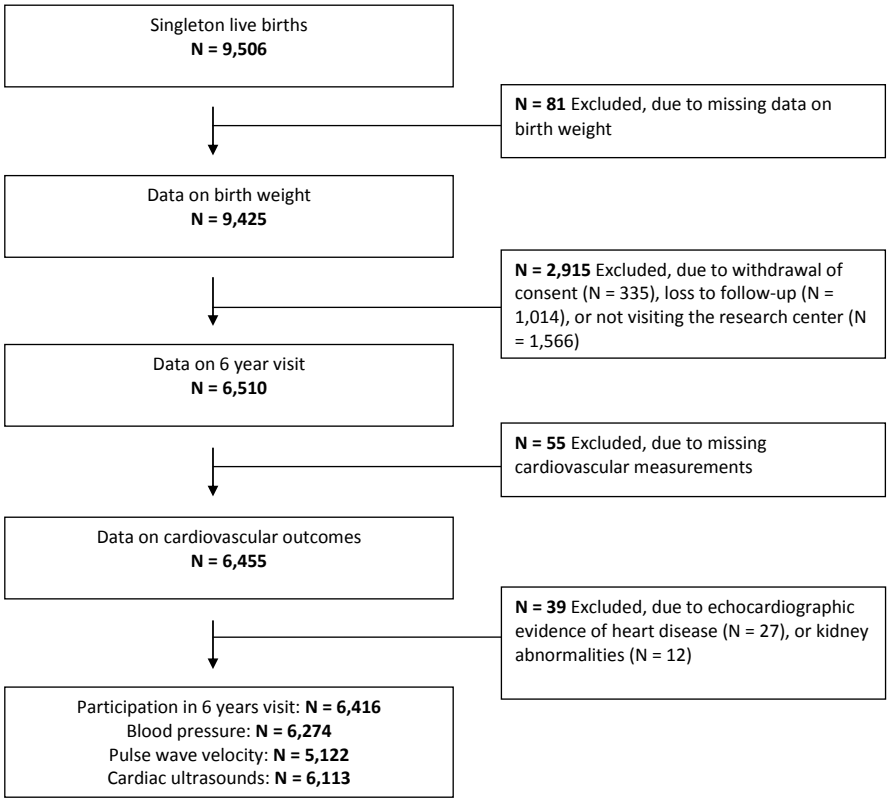


Table S1. Characteristics of study participants after multiple imputation (N = 6,416)

	Imputed
Maternal characteristics	
Age, y, median (95% range)	31.1 (19.8 – 39.9)
Height, cm, mean (SD)	167.5 (7.4)
Weight before pregnancy, kg, mean (SD)	66.5 (12.6)
Body mass index before pregnancy, kg/m^2, mean (SD)	23.7 (4.3)
Parity, % (n)	
0	56.2 (3,607)
≥1	43.8 (2,808)
Missing	-
Educational level mother, % (n)	
Primary or Secondary	54.6 (3,505)
	()
Higher	45.4 (2,910)
Missing	-
Smoking during pregnancy, % (n)	
No	74.5 (4,780)
Yes	25.5 (1,635)
Missing	-
Birth characteristics	
Male sex, % (n)	50.2 (3,221)
Gestational age at birth, wks, median (95% range)	40.1 (35.9 – 42.3)
Birth weight, g, mean (SD)	3,427 (554)
Preterm birth (<37 weeks), % (n)	5.0 (318)
Small for gestational age, % (n)	4.2 (272)
Large for gestational age, % (n)	4.9 (317)
Ethnicity, % (n)	
European	63.9 (4,101)
Non-European	36.1 (2,314)
Missing	-
Breastfeeding during infancy, % (n)	
Ever	92.2 (5,917)
Never	7.8 (498)
Missing	-
Child characteristics at 6 years	
Age, y, mean (SD)	6.0 (5.6 – 7.9)
Height, cm, mean (SD)	119.5 (6.2)
Weight, kg, mean (SD)	23.3 (4.3)
Body mass index, m/kg^2, mean (SD)	16.2 (1.9)
Systolic blood pressure, mmHg, mean (SD)	102.7 (8.1)
Diastolic blood pressure, mmHg, mean (SD)	60.7 (6.7)
Carotid-femoral pulse wave velocity, m/s, mean (SD)	5.5 (0.9)
Left atrial diameter, mm, mean (SD)	25.2 (2.7)
Aortic root diameter, mm, mean (SD)	19.3 (1.8)
Left ventricular mass, g, mean (SD)	53.4 (11.6)
Fractional shortening, %, mean (SD)	35.2 (4.5)

Values are means (SD), medians (95% range) or numbers (%)

*Gestational age <37 weeks at delivery

†Sex specific gestational age adjusted birth weight <5th percentile in study cohort

‡Sex specific gestational age adjusted birth weight >95th percentile in study cohort

Missing values for imputed continuous variables maternal height N = 612, pre-pregnancy weight N = 1,605, pre-pregnancy body mass index = 1,614, child gestational age at birth N = 44, current height N = 8, current weight N = 8, and current body mass index N = 8.

Table S2. Fetal and infant growth characteristics

Growth Characteristics	Boys N = 3,221	Girls N = 3,195	*P*-value
Fetal growth			
Second trimester			
Gestational age (weeks)	20.6 (18.6 – 23.5)	20.5 (18.5 – 23.3)	<0.01
Femur length (mm)	33.5 (3.6)	33.5 (3.5)	0.75
Estimated fetal weight (g)	387 (97)	377 (91)	<0.01
Third trimester			
Gestational age (weeks)	30.4 (28.5 – 33.1)	30.3 (28.3 – 33.0)	<0.01
Femur length (mm)	57.4 (3.1)	57.6 (3.1)	<0.01
Estimated fetal weight (g)	1,633 (260)	1,619 (269)	0.04
Birth			
Gestational age (weeks)	40.1 (35.9 – 42.4)	39.8 (35.7 – 42.1)	0.10
Length (cm)	50.6 (2.4)	49.9 (2.3)	<0.01
Weight (g)	3,489 (567)	3,364 (533)	<0.01
Infant growth			
6 Months			
Age (months)	6.2 (5.2 – 8.2)	6.2 (5.3 – 8.4)	0.55
Length (cm)	68.5 (2.5)	66.7 (2.4)	<0.01
Weight (g)	8,174 (902)	7.594 (832)	<0.01
24 Months			
Age (months)	24.8 (23.4 – 28.3)	24.8 (23.4 – 28.1)	0.37
Height (cm)	88.9 (3.4)	87.7 (3.4)	<0.01
Weight (kg)	13.2 (1.5)	12.7 (1.5)	<0.01

Values are means (SD) or medians (95% range). Differences between boys and girls were compared using independent samples t test

Table S3. Fetal and infant growth characteristics and cardiovascular structures and function at the age of 6 year

	Difference in cardiovascular outcomes expressed as SD (95% Confidence Interval)						
Growth characteristics	**Systolic blood pressure (SD = 8.1 mmHg)**	**Diastolic blood pressure (SD = 6.7 mmHg)**	**Pulse wave velocity (SD = 0.9 m/s)**	**Left atrial diameter (SD = 2.7 mm)**	**Aortic root diameter (SD = 1.8 mm)**	**Left ventricular mass (SD = 11.6 g)**	**Fractional shortening (SD = 4.5 %)**
Second trimester							
Length (SD = 3.6 mm)	0.02 (0.00, 0.05)*	0.00 (-0.02, 0.02)	0.03 (0.00, 0.06)	0.03 (0.00, 0.06)*	0.06 (0.04, 0.09)†	0.06 (0.03, 0.08)†	-0.03 (-0.05, 0.00)
Estimated fetal weight (SD = 94.2 g)	0.03 (0.01, 0.05)*	0.00 (-0.02, 0.02)	0.02 (-0.01, 0.05)	0.05 (0.03, 0.08)†	0.09 (0.06, 0.11)†	0.10 (0.07, 0.12)†	-0.02 (-0.04, 0.01)
Third trimester							
Length (SD = 3.1 mm)	0.00 (-0.03, 0.02)	-0.01 (-0.03, 0.01)	0.03 (0.00, 0.06)	0.06 (0.03, 0.09)†	0.12 (0.10, 0.15)†	0.11 (0.09, 0.14)†	-0.03 (-0.06, -0.01)†
Estimated fetal weight (SD = 264.4 g)	0.01 (-0.01, 0.03)	-0.02 (-0.04, 0.00)*	0.03 (0.00, 0.06)*	0.09 (0.06, 0.11)†	0.16 (0.14, 0.19)†	0.15 (0.13, 0.18)†	-0.02 (-0.05, 0.01)
Birth							
Gestational age (SD = 1.8 weeks)	-0.05 (-0.07, -0.02)†	-0.02 (-0.04, 0.00)	0.00 (-0.03, 0.04)	0.05 (0.02, 0.08)†	0.08 (0.05, 0.11)†	0.07 (0.04, 0.09)†	-0.02 (-0.05, 0.01)
Birth weight (SD = 554 g)	-0.03 (-0.06, -0.01)†	-0.04 (-0.06, -0.02)†	0.01 (-0.02, 0.04)	0.12 (0.09, 0.15)†	0.20 (0.18, 0.23)†	0.20 (0.18, 0.23)†	-0.04 (-0.06, -0.01)†
Gestational age adjusted birth weight (SD = 1.0)	-0.01 (-0.04, 0.01)	-0.03 (-0.05, -0.01)†	0.01 (-0.02, 0.04)	0.11 (0.08, 0.13)†	0.19 (0.16, 0.21)†	0.19 (0.17, 0.22)†	-0.03 (-0.06, -0.01)†
24 months							
Length (SD = 3.4 cm)	0.11 (0.08, 0.14)†	0.04 (0.01, 0.06)†	0.04 (0.01, 0.08)*	0.17 (0.14, 0.20)†	0.25 (0.22, 0.28)†	0.32 (0.29, 0.35)†	0.00 (-0.03, 0.03)
Weight (SD = 1.5 kg)	0.14 (0.11, 0.17)†	0.04 (0.02, 0.06)†	0.00 (-0.04, 0.04)	0.22 (0.19, 0.25)†	0.27 (0.24, 0.30)†	0.36 (0.33, 0.39)†	0.00 (-0.03, 0.03)

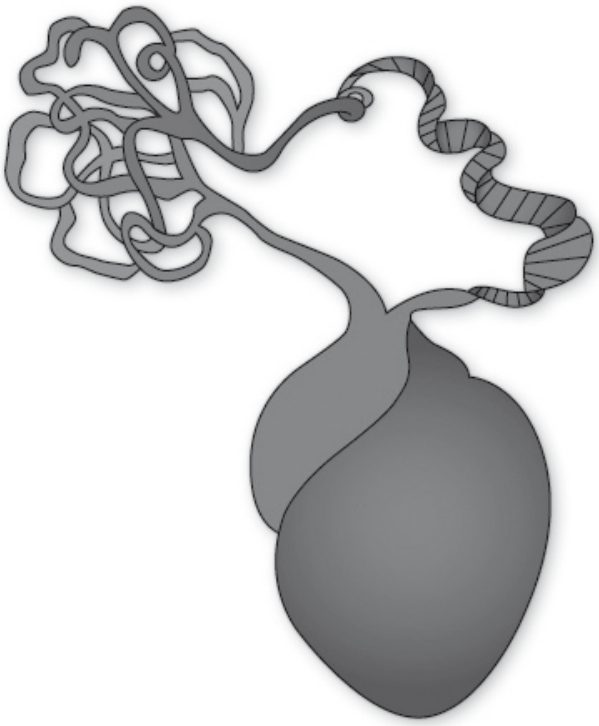
Values are regression coefficients (95% CI) based on multiple linear regression models and reflect the change in SDS of blood pressure, carotid-femoral pulse wave velocity, left cardiac structures and fractional shortening per change in SDS of fetal and infant growth characteristics. Models are adjusted for child sex and current age. Models with ultrasound outcomes are additionally adjusted for ultrasound device and performing sonographer. *P<0.05 †P<0.01

Chapter 3.2

Parental smoking during pregnancy and childhood cardiovascular structures and function

H Rob Taal
Layla L de Jonge
Lennie van Osch-Gevers
Eric AP Steegers
Albert Hofman
Willem A Helbing
Albert J van der Heijden
Vincent WV Jaddoe

Submitted



Abstract

Background: Fetal smoke exposure might lead to fetal developmental adaptations that permanently affect the cardiovascular system. We assessed the associations of both maternal and paternal smoking during pregnancy with childhood cardiovascular structures and function.

Methods: In a prospective cohort study among 5,565 children, we examined whether maternal and paternal smoking during pregnancy are associated with blood pressure, carotid-femoral pulse wave velocity, and left cardiac structures and function in 6 year old children.

Results: As compared to children from non-smoking mothers, children from mothers who smoked more than 10 cigarettes per day had a higher diastolic blood pressure (difference 1.43 mmHg (95%CI: 0.22, 2.63)). Maternal smoking during pregnancy was not associated with childhood carotid-femoral pulse wave velocity or left cardiac structures. Maternal smoking of 10 or more cigarettes per day was associated with a higher fractional shortening in childhood (difference 1.01% (95%CI 0.018, 1.84)). Among mothers who did not smoke during pregnancy, paternal smoking was associated with aortic root diameter, but not with other cardiovascular outcomes.

Conclusion: Maternal smoking during pregnancy might increase childhood diastolic blood pressure and fractional shortening. The stronger effect estimates for maternal smoking compared to paternal smoking might suggest that direct intra-uterine adaptive responses are involved as underlying mechanisms.

Introduction

Adverse fetal exposures might lead to permanent developmental adaptations in the structure and function of the cardiovascular system and other organ systems, and increase the susceptibility of cardiovascular disease and metabolic diseases in later life[1-3]. Maternal smoking during pregnancy is one of the most important, potentially modifiable, adverse fetal exposures[4-5], and is strongly related with an increased risks of low birth weight and preterm birth[5-6]. There is also growing evidence for changes in fetal and childhood cardiovascular structures and function in children born to mothers who smoked during pregnancy, although results seem to be inconsistent[7-10]. Previous studies showed associations of maternal smoking during pregnancy with childhood blood pressure[7-10], but it is not clear whether this association is explained by direct intra-uterine mechanisms or is due to other, unmeasured, confounders. One previous study suggested similar effect sizes for both maternal and paternal smoking on childhood blood pressure[9], indicating that this association might be explained by unmeasured environmental exposures. The extent to which maternal smoking during pregnancy is associated with changes in arterial stiffness, and left cardiac structures and function, which are independent predictors of cardiovascular disease in adulthood[11-12], is not known.

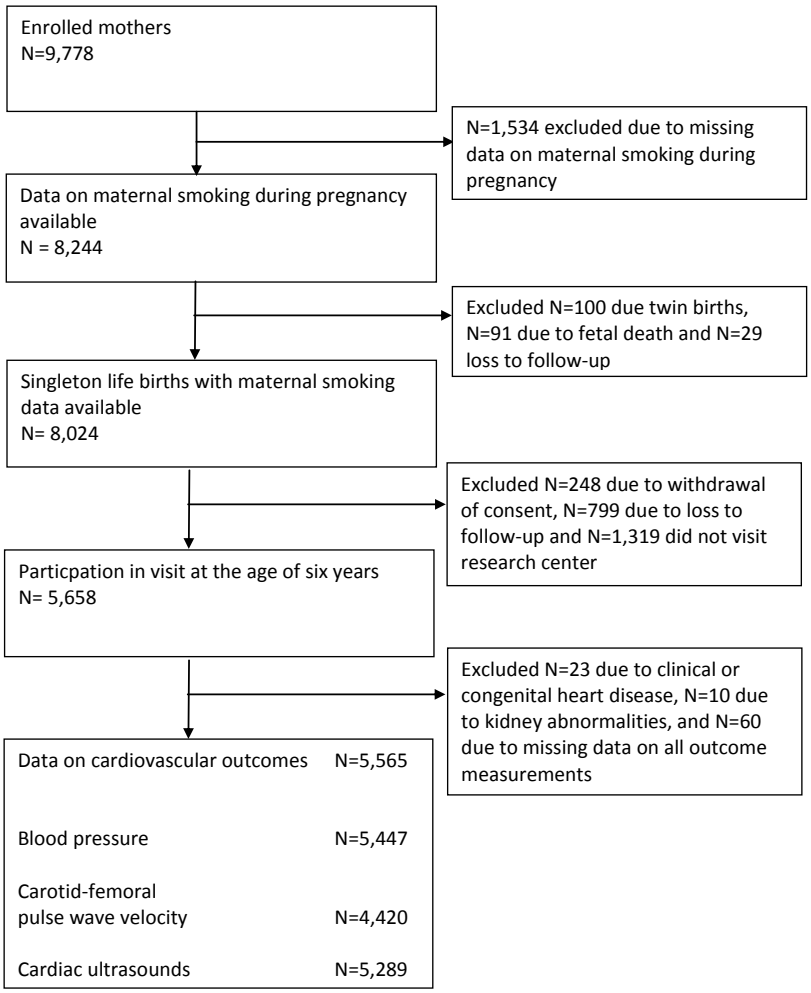
Therefore, we assessed in a large population-based prospective cohort study among 5,565 children, the associations of maternal and paternal smoking during pregnancy with blood pressure, carotid-femoral pulse wave velocity, and left cardiac structures and function in school age children.

Methods

Study design and population for analysis

The study was embedded in the Generation R Study, a population-based, prospective cohort study from fetal life onwards in Rotterdam, the Netherlands[13]**.** Enrolment in the study was aimed at early pregnancy, but was allowed until the birth of the child. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all parents of participants. In total, 9,778 mothers were enrolled in the study, of whom 8,244 (84.3%) provided information about their smoking habits. For the present study, only singleton life births were included (n=8,024), of whom 5,658 (70.5%) children attended the follow-up visit between March 2008 and January 2012. Children with echocardiographic evidence of congenital heart disease or kidney abnormalities were excluded from the study (n=33).

Figure 1. Flowchart



Flowchart of the study population: the Generation R Study Cohort, Rotterdam, the Netherlands.

Blood pressure, cardiac ultrasound or carotid-femoral pulse wave velocity measurements were successfully performed in 5,565 (98.4%) children (see figure 1).

Maternal and paternal smoking during pregnancy

At enrolment, we asked each mother whether she smoked during pregnancy (no smoking; smoking until pregnancy was acknowledged (first trimester only smoking); continued smoking during pregnancy). Mothers who were enrolled before a gestational age of 18 weeks and between 18 and 25 weeks of gestation, also received a second and third trimester questionnaire, respectively. Mothers who reported in the first questionnaire that they smoked during the first trimester only (n=921), but still reported

to smoke in the second or third trimester questionnaire (n=312) were reclassified into the 'continued smoking during pregnancy' category. The same strategy was used for women who reported no smoking in the first questionnaire, but reported smoking in the second or third questionnaire (n=80). Among mothers who smoked, the number of cigarettes smoked daily was categorized as: no maternal smoking, <5 cigarettes/day, 5-9 cigarettes/day, and ≥10 cigarettes/day. Paternal smoking was assessed in the first questionnaire by asking the mother whether the father smoked during pregnancy (yes, no, or do not know). Similar information completed by the father was available in a subset of participants (n=3,558). Agreement between these assessments by the mother and the father was good (sensitivity: 91%; specificity: 95%).

Cardiovascular outcomes in children

Cardiovascular measurements in children at the age of six years, were conducted in a dedicated research center in the Erasmus Medical Center, Rotterdam, the Netherlands. The child was lying quietly, while systolic and diastolic blood pressure was measured at the right brachial artery in supine position, four times with one minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus ™ (Paramus, NJ, USA)[14]. A cuff was selected with a cuff width approximately 40% of the arm circumference and long enough to cover 90% of the arm circumference. More than 95% of the children who visited the research center had 4 successful blood pressure measurements available.

Carotid-femoral pulse wave velocity, the reference method to assess aortic stiffness[12], was assessed using the automatic Complior device (Complior; Artech Medical, Pantin, France) with participants in supine position. The distance between the recording sites at the carotid (proximal) and femoral (distal) artery was measured over the surface of the body to the nearest centimetre. Through piezoelectric sensors placed on the skin, the device collected signals to assess the time delay between the pressure upstrokes in the carotid artery and the femoral artery. Carotid-femoral pulse wave velocity was calculated as the ratio of the distance travelled by the pulse wave and the time delay between the feet of the carotid and femoral pressure waveforms, as expressed in meters per second[15]. To cover a complete respiratory cycle, the mean of at least 10 consecutive pressure waveforms was used in the analyses.

Two-dimensional M-mode echocardiographic measurements were performed using the ATL-Philips Model HDI 5000 (Seattle, WA, USA) or the Logiq E9 (GE Medical Systems, Wauwatosa, WI, USA) devices. The children were examined in a quiet room with the child awake in supine position. Missing echocardiograms were mainly due to restlessness of the child or unavailability of equipment or echocardiographer. Left ventricular mass (LV-mass), aortic root diameter (AOD), left atrial diameter (LAD), and fractional shortening (FS), were measured using methods recommended by the American Society of Echocar-

diography[16]. LVmass was computed using the formula derived by Devereux et al[17]: To assess reproducibility of ultrasound measurements, the intraobserver and interobserver intraclass correlation coefficient were calculated in 30 subjects and varied between 0.85 and 0.99 for the main outcome measures[18].

Covariates

Information on maternal age, parity, educational level, pre-pregnancy body mass index (BMI) and maternal and paternal ethnicity was obtained from questionnaires[19]. Maternal and paternal blood pressure at intake was measured with the validated Omron 907 automated digital oscillometric sphygmanometer (OMRON Healthcare B.V. Hoofddop, the Netherlands)[20]. Child sex, gestational age at birth and birth weight were obtained from midwife and hospital registries. Breastfeeding (yes/no) was assessed using questionnaires. Current height and weight were measured without shoes and heavy clothing at the visit at 6 years, and BMI (kg/m^2) was calculated.

Statistical methods

We assessed differences in baseline characteristics between the categories of maternal smoking during pregnancy using independent samples t-tests and Chi Square tests. We used mixed models to assess the associations of maternal smoking during pregnancy with childhood blood pressure[21]. The mixed-model method fits each of the 4 blood pressure measurements of every child as repeated outcome measures. Next, we used multiple linear regression models to assess the associations of maternal smoking with carotid-femoral pulse wave velocity and left cardiac structures. We also investigated the associations of the quantity of maternal cigarettes smoked with cardiovascular outcomes using similar models. Tests for trend were performed using multiple linear regression models in which the categories of the number of cigarettes smoked were included as a continuous variable, using the non-smoking mothers as reference group. All models were adjusted for maternal age, parity, educational level, ethnicity, pre-pregnancy BMI and blood pressure at intake, and child sex, gestational age at birth, birth weight, breastfeeding status, current age and BMI. Analyses on left cardiac structure and function were additionally adjusted for the ultrasonographer and the ultrasound equipment used. Missing values in covariates (ranging from 0 to 30%), were multiple-imputed, to reduce potential bias associated with missing data[22]. We created five imputed datasets and each dataset was analyzed separately to obtain the effect sizes and standard errors. The results of all 5 imputed analyses were pooled and are presented in this paper. We used similar models to assess the associations of paternal smoking during pregnancy on childhood cardiovascular outcomes among mothers who did not smoke during pregnancy. In these analyses we adjusted for paternal ethnicity and blood pressure at intake. Measures of association are presented with their 95% confidence intervals (CI).

All *P* values are 2-sided. The mixed-models were fitted using the Statistical Analysis System version 9.2 (SAS, Institute Inc., Gary, NC, USA). All other statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics

Subject characteristics are presented in the online supplement (Table 1). The percentage of boys was 50.0%. Children of mothers who continued smoking during pregnancy had a lower birth weight, as compared to children of mothers who did not smoke.

Table 1. Subject Characteristics (n=5,565)

	Smoking during pregnancy		
	Non smoking (n=4159, 74.7%)	Stopped smoking (n=494, 8.9%)	Continued smoking (n=912, 16.4%)
Maternal characteristics			
Age (y)	31.4 (21.9 , 38.6)	30.7 (20.8 , 38.5) †	29.6 (19.4 , 37.7) †
Height (cm)	167.7 (7.4)	168.7 (7.0) †	167.0 (7.1) †
Weight before pregnancy (kg)	64.0 (50.0 , 90.0)	64.0 (51.0 , 90.0)	64.0 (50.0 , 95.0)
Pre-pregnancy body mass index (kg/m^2)	22.7 (18.7 , 32.1)	22.2 (18.6 , 30.5)	22.7 (18.5 , 32.7)
Systolic blood pressure (mmHg)	115.6 (12.3)	116 (11.9)	115.8 (11.9)
Diastolic blood pressure (mmHg)	68.5 (9.5)	67.6 (9.3)	67.1 (9.6) †
Education			
Primary	8.2	7.3	15.4 †
Secondary	40.2	46.5 *	61.6 †
Higher	51.6	46.2	23.0 †
Ethnicity			
Dutch or European	63.8	67.2 *	57.4 †
Non-European	36.2	32.8 *	42.6 †
Paternal characteristics			
Age (y)	33.6 (24.7 , 43.5)	32.5 (23.2 , 43.1) †	32.0 (21.6 , 41.2) †
Height (cm)	182.3 (7.8)	182.9 (8.1)	180.4 (8.1) †
Weight (kg)	83.0 (65.0 , 107.0)	83.0 (64.0 , 108.0)	82.0 (62.0 , 106.0)
Body mass index (kg/m^2)	24.9 (20.4 , 31.1)	24.7 (19.9 , 31.3)	25.2 (20.1 , 31.8)
Systolic blood pressure (mmHg)	130.3 (13.5)	130.1 (14.2)	130.2 (13.6)
Diastolic blood pressure (mmHg)	73.7 (10.5)	72.7 (10.3)	72.8 (11.1)
Smoking, yes (%)	35.6	65.3 †	77.3 †
Birth characteristics			
Males (%)	49.2	45.1	55.2 †

Table 1. Subject Characteristics (n=5,565) (continued)

	Smoking during pregnancy		
	Non smoking (n=4159, 74.7%)	Stopped smoking (n=494, 8.9%)	Continued smoking (n=912, 16.4%)
Gestational age (wk)	40.1 (37.0 , 42.0)	40.1 (37.0 , 42.0)	40.0 (36.4 , 42.1) †
Birth Weight (g)	3460 (543)	3451 (549)	3285 (554) †
Preterm birth (%)	4.5	4.3	6.4
Child characteristics			
Age (y)	6.0 (5.7 , 7.2)	6.0 (5.7 , 7.4)	6.1 (5.7 , 7.8) †
Height (cm)	119.4 (5.9)	119.7 (6.2)	119.6 (6.4)
Weight (kg)	22.4 (18.2 , 30.9)	22.4 (18.2 , 31.7)	23.0 (18.2 , 34.2) †
Body mass index (kg/m^2)	15.8 (13.9 , 19.5)	15.8 (13.8 , 19.6)	16.1 (14.1 , 21.1) †
Mean systolic blood pressure (mmHg)	102.6 (8.2)	102.6 (8.7)	103.7 (8.7) †
Mean diastolic blood pressure (mmHg)	60.5 (6.8)	60.7 (7.2)	61.5 (7.1) †
Pulse wave velocity (m/s)	5.5 (0.9)	5.5 (1.0)	5.5 (0.8)
Left ventricular mass (grams)	53.2 (11.7)	53.4 (11.7)	54.5 (12.4) †
Aortic root diameter (mm)	19.3 (1.8)	19.2 (1.9)	19.4 (1.9)
Left atrial diameter (mm)	25.2 (2.8)	25.0 (2.8)	25.4 (2.8)
Fractional shortening (%)	35.3 (4.5)	35.6 (4.8)	35.7 (4.7) *

* P<0.05, †P<0.01. Values are means (sd) or medians (90% range).

Parental smoking during pregnancy and childhood vascular outcomes

We did not observe differences in systolic and diastolic blood pressure between children from mothers who did not smoke or smoked in first trimester only (Table 2). Continued maternal smoking during pregnancy was not associated childhood systolic blood pressure (difference 0.13 mmHg (95%CI: -0.43, 0.69)), but we observed a positive trend for the association with diastolic blood pressure (difference 0.47 mmHg (95%CI: -0.01, 0.96). We observed a dose-dependent association of the number of cigarettes smoked during the third trimester with childhood diastolic blood pressure (*P* value for trend <0.01), but not with systolic blood pressure. Compared to mothers who did not smoke, mothers who smoked 10 or more cigarettes per day had children with a higher diastolic blood pressure (difference 1.43 mmHg (95%CI: 0.22, 2.63)). Maternal smoking during pregnancy was not associated with childhood carotid-femoral pulse wave velocity. Among children of mothers who did not smoke during pregnancy, paternal smoking was not associated with childhood blood pressure or carotid-femoral pulse wave velocity. Adjustment for the various confounders attenuated the effect sizes of the associations. The difference between the non-adjusted and adjusted models was mainly explained by including maternal educational level and childhood body mass index in the models. We did not observe an interaction of parental smoking with childhood BMI (P>0.05).

Table 2. Parental Smoking during Pregnancy and Blood Pressure and Pulse Wave Velocity in 6 year old Children

	Mean systolic blood pressure (mmHg)	Mean diastolic blood pressure (mmHg)	Pulse wave velocity (m/s)
Maternal smoking during pregnancy			
Non smoking during pregnancy (n=4,070)	Reference	Reference	Reference
Stopped when pregnancy was known (n=481)	-0.27 (-0.98 , 0.44)	-0.09 (-0.51 , 0.70)	-0.05 (-0.14 , 0.05)
Continued smoking during pregnancy (n=896)	0.13 (-0.43 , 0.69)	0.47 (-0.01 , 0.96)	-0.02 (-0.09 , 0.06)
< 5 cigarettes per day (n=354)	0.01 (-0.82 , 0.85)	0.13 (-0.58 , 0.85)	-0.08 (-0.20 , 0.04)
5-9 cigarettes per day (n=217)	-0.50 (-1.56 , 0.57)	0.80 (-0.11 , 1.72)	0.11 (-0.04 , 0.26)
≥10 cigarettes per day (n=129)	0.93 (-0.48 , 2.33)	**1.43 (0.22 , 2.63) ***	0.06 (-0.14 , 0.26)
P-value for trend	0.712	**0.008**	0.525
Paternal smoking			
No (n=2,369)	Reference	Reference	Reference
Yes (n=1,298)	-0.18 (-0.69 , 0.33)	0.12 (-0.39 , 0.52)	0.03 (-0.05 , 0.10)
< 5 cigarettes per day (n=570)	-0.39 (-1.07 , 0.30)	-0.08 (-0.73 , 0.57)	0.00 (-0.09 , 0.10)
5-9 cigarettes per day (n=260)	0.20 (-0.76 , 1.16)	0.69 (-0.13 , 1.51)	0.05 (-0.09 , 0.19)
≥10 cigarettes per day (n=441)	-0.15 (-0.91 , 0.61)	0.01 (-0.57 , 0.60)	-0.01 (-0.11 , 0.10)
P-value for trend	0.741	0.702	0.788

* P<0.05, ** P<0.01. All analyses were adjusted maternal age, parity, educational level, ethnicity, pre-pregnancy body mass index and blood pressure at intake, child sex, gestational age at birth, birth weight, breastfeeding status, current age and body mass index. Values are regression coefficients (95% CI) and reflect the difference in blood pressure (mmHg) or pulse wave velocity (m/s) at the age of 6 years for different categories of maternal smoking during pregnancy. Children of non smoking mothers were used as reference group. The associations of paternal smoking and blood pressure were assessed among children of mothers who did not smoke during pregnancy.

Parental smoking during pregnancy and childhood cardiac outcomes

We did not observe consistent associations of maternal first-trimester only or continued smoking during pregnancy with childhood left cardiac structures (Table 3). Continued maternal smoking during pregnancy of 10 or more cigarettes per day was associated with a higher childhood fractional shortening (difference: 1.01% (95%CI: 0.18, 1.84), as compared to children of mothers who did not smoke during pregnancy). The effect on fractional shortening increased with the number of cigarettes smoked during pregnancy (*P* value for trend: 0.01). Among children of mothers who did not smoke during pregnancy was, paternal smoking during pregnancy was associated with a larger aortic root diameter (difference 0.17 mm (95%CI: 0.05, 0.28)), but not associated with fractional shortening. Adjustment for the various confounders attenuated the effect sizes of the associations.

Table 3. Associations of Parental Smoking during Pregnancy with Cardiac Structures and Function in 6 year old Children

	Left ventricular mass (grams)	Aortic root (mm)	Left atrial diameter (mm)	Fractional shortening (%)
Maternal smoking during pregnancy				
Non smoking during pregnancy (n=3986)	Reference	Reference	Reference	Reference
Stopped when pregnancy was known (n=475)	0.09 (-0.86 , 1.04)	-0.10 (-0.25 , 0.05)	-0.18 (-0.42 , 0.06)	0.18 (-0.23 , 0.60)
Continued smoking during pregnancy (n=881)	0.40 (-0.33 , 1.14)	-0.05 (0.17 , 0.07)	-0.07 (-0.25 , 0.12)	0.26 (-0.06 , 0.58)
< 5 cigarettes per day (n=351)	0.51 (-0.63 , 1.64)	-0.07 (-0.25 , 0.11)	-0.04 (-0.33 , 0.24)	0.17 (-0.32 , 0.67)
5-9 cigarettes per day (n=214)	0.98 (-0.48 , 2.43)	0.00 (-0.23 , 0.23)	-0.20 (-0.56 , 0.17)	0.38 (-0.29 , 1.01)
≥10 cigarettes per day (n=126)	0.13 (-1.80 , 2.07)	-0.00 (-0.31 , 0.31)	0.01 (-0.48 , 0.49)	**1.01 (0.18 , 1.84)** *
P-value for trend	0.271	0.807	0.500	**0.014**
Paternal smoking				
No (n=2,337)	Reference	Reference	Reference	Reference
Yes (n=1,281)	0.20 (-0.49 , 0.89)	**0.17 (0.05 , 0.28)** **	-0.07 (-0.24 , 0.10)	0.07 (-0.22 , 0.37)
< 5 cigarettes per day (n=550)	0.42 (-0.51 , 1.35)	**0.18 (0.03 , 0.33)** *	0.02 (-0.21 , 0.25)	0.06 (-0.34 , 0.46)
5-9 cigarettes per day (n=250)	-0.62 (-1.93 , 0.70)	0.03 (-0.18 , 0.24)	-0.14 (-0.47 , 0.18)	0.18 (-0.38 , 0.75)
≥10 cigarettes per day (n=442)	0.21 (-0.81 , 1.24)	**0.19 (0.03 , 0.35)** *	-0.22 (-0.47 , 0.03)	-0.10 (-0.54 , 0.35)
P-value for trend	0.823	**0.015**	0.098	0.991

*P<0.05, **P<0.01. All analyses were adjusted maternal age, parity, educational level, ethnicity, pre-pregnancy body mass index and blood pressure at intake, child sex, gestational age at birth, birth weight, breastfeeding status, current age and body mass index, ultrasonographer and ultrasound device. Values are regression coefficients (95% CI) and reflect the difference in left cardiac structures and function at the age of six years for different categories of maternal smoking during pregnancy. Children of non smoking mothers were used as reference group. The associations of paternal smoking and left cardiac structures and function were assessed among children of mothers who did not smoke during pregnancy.

Discussion

The results of this population-based cohort study suggest that continued maternal smoking during pregnancy is associated with higher diastolic blood pressure and fractional shortening, but not with left cardiac structures and pulse wave velocity in childhood. Paternal smoking was associated with a larger childhood aortic root diameter.

Strengths and limitations

The main strength of our study is the prospective data collection from early fetal life onwards and the size of the population-based cohort. Our analyses were based on

more than 5,500 children with cardiovascular outcome measurements. The detailed information on maternal and paternal smoking during pregnancy enabled us to assess both trimester specific and dose-response relationships. Blood pressure was measured multiple times with one-minute intervals minimizing measurement error. We used well-described and validated measurements to assess arterial stiffness and cardiac structures and function. Also, we had information about a large number of potential confounders.

We also need to address some limitations. Information about smoking during pregnancy was missing for 15.6% of all mothers. This non-response would lead to biased effect estimates if associations of maternal smoking during pregnancy with cardiovascular outcomes would be different between those mothers included and not included in the analyses. This seems unlikely. Biased estimates in large cohort studies mainly arise from loss to follow-up rather than from a non-response at baseline[23]. Of all children with available data on maternal smoking during pregnancy, 70.5 % participated in the follow-up measurements at the age of six years; 10.0% was lost to follow-up and 3.1% did not provide consent for further follow-up from the age of 6 years onwards. The rest of the mothers and children (16.5%) did provide consent for further follow-up, but did not visit the research center. Overall, mothers who did not visit the research center for follow-up measurements did more frequently smoke during pregnancy and were less educated than the total sample. This selective loss to follow-up possibly might have led to biased effect estimates. Some studies measured cotinine in maternal urine samples to limit misclassification bias[24-25], but this method does not seem to be superior to the use of self-reported smoke exposure, due to low correlations between cotinine levels and smoking behaviour reported by questionnaire[26-27]. Finally, although we have performed adjustment for various potential confounders, residual confounding might still be an issue due to the observational design of the study. We used paternal smoking as a model to explore the role of residual confounding due to unmeasured environmental or family factors.

Parental smoking during pregnancy and childhood cardiovascular outcomes

Several studies have shown an association of maternal smoking during pregnancy with higher blood pressure in children, but results are inconsistent[7-9, 28]. Only some of these studies assessed a dose-response relationship of maternal smoking with childhood blood pressure, which could explain the non-consistent findings. A recently published study in a cohort born in the 1960's, showed that the effect of maternal smoking on childhood blood pressure at the age of 7 years was stronger in children from heavy smoking mothers (>20 cigarettes/day) than in those from mothers who smoked less intensively[28]. This is in line with our findings showing the stronger association with childhood blood pressure in mothers who smoked 10 or more cigarettes per day during pregnancy.

It is not clear whether the association of maternal smoking with childhood blood pressure is explained by direct intra-uterine mechanisms or is due to other, unmeasured, confounders. We used two approaches to explore the role of confounding. First we applied adjustment for multiple potential confounders and second, we assessed the association of both maternal and paternal smoking during pregnancy with cardiovascular outcomes. Adjustment for potential confounders did not fully explain the associations. A recent study by Wen et al. suggested that the association of maternal smoking during pregnancy with childhood blood pressure was mediated by increased postnatal growth[28]. In our study, adjustment of the associations of maternal smoking with childhood blood pressure for childhood BMI only slightly attenuated the effect sizes. We also did not observe interactions between maternal smoking and childhood BMI. Also, our results were independent of the effect of maternal smoking during pregnancy on fetal growth. Our findings of larger effect sizes for the associations for maternal smoking than for paternal smoking would indicate that childhood blood pressure is influenced directly by intra-uterine tobacco exposure. However, we should be careful with concluding intrauterine causal mechanisms since the effect estimates were small, and of borderline significance. Brion et al. showed similar effect sizes for maternal and paternal smoking during pregnancy on childhood blood pressure, suggesting that the association of maternal smoking with childhood blood pressure is explained by unmeasured confounders[9].

Several studies have shown an effect of maternal smoking during pregnancy on other specific measures of vascular function. We have previously shown that maternal smoking during pregnancy is associated with increased fetal-placental arterial resistance in third trimester of pregnancy[10]. A study by Geerts et al. among 259 mothers and children suggested that maternal smoking during pregnancy was associated with a thicker carotid artery intima media and a lower arterial distensibility at the age of 5 years[29]. We did not find an association of maternal smoking during pregnancy with childhood carotid-femoral pulse wave velocity. Both carotid-femoral pulse wave velocity and arterial distensibility are markers of arterial stiffness and a risk factor for cardiovascular disease[12]. The difference in results might be explained by different measurements techniques and localizations. Further studies focused on local and general pulse wave velocity measurements are needed.

Lampl et al. examined in 34 fetuses, the association of maternal smoking with repeatedly measured fetal cardiac volume and showed that cardiac growth was diminished in fetuses of mothers who smoked during pregnancy[30]. In the current study, we did not find associations of smoking status of the mother with left ventricular mass and aortic root and left atrial diameter. Surprisingly, paternal smoking during pregnancy was associated with a larger aortic root diameter. We cannot explain these finding by biological plausible underlying mechanisms. This association might be explained by

residual confounding or by a chance finding. We did find a dose-dependent association of maternal smoking during pregnancy with childhood fractional shortening. We cannot explain this finding, but it might be due to a higher after load due to a higher blood pressure. Paternal smoking during pregnancy was not associated with fractional shortening. Further research is needed to assess if these changes in fractional shortening persist in later life and have effects on cardiac development.

The biological mechanisms underlying the associations of fetal smoke exposure with childhood cardiovascular outcomes are not known. Various smoking related substances might be involved. Nicotine is an important teratogen and induces vasoconstriction which leads to reduced placental blood flow and lower oxygen levels in the fetus[31]. Fetal vasoconstriction and impaired blood flow may lead to suboptimal hemodynamic stimulus for placental and fetal vascular development[32-33]. In time, these changes in development might result in a higher blood pressure and other cardiovascular changes in later life. Other toxins from maternal smoking, such as carbon monoxide and cadmium might also lead to reduced placental and fetal perfusion and cardiovascular responses[34-35]. Carbon monoxide is rapidly absorbed in the blood where it binds to hemoglobin and forms carboxyhaemoglobin which results in fetal hypoxia and animal studies have shown a negative effect on cardiac maturation[34]. Cadmium is known to accumulate in the placenta is suggested to inhibit the activity of 11-B-hydroxysteroid dehydrogenase type 2. Reduced activity of this enzyme might lead to fetal growth restriction and vascular adaptations[35]. Further research is needed to assess possible mechanisms underlying the associations between maternal smoking during pregnancy and cardiovascular function in later life.

Conclusions

In conclusion, we found that maternal smoking during pregnancy might increase childhood diastolic blood pressure and fractional shortening. The stronger effect estimates for maternal smoking compared to paternal smoking might suggest that direct intrauterine adaptive responses are involved as underlying mechanisms. This study adds to the increasing body of evidence that maternal smoking during pregnancy has adverse effects on childhood and adult cardiovascular health. Maternal smoking during pregnancy is potentially modifiable and presents a possible opportunity for prevention of cardiovascular disease in later life. Further research is needed to assess the fetal effects of smoking on postnatal cardiovascular development and to establish whether reduction of maternal smoking during pregnancy also leads to a better cardiovascular health in during the life course.

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Chapter 3.3

Parental distress during pregnancy and cardiovascular development in childhood

H Rob Taal
Layla L de Jonge
Henning Tiemeier
Lennie van Osch-Gevers
Albert Hofman
Frank C Verhulst
Willem A Helbing
Albert J van der Heijden
Vincent WV Jaddoe

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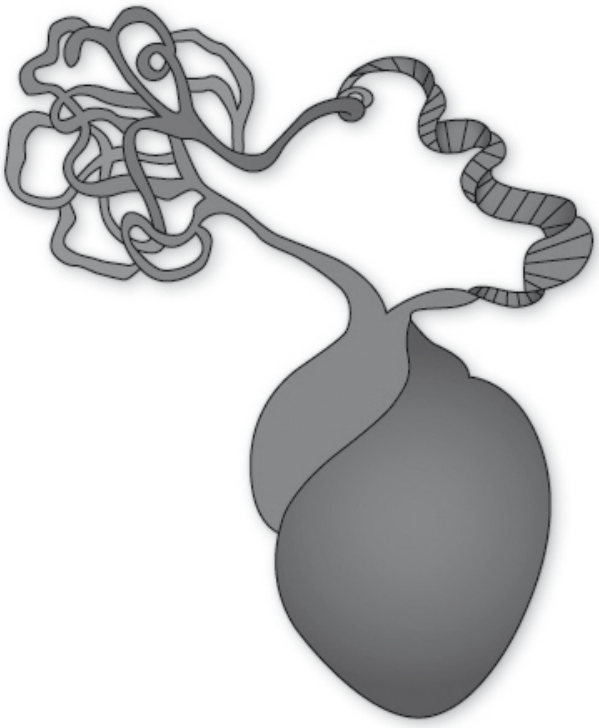


Abstract

Background: Maternal psychological distress during pregnancy might lead to higher fetal cortisol exposure, which subsequently leads to fetal cardiovascular developmental adaptations and cardiovascular dysfunction in later life. We examined whether maternal and paternal psychological distress were associated with the cardiovascular outcome measurements in school age children.

Methods: In a population-based prospective cohort study among 4,831 children, we assessed maternal and paternal psychological distress during pregnancy by questionnaire, using the Brief Symptom Inventory. At the child age of six years, we performed blood pressure and carotid- femoral pulse wave velocity measurements, and M-mode measurements of left cardiac structures and fractional shortening.

Results: We did not observe associations of high maternal and paternal psychological symptom scores with childhood blood pressure and carotid-femoral pulse wave velocity after adjustment for potential confounders. Maternal overall psychological symptoms were associated with a lower childhood left ventricular mass (difference -1.10 grams (95% confidence interval -2.13 to -0.07) between mothers with high scores and normal scores), but not with other cardiac structures and fractional shortening. Paternal overall psychological symptoms showed a similar association with childhood left ventricular mass (difference -1.34 grams (95% confidence interval -3.69 to 1.02) between fathers with high scores and normal scores).

Conclusion: Our results do not support the hypothesis that maternal psychological distress affects cardiovascular development in early life. Similar associations of maternal and paternal psychological distress with left ventricular mass suggest that these associations could be due to unmeasured social and environmental factors, rather than direct intra-uterine effects.

Introduction

Adverse fetal exposures may lead to permanent developmental adaptations in the structure and function of the cardiovascular system and other organ systems[1]. These adaptations may be beneficial on short-term, but increase the susceptibility of cardiovascular disease and metabolic diseases in later life[1]. This hypothesis is largely based on studies showing associations of low birth weight with high blood pressure, cardiovascular disease and its risk factors[2-4]. Maternal psychological distress during pregnancy might be one of the adverse fetal exposures leading to fetal developmental adaptations[5], possibly by dysregulation of the maternal hypothalamic-pituitary-adrenal (HPA) axis[6], and higher fetal cortisol exposure[6]. Previous animal and human studies have shown that excessive exposure to exogenous glucocorticoids is associated with higher blood pressure in the offspring[7-8]. Mild endogenous elevations in cortisol levels, e.g. due to maternal psychological distress during pregnancy, might also lead to developmental adaptations[9] and affect cardiovascular function in the offspring, but the number of studies is limited[10]. Any association of maternal pscyhological distress with childhoodblood pressure could be explained by direct intra-uterine mechanisms of high fetal cortisol levels, or by other environmental factors. Similar effect sizes for maternal and paternal psychological distress on childhood outcomes would indicate that associations are due to unmeasured shared social and envrionmental factors[11].

Therefore, we assessed in a large population-based prospective cohort study among 4,831 children, the associations of maternal and paternal psychological distress with blood pressure, carotid-femoral pulse wave velocity and left cardiac structures and function in children at the age of six years.

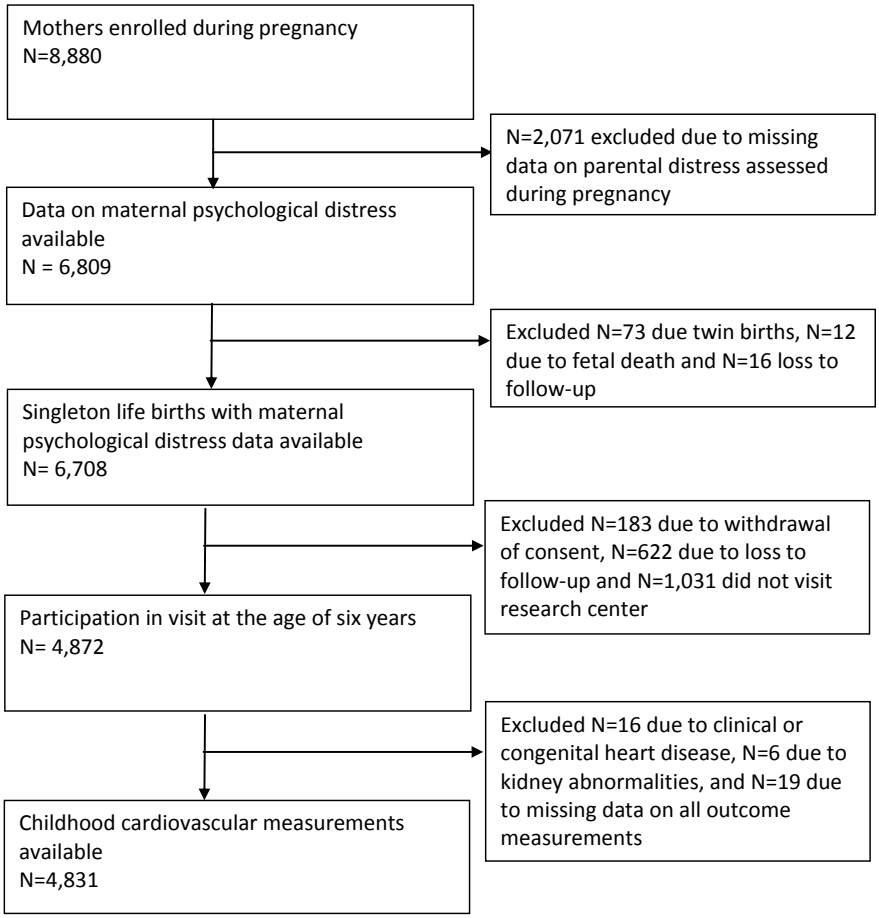
Methods

Study design and population

The study was embedded in the Generation R Study, a population-based, prospective cohort study from fetal life onwards in Rotterdam, the Netherlands[12]. Enrolment in the study was aimed at early pregnancy, but was possible until birth of the child. Information about maternal psychological distress during pregnancy was collected in the second trimester of pregnancy. Between March 2008 and January 2012, all participating children and their mothers were invited to a dedicated research center, to participate in detailed follow-up measurements at the median age of 6 years (95% range 5.6 to 7.4 years). The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all parents of participants.

In total, 8,880 mothers were enrolled in the study during pregnancy, of whom 6,809 (75.5%) provided information about psychological symptoms during pregnancy. For the present study, only singleton life births were included (n=6,708), of whom 4,872 (72.5%) children attended the follow-up visit between January 2009 and January 2012. Children with clinical or echocardiographic evidence of congenital heart disease or kidney abnormalities were excluded from the study (n=22). Blood pressure, carotid-femoral pulse wave velocity or cardiac ultrasound measurements were successfully performed in 4,831 children (see Figure 1).

Figure 1. Flowchart



Flowchart of the study population: the Generation R Study Cohort, Rotterdam, the Netherlands.

Parental psychological distress

Information on maternal and paternal psychological distress was obtained by postal questionnaires that were returned at around 20 weeks of gestation using the Brief Symptom Inventory and the subscale General Functioning of the Family Assessment Device[13]. Mother and father each completed the questionnaires. The Brief Symptom Inventory is a validated self-report questionnaire with 53 items[13]. These items define a broad spectrum of psychological symptoms in the preceding 7 days. A global index (Global Severity Index) and 3 symptom scales (Depression, Anxiety, and Hostility) were defined[13]. The Global Severity Index is a measure of current level or depth of the symptoms, and denotes overall psychological symptoms. Each item was rated on five-point uni-dimensional scales ranging from '0' (not at all) to '4' (extremely). A score is provided for each symptom scale by summing the item scores of each scale and dividing the results by the number of endorsed symptoms. This resulted in a range of scores from 0 to 4. Higher scores on these scales represented an increased occurrence of overall psychological symptoms, symptoms of depression, anxiety, or hostility. According to previous studies, mothers were categorized as being sensitive for 'clinically' significant psychological symptoms (yes/no) when having a score above 0.71 on the overall psychological symptoms scale, above 0.80 on the depression scale, and above 0.71 on the anxiety scale[14]. Fathers were categorized as being sensitive for 'clinically' significant psychological symptoms (yes/no) when having a score above 0.66 on the overall psychological symptoms scale, above 0.71 on the depression scale, and above 0.65 on the anxiety scale[14]. In the current study, internal consistencies (Cronbach's alpha) for the different scales of the mother and the father ranged from 0.72 to 0.95 and from 0.61 to 0.93 respectively. Family stress was assessed by the subscale General Functioning of the Family Assessment Device. General Functioning is a validated overall self-report measure of health and pathology of a family and consists of a 12 items measure[15]. Half of the items describe health functioning, e.g. 'In times of crisis, we can turn to each other for support'. The other half describes unhealthy items, e.g. 'There are a lot of unpleasant and painful feelings in our family'. Both the mother and the father were asked to rate how well each item described their family by selecting from four different responses: strongly agree, agree, disagree, or strongly disagree. The item scores were summed and divided by 12, yielding a total score from 1 to 4. A general functioning score >2.17 (cut-off) denotes high family stress[15]. The internal consistency of maternal and paternal general functioning was α=0.89 and α=0.85 respectively.

Childhood cardiovascular measurements

All cardiovascular measurements in childhood were performed in a dedicated research center in the Erasmus Medical Center, Rotterdam, the Netherlands. The child was lying quietly, while systolic and diastolic blood pressure was measured at the right brachial

artery in supine position, four times with one minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus ™ (Paramus, NJ, USA)[16]. A cuff was selected with a cuff width approximately 40% of the arm circumference and long enough to cover 90% of the arm circumference. More than 90% of the children who visited the research center had four successful blood pressure measurements available.

Carotid-femoral pulse wave velocity, the reference method to assess aortic stiffness[17], was assessed using the automatic Complior device (Complior; Artech Medical, Pantin, France) with participants in supine position. The distance between the recording sites at the carotid (proximal) and femoral (distal) artery was measured over the surface of the body to the nearest centimeter. Through piezoelectric sensors placed on the skin, the device collected signals to assess the time delay between the pressure upstrokes in the carotid artery and the femoral artery. Carotid-femoral pulse wave velocity was calculated as the ratio of the distance travelled by the pulse wave and the time delay between the feet of the carotid and femoral pressure waveforms, as expressed in meters per second[18]. To cover a complete respiratory cycle, the mean of at least 10 consecutive pressure waveforms was used in the analyses. Recently, it has been shown that a similar measure, carotid-radial pulse wave velocity, can be measured reliably measured with good reproducibility in a large pediatric population based cohort[19].

Two-dimensional M-mode echocardiographic measurements were performed using the ATL-Philips Model HDI 5000 (Seattle, WA, USA) or the Logiq E9 (GE Medical Systems, Wauwatosa, WI, USA) devices. The majority (77%) of all echocardiographic measurements were measured by three sonographers, and were supervised by a paediatric cardiologist (L.v.O.-G.). The children were examined in a quiet room with the child awake in supine position. Missing echocardiograms were mainly due to restlessness of the child or unavailability of equipment or sonographer. Left atrial diameter (LAD), interventricular end-diastolic septal thickness (IVSTD), left ventricular end-diastolic diameter (LVEDD), left ventricular end-diastolic posterior wall thickness (LVPWTD), interventricular end-systolic septal thickness (IVSS), left ventricular end-systolic diameter (LVESD), left ventricular end-systolic posterior wall thickness (LVPWTS), aortic root diameter (AOD) and fractional shortening (FS) as a quantification of global left ventricular systolic function, were measured using methods recommended by the American Society of Echocardiography[20]. Left ventricular mass (LV mass) was computed using the formula derived by Devereux et al[21]:

LV mass = $0.80 \times 1.04((IVSTD + LVEDD + LVPWTD)^3 - (LVEDD)^3) + 0.6$. To assess reproducibility of ultrasound measurements, the intraobserver and interobserver intraclass correlation coefficient were calculated in 30 subjects and varied between 0.85 and 0.99 for the main outcome measures[22].

Covariates

Information on maternal age, parity, educational level, smoking habits during pregnancy, ethnicity, pre-pregnancy body mass index (BMI) and paternal ethnicity was obtained from questionnaires. Maternal education was defined as highest followed education according to the classification of Statistics Netherlands and categorized in primary, secondary and higher[23]. Maternal and paternal blood pressure at intake were measured with the validated Omron 907 automated digital oscillometric sphygmanometer (OMRON Healthcare B.V. Hoofddop, the Netherlands)[24]. Date of birth, child sex, gestational age at birth and birth weight were obtained from midwife and hospital registries. Breastfeeding (yes/no) was assessed using questionnaires. Current height and weight were measured without shoes and heavy clothing at the visit at six years, and BMI (kg/m^2) was calculated.

Statistics

We used mixed models to assess associations maternal psychological distress scales (dichotomized based on the clinical cut-offs and as continuous) during pregnancy and blood pressure[25]. The mixed-model method fits each of the as many as four blood pressure measurements of every child as repeated outcome measures. One of the advantages of this approach, compared to using the average measure for each child as an outcome, is that subjects with the maximum number of blood pressure measurements available and the least individual variability in their blood pressure measurements are assigned the highest weight in the analysis[26-27]. We used multiple linear regression models to assess the association of maternal psychological distress scales (dichotomized based on the clinical cut-offs and as continuous) and carotid-femoral pulse wave velocity and left cardiac structures. All analyses were adjusted for maternal age, parity, educational level, ethnicity and pre-pregnancy BMI, and child sex, gestational at birth, birth weight, breastfeeding and age and BMI at the six year visit. Analyses on left cardiac structure were additionally adjusted for the performing sonographer and ultrasound device. We used similar models to assess the associations of paternal psychological distress scales with cardiovascular outcomes, adjusted for paternal ethnicity and blood pressure instead of the corresponding maternal variables. Missing values in covariates (ranging from 0 to 37%), were multiple-imputed, to reduce potential bias associated with missing data[28]. We created five imputed datasets and each dataset was analyzed separately to obtain the effect sizes and standard errors. The results of all five imputed analyses were pooled and are presented in this paper. Measures of association are presented with their 95% confidence intervals (CI). The mixed-models were fitted using the Statistical Analysis System version 9.2 (SAS, Institute Inc., Gary, NC, USA). All other statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics

Subject characteristics are presented in Table 1. The percentage of boys was 49.7%. The mean birth weight was 3441 grams (SD: 546 grams). Mean systolic blood pressure and diastolic blood pressure in six year old children was 102.6 mmHg (SD: 8.2 mmHg) and 60.7 mmHg (SD: 6.9 mmHg) respectively.

Parental distress and childhood vascular outcomes

Using dichotomized symptom scales, high maternal overall psychological symptoms were associated with a higher childhood systolic and diastolic blood pressure in unadjusted models (differences 0.86 mmHg (95% CI 0.11 to 1.62) and 0.72 mmHg (95% CI 0.08 to 1.36) respectively (Table 2)). High family stress reported by the mother was positively associated with a higher childhood diastolic blood pressure (difference 0.75 mmHg (95% CI 0.20 to 1.31), but not with childhood systolic blood pressure. After adjustment for various potential confounders, these associations attenuated towards the null and did not remain significant. High scores on maternal depression, anxiety or hostility symptoms were not associated with childhood systolic or diastolic blood pressure. We investigated paternal psychological distress in mothers who scored lower than the clinical cut-off for the respective symptom scales. High paternal overall psychological symptoms were associated with a higher childhood diastolic blood pressure in the unadjusted analyses, but these effects did not remain significant after adjustment. Neither maternal nor paternal psychological distress scores were associated with childhood carotid-femoral pulse wave velocity.

Parental distress and childhood cardiac outcomes

Using dichotomized symptom scales, high maternal overall psychological symptom scores were associated with a lower childhood left ventricular mass (Table 3). After adjustment, the association attenuated but remained statistically significant (difference -1.10 grams (95% CI -2.13 to -0.07)). High scores on maternal depression and anxiety symptoms, but not hostility symptoms, tended to be associated with a lower childhood left ventricular mass (difference -0.73 grams (95% CI -1.77 to 0.30) and -0.97 (95% CI -1.94 to -0.01) respectively) in the adjusted models. Also, high maternal hostility symptoms were associated with a smaller childhood left atrial diameter (difference -0.27 (95% CI -0.46 to -0.08)). In adjusted models, high paternal overall psychological symptoms, depressions symptoms and anxiety symptom scores, showed similar effect sizes with cardiac outcomes as compared to maternal psychological symptom scores, although these associations were not statistically significant. High paternal hostility symptoms were associated with a lower childhood left ventricular mass (difference -1.66 (95% CI

Table 1. Subject characteristics

Maternal characteristics	(N=4,831)
Age (y)	31.2 (21.5 – 38.2)
Height (cm)	168.0 (7.3)
Weight before pregnancy (kg)	64.0 (51.0 – 90.0)
Pre-pregnancy body mass index (kg/m^2)	22.7 (18.7 – 32.0)
Systolic blood pressure (mmHg)	115.8 (12.1)
Diastolic blood pressure (mmHg)	68.2 (9.5)
Smoking during pregnancy, yes (%)	25.4
Breastfeeding, yes (%)	92.5
Parity, multipara (%)	41.0
Education	
Primary	7.9
Secondary	43.3
Higher	48.8
Ethnicity	
European	65.4
Non-European	34.6
Paternal characteristics	
Age (y)	33.1 (23.8 – 42.8)
Height (cm)	182 (8.0)
Weight (kg)	83.0 (65.0 – 107.0)
Body mass index (kg/m^2)	22.6 (18.7 – 32.0)
Systolic blood pressure (mmHg)	130.4 (13.4)
Diastolic blood pressure (mmHg)	73.5 (10.5)
Smoking, yes (%)	45.5
Ethnicity	
Dutch or European	61.4
Non-European	38.6
Birth characteristics	
Males (%)	49.7
Gestational age (wk)	40.2 (37.1 – 42.1)
Birth Weight (g)	3441 (546)
Preterm birth (%)	4.6
Child characteristics	
Age (y)	6.0 (5.7 – 7.2)
Height (cm)	119.5 (6.1)
Weight (kg)	23.3 (18.2 – 31.2)
Body mass index (kg/m^2)	15.9 (13.9 – 19.7)
Mean systolic blood pressure (mmHg)	102.6 (8.2)
Mean diastolic blood pressure (mmHg)	60.7 (6.9)
Pulse wave velocity (m/s)	5.5 (1.0)
Left ventricular mass (g)	53.6 (11.8)
Aortic root diameter (mm)	19.3 (1.8)
Left atrial diameter (mm)	25.2 (2.7)
Fractional shortening (%)	35.3 (4.5)

Values are means (SD), medians (90% range), or percentage

Table 2. Maternal and paternal distress and blood pressure and pulse wave velocity in six year old children

		Systolic blood pressure (mmHg)		Diastolic blood pressure (mmHg)		Carotid-femoral pulse wave velocity (m/s)	
	N	Crude model [a]	Adjusted model [b]	Crude model [a]	Adjusted model [b]	Crude model [a]	Adjusted model [b]
Maternal psychological distress							
Overall psychological symptoms	4,607	**0.86 (0.11 , 1.62) ***	0.16 (-0.61 , 0.93)	**0.72 (0.08 , 1.36) ***	0.04 (-0.62 , 0.70)	0.05 (-0.05 , 0.16)	0.00 (-0.11 , 0.11)
Depression symptoms	4,603	0.35 (-0.41 , 1.12)	-0.18 (-0.95 , 0.58)	0.52 (-0.13 , 1.16)	-0.04 (-0.70 , 0.63)	0.08 (-0.03 , 0.18)	0.04 (-0.07 , 0.15)
Anxiety symptoms	4,606	0.51 (-0.21 , 1.23)	-0.09 (-0.81 , 0.63)	0.50 (-0.11 , 1.11)	-0.07 (-0.69 , 0.55)	0.02 (-0.08 , 0.12)	-0.01 (-0.11 , 0.09)
Hostility symptoms	4,596	0.11 (-0.46 , 0.67)	-0.42 (-0.99 , 0.15)	0.26 (-0.21 , 0.74)	-0.22 (-0.71 , 0.27)	0.00 (-0.08 , 0.08)	-0.03 (-0.11 , 0.05)
Family stress	4,659	0.57 (-0.09 , 1.23)	0.21 (-0.43 , 0.86)	**0.75 (0.20 , 1.31) ***	0.45 (-0.11 , 1.01)	0.08 (-0.01 , 0.17)	0.06 (-0.04 , 0.16)
Paternal psychological distress among mothers with low scores							
Overall psychological symptoms	3,072	0.61 (-1.04 , 2.26)	0.33 (-1.28 , 1.95)	**1.54 (0.13 , 2.95) ***	1.24 (-0.17 , 2.64)	0.13 (-0.13 , 0.38)	0.12 (-0.14 , 0.38)
Depression symptoms	3,070	0.97 (-0.62 , 2.56)	0.77 (-0.79 , 2.33)	1.26 (-0.10 , 2.62)	0.94 (-0.41 , 2.30)	0.03 (-0.21 , 0.26)	0.02 (-0.22 , 0.25)
Anxiety symptoms	3,018	0.06 (-1.04 , 1.17)	-0.08 (-1.16 , 1.00)	0.23 (-0.71 , 1.17)	0.07 (-0.86 , 1.00)	0.07 (-0.08 , 0.23)	0.08 (-0.08 , 0.24)
Hostility symptoms	2,745	0.14 (-0.94 , 1.21)	-0.15 (-1.21 , 0.90)	0.35 (-0.57 , 1.25)	-0.01 (-0.92 , 0.90)	0.06 (-0.10 , 0.22)	0.03 (-0.13 , 0.19)
Family stress	2,659	**0.95 (0.04 , 1.86) ***	0.46 (-0.44 , 1.35)	0.16 (-0.62 , 0.94)	-0.22 (-1.00 , 0.57)	-0.04 (-0.17 , 0.10)	-0.04 (-0.17 , 0.10)

*P<0.05, †P<0.01. [a] The crude model is adjusted for child sex and age at measurement. [b] The adjusted model was additionally adjusted for maternal age, parity, educational level, smoking habits during pregnancy, maternal or paternal ethnicity, pre-pregnancy body mass index and maternal or paternal blood pressure at intake, gestational age at birth, birth weight, breastfeeding status, and body mass index at the age of six years. Values are regression coefficients (95% CI) and reflect the difference in blood pressure (mmHg) or carotid-femoral pulse wave velocity (m/s) for reporting high scores on the different psychological symptom scales from a mixed-model or linear regression model.

Table 3. Maternal and paternal distress and cardiac structures and function in six year old children

		Left ventricular mass (grams)		Aortic root diameter (mm)		Left atrial diameter (mm)		Fractional shortening (%)	
	N	Crude model[a]	Adjusted model[b]	Crude model[a]	Adjusted model[b]	Crude model[a]	Adjusted model[b]	Crude model[a]	Adjusted model[b]
Maternal psychological distress									
Overall psychological symptoms	4,473	**-1.31 (-2.38 , -0.24) ***	**-1.10 (-2.13 , -0.07) ***	-0.04 (-0.20 , 0.13)	-0.01 (-0.18 , 0.16)	-0.05 (0.31 , 0.21)	-0.17 (-0.43 , 0.09)	0.20 (-0.23 , 0.63)	-0.08 (-0.53 , 0.37)
Depression symptoms	4,467	**-1.25 (-2.33 , -0.17) ***	-0.73 (-1.77 , 0.30)	-0.06 (-0.23 , 0.11)	-0.01 (-0.18 , 0.16)	-0.09 (-0.35 , 0.17)	-0.15 (-0.41 , 0.11)	**0.46 (0.02 , 0.89) ***	0.24 (-0.21 , 0.69)
Anxiety symptoms	4,471	-0.99 (-2.01 , 0.04)	**-0.97 (-1.94 , -0.01) ***	0.05 (-0.11 , 0.21)	0.06 (-0.10 , 0.21)	-0.00 (-0.25 , 0.24)	-0.13 (-0.38 , 0.11)	**0.59 (0.17 , 1.00) †**	0.35 (-0.08 , 0.77)
Hostility symptoms	4,462	-0.50 (-1.30 , 0.31)	-0.34 (-1.11 , 0.44)	-0.01 (-0.14 , 0.12)	-0.00 (-0.13 , 0.12)	-0.18 (-0.38 , 0.01)	**-0.27 (-0.46 , -0.08) †**	0.25 (-0.08 , 0.58)	0.06 (-0.28 , 0.40)
Family stress	4,518	0.14 (-0.80 , 1.07)	0.35 (-0.53 , 1.22)	0.02 (-0.13 , 0.17)	0.04 (-0.11 , 0.18)	-0.12 (-0.34 , 0.11)	-0.18 (-0.40 , 0.04)	0.05 (-0.33 , 0.43)	-0.06 (-0.44 , 0.32)
Paternal psychological distress among mothers with low scores									
Overall psychological symptoms	2,971	-1.23 (-3.76 , 1.29)	-1.34 (-3.69 , 1.02)	0.04 (-0.37 , 0.44)	0.05 (-0.34 , 0.44)	-0.57 (-1.19 , 0.04)	**-0.68 (-1.26 , -0.09) ***	-0.15 (-1.19 , 0.89)	-0.28 (-1.32 , 0.76)
Depression symptoms	2,971	-1.16 (-3.57 , 1.25)	-0.73 (-2.97 , 1.51)	-0.22 (-0.61 , 0.17)	-0.12 (-0.49 , 0.25)	-0.49 (-1.08 , 0.09)	-0.51 (-1.07 , 0.05)	-0.12 (-1.10 , 0.87)	-0.21 (-1.20 , 0.77)
Anxiety symptoms	2,919	-1.25 (-2.92 , 0.41)	-1.25 (-2.80 , 0.30)	-0.03 (-0.30 , 0.23)	-0.03 (-0.29 , 0.23)	-0.16 (-0.57 , 0.25)	-0.19 (-0.57 , 0.20)	-0.20 (-0.87 , 0.48)	-0.25 (-0.92 , 0.43)
Hostility symptoms	2,655	**-2.52 (-4.16 , 0.89) †**	**-1.66 (-3.18 , -0.13) ***	-0.03 (-0.29 , 0.23)	0.08 (-0.17 , 0.33)	-0.17 (-0.57 , 0.23)	-0.09 (-0.47 , 0.29)	**0.73 (0.07 , 1.40) ***	0.66 (-0.01 , 1.33)
Family stress	2,578	-0.09 (-1.52 , 1.35)	-0.42 (-1.77 , 0.92)	-0.02 (-0.25 , 0.21)	-0.07 (-0.29 , 0.15)	0.15 (-0.20 , 0.49)	0.01 (-0.33 , 0.34)	-0.27 (-0.71 , 0.27)	-0.40 (-0.98 , 0.18)

*P<0.05, †P<0.01. [a] The crude model is adjusted for child sex, age at measurement, sonographer and ultrasound device. [b] The adjusted model was additionally adjusted for maternal age, parity, educational level, smoking habits during pregnancy, maternal or paternal ethnicity, pre-pregnancy body mass index and maternal or paternal blood pressure at intake, gestational age at birth, birth weight, breastfeeding status and body mass index at the age of six years. Values are regression coefficients (95% CI) and reflect the difference in left cardiac structures at the age of six years for reporting high scores on the different psychological symptom scales from a linear regression model.

-3.18 to -0.13)). In the adjusted models, maternal and paternal psychological distress was not associated with childhood aortic root diameter and fractional shortening.

Discussion

Results of this population-based cohort study suggest that maternal and paternal psychological distress during pregnancy are not associated with childhood blood pressure and carotid-femoral pulse wave velocity. Both higher maternal and paternal psychological distress were associated with lower childhood left ventricular mass.

Strength and limitations

The main strength of our study is the prospective data collection from fetal life onwards and the size of the population-based cohort. Our analyses were based on more than 4,500 children with cardiovascular outcome measurements. As far as we know, this is the largest study, investigating the associations between parental distress and cardiovascular outcomes in childhood. The assessment of maternal as well as paternal distress, allowed us to explore whether the associations are likely due to an intra-uterine effects of maternal psychological distress or rather due to shared environmental factors between the mother and the father. Blood pressure was measured multiple times with one-minute intervals minimizing measurement error. We used well-described and validated measurements to assess arterial stiffness and cardiac structures and function. Also, we had information about a large number of potential confounders. However, some limitations need to be addressed. We measured maternal psychological distress at one time-point during pregnancy. Unfortunately, we do not have information on persistence and intensity of psychological symptoms throughout the pregnancy. Some studies have suggested that the timing of psychological distress during pregnancy might be important for fetal programming effects[29]. Information about maternal psychological distress was missing for 24.5% of all mothers. This non-response at baseline would lead to biased effect estimates if the associations of maternal psychological distress during pregnancy with cardiovascular outcomes would be different in mothers who were not included in these analyses, compared to the mothers who are included in this study. This seems unlikely. Furthermore, it has been described that biased estimates in large cohort studies mainly arise from loss to follow-up rather than non-response at baseline[30]. Of all children with available data on maternal psychological distress, 72.5% participated in the follow-up measurements around the age of six years; 9.3% was loss to follow-up and 2.7% did not provide consent for further follow-up from the age of six years onwards. The remainder of the mothers and children without measurements available did provide consent for further follow-up but were not able to visit the research center. Overall,

mothers from children who did not visit the research center, had higher psychological distress scores, smoked more frequently and were less well educated than the total sample. This selective loss to follow-up might have led to biased effect estimates and a loss of statistical power. Assessing parental psychological distress by questionnaire might lead to misclassification bias, due to underreporting of psychological distress. In general, this misclassification most likely would lead to an underestimation of the effect of parental psychological distress on cardiovascular outcomes. Finally, although we have performed adjustment for various potential confounders, residual confounding might still be an issue due to the observational design of the study.

Parental distress and childhood vascular outcomes

Several studies in both animals and humans have shown that fetal exposure to high levels of cortisol, e.g. due to administration of therapeutic drugs, is associated with a higher blood pressure in later life[7-8]. We hypothesized that maternal psychological distress would lead to an increased fetal exposure to cortisol, which could lead to a higher blood pressure or increased carotid-femoral pulse wave velocity. Studies of normal variation in endogenous cortisol levels and the effect on blood pressure in later life have been scarce. A small study by Rondo et al. among 130 mothers and children aged five to seven years, investigated the association of maternal cortisol levels in third trimester of pregnancy with childhood blood pressure. Although this study did not provide exact effect estimates, the authors mention in the discussion that maternal cortisol levels were not associated with childhood blood pressure[10]. In our study, we did not find associations of maternal or paternal psychological distress scales with childhood blood pressure after adjustment for potential confounders in the largest sample so far. Our findings suggest that social and environmental factors might explain the previously suggested association. However, we have to acknowledge that maternal stress assessment was different in timing and instrument between the studies, which might explain the inconsistent results. It might also be that the maternal psychological distress does not lead to a sufficient rise in fetal cortisol levels in our population. Furthermore, effects might not be present at this age, but might become apparent in later life. The mechanism underlying the association of overexposure to cortisol with a higher blood pressure in the offspring remains unclear, but several mechanisms have been implicated. In animal studies, prenatal exposure to cortisol has been associated with micro vascular dysfunction and affected functioning of the renin-angiotensin system and the HPA-axis in the offspring, with possible subsequent effects on blood pressure in later life[31-32]. The study by Rondo et al. did suggest that maternal cortisol concentrations were positively associated with systemic vascular resistance, as measured by analyzing the shape of arterial pressure wave produced by the heart beat[10]. In our study, we did not find any association of maternal psychological distress scales with carotid-femoral pulse wave velocity around

the same age. Further research is needed to assess the effect of maternal psychological distress and cortisol levels on different measures of childhood vascular function, and to assess whether differences arise in later life.

Parental distress and childhood cardiac outcomes

Not much is known on the effect of maternal psychological distress on cardiac development. There have been several animal studies investigating the effect of fetal cortisol exposure on the developing heart[33-34]. In sheep fetuses it is shown that cortisol stimulates cell cycle activity of cardiomyocytes[33]. Furthermore, neonatal exposure to glucocorticoids has been associated with a lower heart weight in rats at the age of 4 weeks and at older age, with depressed systolic function with compensatory dilatation to maintain normal cardiac output[34]. In line with this study, we have found that maternal psychological distress is associated with a lower left ventricular mass. However, we observed that paternal psychological distress showed similar associations, suggesting these associations are not due to direct intrauterine mechanisms. In contrast, Chen et al. found in a retrospective study among 116 children, that children, whose mothers received repeated courses of antenatal corticosteroids, did not have different left ventricular mass and fractional shortening compared to age, sex and ethnicity matched control children who did not receive antenatal corticosteroids[35]. We also did not find consistent associations with other cardiac structures and fractional shortening. Residual confounding might be an issue; e.g. we did not take into account physical activity of the children. High parental psychological distress is associated with lower physical activity in children, which in turn is associated with a lower left ventricular mass[36-37]. This could explain (part) of the association of parental distress and left ventricular mass found in this study. Further research is needed to confirm these findings, identify exact underlying mechanisms and to assess whether these changes persist in later life.

Conclusions

Our study suggests that maternal psychological distress during pregnancy is not associated with childhood blood pressure and carotid-femoral pulse wave velocity. We have found evidence that maternal psychological distress is associated with left ventricular mass. However, similar effect sizes were found for paternal distress, indicating that these associations might be due to other shared environmental factors. Further research is needed to investigate the exact underlying mechanisms and the long-term effects of parental psychological distress on cardiovascular development.

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Chapter 3.4

Maternal diet during pregnancy and childhood blood pressure

Leontine CL van den Hil
H Rob Taal
Layla L de Jonge
Denise HM Heppe
Eric AP Steegers
Albert Hofman
Albert J van der Heijden
Vincent WV Jaddoe

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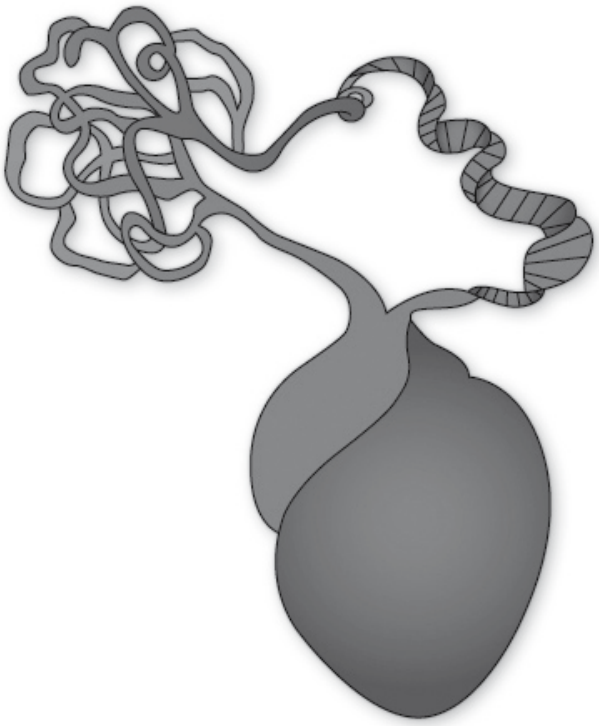

Abstract

Background: Suboptimal maternal dietary intake during pregnancy might lead to fetal cardiovascular adaptations and higher blood pressure in the offspring. The aim of this study was to investigate the associations of maternal first trimester dietary intake with blood pressure in children at the age of six years.

Methods: We assessed first trimester maternal daily dietary intake by a food frequency questionnaire and measured folate, homocysteine and vitamin B12 concentrations in blood, in a population-based prospective cohort study among 2,863 mothers and children. Childhood systolic and diastolic blood pressure was measured using a validated automatic sphygmomanometer.

Results: Higher maternal iron intake tended to be associated with a lower childhood systolic blood pressure (-0.30 mmHg per standard deviation increase in iron intake (95% CI: -0.61 to 0.01)). Also, higher maternal vitamin B12 concentrations were associated with a higher diastolic blood pressure (0.31 mmHg per standard deviation increase in vitamin B12 (95% CI: 0.06 to 0.56)). After taking into account multiple testing, none of the associations were statistically significant. Maternal first trimester folate and homocysteine concentrations were not associated with childhood blood pressure.

Conclusion: Results from this study suggest that maternal iron intake and vitamin B12 concentrations during first trimester of pregnancy might affect childhood blood pressure, although the effect estimates were small and were not significant after correction for multiple testing. Further studies are needed to replicate these findings, to elucidate the underlying mechanisms and to assess whether these differences in blood pressure persist in later life.

Introduction

Suboptimal maternal and fetal nutrition might lead to fetal cardiovascular developmental adaptations and subsequent cardiovascular disease in later life[1-3]. Support for this hypothesis is largely based on experimental animal studies and historical cohort studies in humans showing associations of maternal exposure to extreme famine during pregnancy with development of hypertension in later life[4-6]. A disbalance in nutrient intake during pregnancy might also be an explanation for the development of hypertension[4]. Not much is known about the associations of less extreme variations in maternal dietary intake with cardiovascular disease in the offspring. Several studies focused on the associations of maternal micro- or macronutrient intake with childhood blood pressure but the results are inconclusive[7-17]. Two previous studies suggested that higher maternal fat intake during pregnancy was associated with diastolic blood pressure in the offspring, although the studies observed opposite effects[7-8]. Also, maternal intake of micronutrients during pregnancy, such as calcium, sodium and iron has been suggested to be associated with blood pressure in children, but results were not consistent[9-11, 13-15]. Other micronutrients, including folate, homocysteine and vitamin B12, might affect vascular development[18]. High homocysteine and low folate and levels are associated with endothelial dysfunction[19-20] and might subsequently lead to higher blood pressure in later life. Since vitamin B12 lower homocysteine levels, this might also influences blood pressure.

We assessed in a large population-based prospective cohort study among 2,863 Dutch mothers and their children, the associations of maternal daily total energy intake, intake of macronutrients and micronutrients in first trimester of pregnancy with blood pressure at the age of six years. We also assessed whether first trimester folate, homocysteine and vitamine B12 concentrations in maternal blood were associated with childhood blood pressure.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, The Netherlands[21-22]. Enrolment in the study was aimed at early pregnancy, but was allowed until the birth of the child. Information about maternal diet and other life style related variables during pregnancy were collected at enrolment. At the age of six years, all participating children and their mothers were invited to a dedicated research center, to participate in detailed hands on measurements. The study has been approved by the Medical Ethics Committee of

the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all parents of participants. The present analysis was limited to Dutch mothers, since the food frequency questionnaire was validated for assessment of dietary intake in a Dutch population. We selected mothers who were enrolled in the study before a gestational age of 25 weeks, because we aimed to assess dietary intakes in the first trimester of pregnancy. In total 4,032 Dutch mothers were enrolled before a gestational age of 25 weeks with a median of 13.6 weeks of gestation (90% range 11.5-20.2 weeks) (see Figure 1). Data on maternal dietary intake or biomarker concentrations were available in 3,960 (=98%) of these mothers. We excluded multiple births (n=104) and stillbirths (n=27) from the analyses, leaving 3,827 Dutch mothers. Of the 3,827 singleton life born children, 2,949 (77%) children attended the follow-up visit at the age of six years. Blood pressure was successfully measured in 2,863 (97%) of the children who visited the research center.

Maternal daily dietary intake

We assessed maternal dietary intake at enrolment in the study in Dutch mothers using a modified version of the validated semi quantitative food frequency questionnaire (FFQ) of Klipstein-Grobusch et al.[23]. The FFQ considered food intake over the prior three months, thereby covering the dietary intake in first trimester of pregnancy. The FFQ consisted of 293 items structured according to meal pattern. Questions included consumption frequency, portion size, preparation method, and additions. Portion sizes were estimated using Dutch household measures and photographs of foods showing different portion sizes[24]. We used the Dutch food composition table for calculating daily intake of nutritional values[25].

Folic acid supplement intake

Information on folic acid supplement use (0.4-0.5 mg) and the initiation of supplementation was obtained by questionnaires at enrolment of the study. We categorized folic acid supplement use into three groups: 1) periconceptional use, 2) start when pregnancy was known or 3) no use during pregnancy. Self-reported folic acid use was validated in a subgroup by serum folate levels in the first trimester, i.e. before 12 weeks of gestation. Within the group of mothers who reported using folic acid supplements (n=204), the median of serum folate was 23.5 (range 4.3-45.3) nmol/l, whereas the median serum folate concentrations of mothers who did not report folic acid supplement use (n=68) was 11.1(range 4.7-29.6) nmol/l. The difference in distribution function (Mann-Whitney test) was statistically significant ($P<0.001$)[26]. Information about folic acid supplement use was available in 2,505 subjects (87.5%).

Folate, homocysteine and vitamine B12 concentrations

In early pregnancy (median 12.9 weeks of gestation, 90% range 10.6-16.8) venous samples were drawn and stored at room temperature before being transported to the regional laboratory for processing and storage for future studies. Processing was aimed to be completed within a maximum of three hours after venous puncture. The samples were centrifuged and thereafter stored at -80°C[21]. To analyze folate, homocysteine and vitamin B12 concentrations, EDTA plasma samples (folate, homocysteine) and serum samples (vitamin B12) were picked and transported to the Department of Clinical Chemistry at the Erasmus University Medical Center, Rotterdam in 2008. Folate, homocysteine and vitamin B12 concentrations were analyzed using an immunoelectrochemoluminence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands). The between-run coefficients of variation for plasma folate were 8.9% at 5.6 nmol/L, 2.5% at 16.6 nmol/L, and 1.5% at 33.6 nmol/L, with an analytic range of 1.8-45.3 nmol/L. The same coefficients of variation for plasma homocysteine were 3.1% at 7.2 µmol/L, 3.1% at 12.9 µmol/L, and 2.1% at 26.1 µmol/L, with an analytic range of 1-50 µmol/L. This coefficient of variation for serum vitamin B12 was 3.6% at 142 pmol/L, 7.5% at 308 pmol/L, and 3.1% at 633 pmol/L, with an analytic range of 44-1476 pmol/L. Plasma concentrations of maternal folate, homocysteine and serum concentrations of vitamin B12 in first trimester of pregnancy were available in 2,305 (80.5%) of the mothers of the children included in this study. Missing data were mainly due to logistical reasons.

Blood pressure measurements

Blood pressure measurements in children were conducted around the age of six years in a dedicated research center in the Erasmus Medical Center, Rotterdam, the Netherlands. The child was lying quietly, while systolic blood pressure and diastolic blood pressure were measured at the right brachial artery in supine position, four times with one minute intervals. A cuff was selected with a cuff width approximately 40% of the arm circumference and long enough to cover 90% of the arm circumference. We used the validated automatic sphygmomanometer Datascope Accutorr Plus™ (Paramus, NJ, USA)[27]. Of all children visiting the research center, 91.3% had four successful blood pressure measurements available.

Covariates

Information on maternal age, pre-pregnancy body mass index (BMI), parity, alcohol use and smoking habits during pregnancy and educational level was obtained from questionnaires. Maternal education was defined as highest followed education according to the classification of Statistics Netherlands and categorized in primary, secondary and higher[28]. Child sex, gestational age at birth and birth weight were obtained from midwife and hospital registries. Breastfeeding (ever/never) was assessed using questionnaires.

Current height and weight were measured without shoes and heavy clothing at the visit at six years, and BMI (kg/m^2) was calculated.

Statistical methods

Differences in child characteristics at the age of six years between boys and girls were assessed using t-test and χ^2-tests for independent samples. Maternal dietary intake variables were categorized into quintiles. This approach was used for all dietary exposures (total energy, carbohydrate, fat, protein intake and the protein-carbohydrate ratio; calcium, iron and sodium intake; folate, homocysteine and vitamin B12 concentrations). We used mixed models to assess associations between predictors and blood pressure[29]. The mixed-model method fits each of the as many as 4 blood pressure measurements of every child as repeated outcome measures. An advantage of this modelling approach over using the average measure of blood pressure for each child as an outcome is that children with more measurements and less variability in their measurements are assigned more weight than those with fewer measurements, more variability, or both[9, 30]. We used similar mixed-models to assess the association of folic acid supplement use with blood pressure at the age of six years. All analyses were adjusted for child's sex, gestational age at intake and age at blood pressure measurement (crude model). Potential covariates were selected based on previous literature[31-32]. We assessed crude associations, adjusted for age and gender, of possible covariates with childhood blood pressure. Only the covariates which were significantly associated with systolic or diastolic blood pressure in our study population were included in our fully adjusted model. The fully adjusted model included maternal age, pre-pregnancy body mass index, alcohol use and smoking during pregnancy, educational level, gestational age at birth, birth weight, current body mass index and month in which blood pressure measurement was taken. Tests for trends were conducted by using the maternal dietary intake variables as continuous variable in the linear mixed-models. We provided the effect per standard deviation increase in the dietary intake variable. In the macronutrient analyses, we used the energy partition method to adjust for energy intake of the other macronutrients, since total energy intake and macronutrient intakes were strongly correlated[33]. The actual energy derived from the macronutrients was used in the analyses. In the micronutrient analyses, we used the residual nutrient method, and additionally adjusted for total energy intake[33].

Missing values in covariates (ranging from 0 to 15%) were multiple-imputed to reduce potential bias associated with missing data[34]. We created five imputed datasets and each dataset was analyzed separately to obtain the effect sizes and standard errors. The results of all five imputed analyses were pooled and are presented in this paper. In this study we investigated maternal macronutrient intake, micronutrient intake and maternal biomarker concentrations in pregnancy. Within these exposure groups, variables are

correlated. To take into account multiple testing, we applied a Bonferroni correction and considered a P-value lower than 0.017 (0.05/3 (three exposure groups)) as statistically significant.

The mixed-models were fitted using the Statistical Analysis System version 9.2 (SAS, Institute Inc., Gary, NC, USA). All other statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Table 1 presents the maternal and birth subject characteristics. The mean intake of total energy was 8,982.8 (SD: 2,117.3) kj/day, of calcium 1,222 (SD: 420) milligrams/day, of iron 12.1 (SD 3.3) milligrams/day and of sodium 3,314 (SD: 931) milligrams/day. The mean birth weight was 3,497 (SD: 548) grams. Child characteristics at the age of six years for boys and girls separately are shown in table 2. Systolic blood pressure and diastolic blood pressure were significantly higher in girls compared to boys ($p<0.05$).

Table 1. Maternal and birth characteristics: the Generation R Study Cohort, Rotterdam, Netherlands

	Not imputed	Imputed
Maternal characteristics		
Age	31.8 (22.2-39.6)	
Height (cm)	170.8 (6.4)	
Weight (kg)	68.1 (12.3)	68.1 (12.2)
Body mass index (kg/m^2)	22.3 (18.2-34.3)	22.4 (18.2-34.5)
Parity ≥1 (%)	38.4	38.4
Alcohol use (%)		
No	31.8	31.9
First trimester only	16.7	16.5
Continued	51.5	51.6
Smoking during pregnancy (%)		
Never	75.1	75.6
First trimester only	9.6	9.6
Continued	15.3	14.8
Educational level (%)		
Primary	2.5	2.5
Secondary	36.6	36.6
Higher	60.9	60.9
Energy intake (kj/day)	8,982.8 (2,117.3)	
Carbohydrate intake (grams/day)	262.2 (74.1)	
% of total energy intake	48.3 (37.2-61.3)	
Fat intake (grams/day)	86.5 (24.0)	
% of total energy intake	36.4 (25.3-46.4)	
Protein intake (grams/day)	79.7 (18.9)	
% of total energy intake	14.9 (10.7-19.9)	

Table 1. Maternal and birth characteristics: the Generation R Study Cohort, Rotterdam, Netherlands (continued)

	Not imputed	Imputed
Calcium intake (milligrams/day)	1,222.3 (419.9)	
Iron intake (milligrams/day)	12.1 (3.3)	
Sodium intake (milligrams/day)	3,314.3 (931.3)	
Folic acid use (%)		
No	9.6	
Started when pregnancy was known	33.7	
Started periconceptional	56.7	
Folate levels (nmol/l)	20.2 (8.7)	
Homocystein levels (umol/l)	7.3 (2.1)	
Vitamin B12 levels (pmol/l)	193.2 (87.4)	
Birth characteristics		
Birth weight (grams)	3,498 (548)	
Gestational age at birth (weeks)	40.3 (35.9-42.3)	
Sex (males %)	49.8	
Breastfeeding (never)	8.7	9.2

Values are means (Standard deviation), medians (95% range) or percentages. Only covariates with missing data were multiple-imputed.

Table 2. Child characteristics: the Generation R Study Cohort, Rotterdam, Netherlands

	Boys (n= 1,427)	Girls (n=1,436)
Age (years)	6.0 (5.6-7.5)	6.0 (5.6-7.1)
Height (cm)	119.9 (5.7)	119.1 (5.6)
Weight (kg)	23.0 (3.5)	22.7 (3.7)
Underweight (%)	6.4	4.0
Normal weight (%)	84.6	82.6
Overweight (%)	9.0	13.3
Body mass index (kg/m^2)	15.7 (13.7-18.9)	15.6 (13.7-19.8)
Systolic blood pressure (mmHg)	102.2 (7.3)	103.3 (8.2)*
Diastolic blood pressure (mmHg)	60.2 (6.4)	61.6 (6.5)*

Values are means (standard deviation), medians (95% range) or percentages.

Maternal total daily energy intake and daily intake of carbohydrates, fat and proteins were not associated with childhood systolic and diastolic blood pressure (Table 3). The protein-carbohydrate intake ratio was not associated with childhood blood pressure (Table 3). After adjustment for confounders, maternal intake of iron tended to be inversely associated with childhood systolic blood pressure (-0.30 mmHg per standard deviation increase in iron intake (95%CI: -0.61 to 0.01), *p*-value for trend 0.06), but not with diastolic blood pressure (Table 4). Maternal intake of calcium and sodium was not consequently associated with childhood systolic or diastolic blood pressure.

We did not find an association of folic acid supplement use during pregnancy with childhood systolic or diastolic blood pressure (Table 5). The associations of maternal first trimester folate, homocysteine and vitamin B12 concentrations with childhood blood

Table 3. Associations of maternal macronutrients intake during pregnancy with blood pressure at the age of six years in a Dutch population

	Systolic blood pressure (mmHg)		Diastolic blood pressure (mmHg)	
	Crude model	**Adjusted model**	**Crude model**	**Adjusted model**
Macronutrients (n=2,554)				
Total calorie intake (Kjoule)				
First quintile	*Reference*	*Reference*	*Reference*	*Reference*
Second quintile	0.07 (-0.87 to 1.01)	0.09 (-0.83 to 1.00)	0.53 (-0.25 to 1.31)	0.55 (-0.23 to 1.32)
Third quintile	-0.15 (-1.08 to 0.79)	0.04 (-0.87 to 0.96)	0.11 (-0.67 to 0.89)	0.19 (-0.58 to 0.96)
Fourth quintile	-0.44 (-1.38 to 0.50)	-0.17 (-1.09 to 0.75)	-0.20 (-0.99 to 0.58)	-0.10 (-0.88 to 0.68)
Fifth quintile	-0.14 (-1.08 to 0.80)	-0.03 (-0.95 to 0.90)	0.30 (-0.48 to 1.09)	0.33 (-0.45 to 1.11)
Tren[d] #	-0.04 (-0.34 to 0.26)	0.02 (-0.27 to 0.31)	0.06 (-0.19 to 0.31)	0.07 (-0.17 to 0.32)
Carbohydrate intake (Kjoule)				
First quintile	*Reference*	*Reference*	*Reference*	*Reference*
Second quintile	-0.00 (-0.94 to 0.94)	0.20 (-0.75 to 1.14)	0.49 (-0.29 to 1.27)	0.52 (-0.28 to 1.31)
Third quintile	0.41 (-0.53 to 1.35)	0.61 (-0.38 to 1.60)	0.39 (-0.39 to 1.17)	0.33 (-0.50 to 1.16)
Fourth quintile	-0.14 (-1.08 to 0.80)	0.06 (-0.99 to 1.11)	-0.10 (-0.88 to 0.68)	-0.20 (-1.09 to 0.68)
Fifth quintile	0.18 (-0.75 to 1.12)	0.37 (-0.76 to 1.50)	0.65 (-0.13 to 1.44)	0.47 (-0.48 to 1.42)
Trend #	0.07 (-0.23 to 0.36)	0.11 (-0.26 to 0.49)	0.15 (-0.10 to 0.40)	0.10 (-0.21 to 0.41)
Fat intake (Kjoule)				
First quintile	*Reference*	*Reference*	*Reference*	*Reference*
Second quintile	0.09 (-0.85 to 1.02)	0.19 (-0.76 to 1.14)	0.47 (-0.31 to 1.25)	0.36 (-0.44 to 1.16)
Third quintile	-0.44 (-1.38 to 0.50)	-0.24 (-1.27 to 0.79)	0.19 (-0.59 to 0.98)	0.06 (-0.81 to 0.93)
Fourth quintile	-0.55 (-1.50 to 0.39)	-0.27 (-1.37 to 0.82)	-0.44 (-1.23 to 0.34)	-0.68 (-1.61 to 0.25)
Fifth quintile	-0.18 (-1.12 to 0.75)	0.19 (-1.05 to 1.43)	0.18 (-0.61 to 0.96)	-0.10 (-1.15 to 0.94)
Trend #	-0.12 (-0.42 to 0.17)	0.02 (-0.41 to 0.44)	-0.07 (-0.32 to 0.18)	-0.21 (-0.57 to 0.14)
Protein intake (Kjoule)				
First quintile	*Reference*	*Reference*	*Reference*	*Reference*
Second quintile	-0.75 (-1.68 to 0.19)	-0.62 (-1.58 to 0.34)	-0.19 (-0.97 to 0.59)	-0.04 (-0.85 to 0.77)
Third quintile	-0.87 (-1.81 to 0.07)	-0.68 (-1.71 to 0.36)	-0.58 (-1.36 to 0.20)	-0.32 (-1.19 to 0.55)
Fourth quintile	-0.60 (-1.54 to 0.34)	-0.34 (-1.48 to 0.80)	-0.30 (-1.08 to 0.49)	0.06 (-0.91 to 1.02)
Fifth quintile	-0.56 (-1.50 to 0.38)	-0.45 (-1.76 to 0.84)	-0.02 (-0.81 to 0.76)	0.35 (-0.75 to 1.44)
Trend #	-0.15 (-0.43 to 0.17)	-0.12 (-0.57 to 0.33)	0.04 (-0.21 to 0.28)	0.21 (-0.17 to 0.59)
Protein-Carbohydrate ratio				
First quintile	*Reference*	*Reference*	*Reference*	*Reference*
Second quintile	-1.11 (-2.05 to -0.17)*	-0.90 (-1.82 to 0.02)	-0.63 (-1.42 to 0.14)	-0.38 (-1.16 to 0.40)
Third quintile	-0.88 (-1.81 to 0.05)	-0.54 (-1.47 to 0.39)	-0.80 (-1.58 to -0.02)*	-0.47 (-1.25 to 0.31)
Fourth quintile	-0.87 (-1.81 to 0.06)	-0.50 (-1.43 to 0.43)	-0.64 (1.42 to 0.14)	-0.22 (-1.01 to 0.57)
Fifth quintile	-0.99 (-1.93 to -0.06)*	-0.65 (-1.58 to 0.29)	-0.61 (-1.39 to 0.17)	-0.21 (-1.00 to 0.58)
Trend #	-0.21 (-0.51 to 0.08)	-0.11 (-0.41 to 0.19)	-0.16 (-0.41 to 0.09)	-0.05 (-0.30 to 0.20)

Values are regression coefficients (95% CI) and reflect the difference in blood pressure (mmHg) at the age of six years for each quintile of macronutrient intake (kj derived from macronutrients). The crude model is adjusted for child sex and age at blood pressure measurement. The adjusted model is additionally adjusted for maternal age, pre-pregnancy body mass index, alcohol use and smoking during pregnancy, educational level, gestational age at birth, birth weight, current body mass index and month in which blood pressure measurement was taken and additionally adjusted for the energy from the other macronutrients following the energy partition method [(1)].

#Tests for trends were conducted by using the maternal dietary intake variables as continuous variable in the linear mixed-models. Values are regression coefficients (95% CI) and reflect the difference in blood pressure (mmHg) per standard deviation increase in macronutrient intake.

Table 4. Associations of maternal micronutrients intake during pregnancy with blood pressure at the age of six years in a Dutch population

	Systolic blood pressure (mmHg)		Diastolic blood pressure (mmHg)	
	Crude model	**Adjusted model**	**Crude model**	**Adjusted model**
Micronutrients (n=2,554)				
Calcium				
First quintile	*Reference*	*Reference*	*Reference*	*Reference*
Second quintile	0.44 (-0.50 to 1.37)	0.47 (-0.45 to 1.40)	0.80 (0.02 to 1.58)*	0.90 (0.13 to 1.68)*
Third quintile	0.10 (-0.83 to 1.04)	0.25 (-0.68 to 1.18)	0.29 (-0.49 to 1.07)	0.52 (-0.26 to 1.30)
Fourth quintile	-0.25 (-1.18 to 0.69)	-0.05 (-0.98 to 0.87)	-0.34 (-1.12 to 0.44)	-0.10 (-0.88 to 0.68)
Fifth quintile	0.32 (-0.62 to 1.26)	0.38 (-0.54 to 1.31)	0.75 (-0.04 to 1.53)	0.92 (0.14 to 1.71)*
Trend #	0.09 (-0.21 to 0.38)	0.10 (-0.20 to 0.39)	0.15 (-0.10 to 0.40)	0.20 (-0.04 to 0.45)
Iron				
First quintile	*Reference*	*Reference*	*Reference*	*Reference*
Second quintile	-0.05 (-0.99 to 0.88)	0.31 (-0.63 to 1.25)	0.32 (-0.47 to 1.10)	0.63 (-0.16 to 1.43)
Third quintile	-0.57 (-1.51 to 0.37)	-0.06 (-1.02 to 0.90)	0.18 (-0.60 to 0.96)	0.70 (-0.11 to 1.51)
Fourth quintile	-0.94 (-1.87 to -0.00)*	-0.34 (-1.31 to 0.62)	-0.22 (-1.00 to 0.56)	0.32 (-0.49 to 1.13)
Fifth quintile	-1.38 (-2.32 to -0.44)**	-0.76 (-1.73 to 0.21)	-0.71 (-1.50 to 0.07)	-0.14 (-0.96 to 0.68)
Trend #	-0.48 (-0.78 to -0.18)**	-0.30 (-0.61 to 0.01)	-0.23 (-0.47 to 0.02)	-0.06 (-0.32 to 0.20)
Sodium				
First quintile	*Reference*	*Reference*	*Reference*	*Reference*
Second quintile	-0.71 (-1.65 to 0.23)	-0.57 (-1.50 to 0.35)	-0.41 (-1.19 to 0.38)	-0.34 (-1.12 to 0.44)
Third quintile	-0.98 (-1.92 to -0.04)*	-0.90 (-1.82 to 0.02)	-0.49 (-1.27 to 0.30)	-0.44 (-1.22 to 0.33)
Fourth quintile	-0.17 (-1.10 to 0.77)	-0.24 (-1.16 to 0.68)	-0.11 (-0.89 to 0.67)	-0.12 (-0.89 to 0.66)
Fifth quintile	-0.08 (-1.02 to 0.85)	-0.08 (-1.00 to 0.85)	-0.30 (-1.08 to 0.48)	-0.27 (-1.04 to 0.52)
Trend #	0.07 (-0.22 to 0.37)	0.06 (-0.24 to 0.35)	-0.01 (-0.25 to 0.24)	-0.00 (-0.25 to 0.24)

Values are regression coefficients (95% CI) and reflect the difference in blood pressure (mmHg) at the age of six years for each quintile of micronutrient intake. The crude model is adjusted for child sex and age at blood pressure measurement. The adjusted model is additionally adjusted for maternal age, pre-pregnancy body mass index, alcohol use and smoking during pregnancy, educational level, gestational age at birth, birth weight, current body mass index and month in which blood pressure measurement was taken.

Table 5. Associations of maternal folic acid supplement use during pregnancy with blood pressure at the age of six years in a Dutch population

	Systolic blood pressure (mmHg)		Diastolic blood pressure (mmHg)	
	Crude model	**Adjusted model**	**Crude model**	**Adjusted model**
Folic acid supplement use (n=2,379)				
No (n=227)	0.64 (-0.34 to 1.61)	0.09 (-0.90 to 1.07)	0.46 (-0.35 to 1.27)	-0.03 (-0.85 to 0.79)
Started when pregnancy was known (n=797)	0.04 (-0.59 to 0.66)	-0.04 (-0.68 to 0.60)	-0.40 (-0.92 to 0.12)	-0.52 (-1.05 to 0.04)
Started periconceptional (n=1,355)	*Reference*	*Reference*	*Reference*	*Reference*

*P<0.05

Values are regression coefficients (95% CI) and reflect the difference in blood pressure (mmHg) at the age of six years for each group of maternal folic acid supplement use. The crude model is adjusted for child sex and age at blood pressure measurement. The adjusted model is additionally adjusted for maternal age, pre-pregnancy body mass index, alcohol use and smoking during pregnancy, educational level, gestational age at birth, birth weight, current body mass index and month in which blood pressure measurement was taken

Table 6. Associations of maternal first trimester folate, homocysteine and vitamin B12 levels with blood pressure at the age of six years in a Dutch population

	Systolic blood pressure (mmHg)		Diastolic blood pressure (mmHg)	
	Crude model	**Adjusted model**	**Crude model**	**Adjusted model**
Maternal folate (n=2,266)				
First quintile	0.68 (-0.26 to 1.63)	0.27 (-0.68 to 1.23)	0.46(-0.33 to 1.25)	0.14 (-0.65 to 0.94)
Second quintile	0.50 (-0.44 to 1.44)	0.41 (-0.52 to 1.35)	0.46 (-0.33 to 1.24)	0.41 (-0.38 to 1.18)
Third quintile	-0.36 (-1.29 to 0.57)	-0.35 (-1.27 to 0.58)	-0.22 (-1.00 to 0.56)	-0.19 (-0.96 to 0.59)
Fourth quintile	0.46 (-0.47 to 1.39)	0.36 (-0.56 to 1.29)	0.60 (-0.18 to 1.38)	0.56 (-0.21 to 1.34)
Fifth quintile	*Reference*	*Reference*	*Reference*	*Reference*
Trend #	0.06 (-0.24 to 0.35)	0.11 (-0.18 to 0.40)	-0.03 (-0.28 to 0.21)	0.03 (-0.21 to 0.27)
Maternal homocysteine (n=2,244)				
First quintile	*Reference*	*Reference*	*Reference*	*Reference*
Second quintile	0.35 (-0.55 to 1.26)	0.23 (-0.66 to 1.12)	-0.07 (-0.83 to 0.68)	-0.16 (-0.90 to 0.59)
Third quintile	0.42 (-0.50 to 1.35)	0.22 (-0.69 to 1.13)	-0.21 (-0.99 to 0.56)	-0.33 (-1.09 to 0.43)
Fourth quintile	0.25 (-0.68 to 1.19)	0.08 (-0.83 to 0.99)	-0.19 (-0.96 to 0.58)	-0.28 (-1.04 to 0.48)
Fifth quintile	0.58 (-0.35 to 1.51)	0.08 (-0.84 to 1.00)	-0. 23 (-1.01 to 0.54)	-0.56 (-1.33 to 0.21)
Trend #	-0.00 (-0.30 to 0.29)	-0.00 (-0.29 to 0.28)	-0.19 (-0.43 to 0.05)	-0.18 (-0.42 to 0.06)
Maternal vitamin B12 (n=2,158)				
First quintile	-0.07 (-1.03 to 0.88)	-0.50 (-1.46 to 0.45)	-0.44 (-1.24 to 0.35)	-0.75 (-1.54 to 0.05)
Second quintile	-0.75 (-1.70 to 0.21)	-0.96 (-1.91 to -0.02)*	-0.70 (-1.50 to 0.09)	-0.88 (-1.66 to -0.09)*
Third quintile	-0.42 (-1.37 to 0.54)	-0.48 (-1.42 to 0.47)	-0.38 (-1.18 to 0.41)	-0.42 (-1.21 to 0.36)
Fourth quintile	-0.11 (-1.06 to 0.85)	-0.24 (-1.18 to 0.71)	-0.15 (-0.95 to 0.64)	-0.24 (-1.03 to 0.55)
Fifth quintile	*Reference*	*Reference*	*Reference*	*Reference*
Trend #	0.06 (-0.24 to 0.36)	0.18 (-0.11 to 0.48)	0.22 (-0.03 to 0.47)	0.31 (0.06 to 0.56)*

*P<0.05

Values are regression coefficients (95% CI) and reflect the difference in childhood blood pressure (mmHg) at the age of six years for each quintile of biomarker concentrations. The highest quintile in maternal folate and vitamin B12 levels and the lowest quintile in homocysteine levels were used as reference groups.

The crude model is adjusted for child sex and age at blood pressure measurement. The adjusted model is additionally adjusted for maternal age, pre-pregnancy body mass index, alcohol use and smoking during pregnancy, educational level, gestational age at birth, birth weight, current body mass index and month in which blood pressure measurement was taken.

#Tests for trends were conducted by using the maternal dietary intake variables as continuous variable in the linear mixed-models. Values are regression coefficients (95% CI) and reflect the difference in blood pressure (mmHg) per standard deviation increase in micronutrient intake.

pressure are presented in Table 6. We found no consistent associations of maternal first trimester folate and homocysteine concentrations with childhood systolic blood pressure and diastolic blood pressure in six year old children. Lower maternal first trimester vitamin B12 concentrations were associated with lower childhood diastolic blood

pressure, but not systolic blood pressure (difference 0.31 mmHg per standard deviation increase in vitamin B12 concentration (95%CI: 0.06 to 0.56), *p*-value for trend 0.02), but not with systolic blood pressure. However, the associations between iron intake and vitamin B12 concentrations with childhood blood pressure did not reach the significance threshold after adjustment for multiple testing.

Discussion

Results from this population-based prospective cohort study suggest that maternal daily macronutrient and micronutrient intake in first trimester of pregnancy is not associated with childhood blood pressure at the age of six years. We observed that higher iron intake and vitamin B12 concentrations may be associated with a lower childhood systolic and higher diastolic blood pressure respectively, but we should also consider the effect of multiple testing. After multiple testing correction none of the results remained significant.

Strengths and limitations

The main strength of our study is the prospective design from early life onwards and the large sample size of this population-based cohort. To our knowledge, this is one of the largest prospective studies that examined the associations between daily maternal intake of micronutrients and macronutrients in first trimester of pregnancy with childhood blood pressure. We used a food frequency questionnaire, previously validated in a Dutch population[23], to assess dietary intake of the mothers. Although we have used a validated questionnaire, misclassification of dietary intake can still occur, which might have led to an underestimation of the effect estimates. We measured blood pressure in six year old children using a validated automatic sphygmanometer[27] and acquired multiple blood pressure measurements to minimize measurement error. Furthermore, information about a large number of potential confounders was available. However, some limitations need to be addressed. Of all mothers included before a gestational age of 25 weeks, information on maternal dietary intake was missing for only 1.4% of all mothers. This non-response would lead to biased effect estimates if the associations of maternal dietary intake and childhood blood pressure would be different between mothers included and not included in the analyses. This seems unlikely because biased estimates in large cohort studies mainly arise from loss to follow-up rather than from a non-response at baseline[35]. Of all Dutch singleton life born children with available data on maternal dietary intake during first trimester of pregnancy, 74.3% participated in the follow-up measurements at the age of six years. Overall, mothers who did not visit the research center were younger, less well educated, smoked more frequently and used

less alcohol during pregnancy. This selective loss to follow-up might have led to biased effect estimates. We also have to acknowledge that our study population included in the analyses, is comprised of relatively healthy women with a higher proportion of folic acid suppelment use compared to other populations. This might have resulted in smaller observed differences and might limit generalizabilty to other populations. Also, maternal dietary intake was only assessed in first trimester of pregnancy, while dietary intake might change during pregnancy. Although it has been demonstrated that maternal nutritional intake did not change significantly during pregnancy[36], second or third trimester maternal diet might be associated with cardiovascular development, and influence childhood blood pressure. Unfortunately, we were not able to assess these associations in the current study. Finally, although we have performed adjustment for various potential confounders, residual confounding might still be an issue due to the observational design of the study.

Maternal macronutrient intake

Low birth weight is associated with higher blood pressure and cardiovascular disease in later life[37-38]. It has been suggested that these associations might be explained by suboptimal maternal diet[1-3]. Historical cohort studies in the Netherlands and China showed that maternal exposure to famine is associated with a higher blood pressure in adult offspring[4, 39]. The effect of normal variation of maternal daily energy intake and macronutrient intake on childhood blood pressure has been examined in several studies, but showed inconsistent results[7-8, 16]. Consistent with previous studies, we did not observe associations of maternal total energy intake during pregnancy and childhood blood pressure[8, 40]. As suggested by Roseboom et al., it might be that an imbalance of maternal macronutrients, rather than total energy intake, is associated with childhood blood pressure[4]. Using the energy partition method[33], we did not observe associations of maternal daily carbohydrate, fat or protein intake with childhood blood pressure. Two smaller studies from Finland and the Philippines previously showed conflicting results regarding the associations of maternal fat intake with childhood blood pressure[7-8]. Our results suggest there is no association of maternal fat intake with childhood blood pressure. Also, a previous study showed that a higher maternal protein intake is associated with a lower blood pressure in boys, but these findings were not confirmed in other studies[8, 40-41]. The inconsistent results might be explained by differences in study populations.

Maternal calcium, iron and sodium intake

Several studies, including follow-up studies of randomized clinical trials, have shown an association of calcium supplement use during pregnancy with lower blood pressure in children[11, 14]. However, maternal calcium intake from normal dietary intake was not

associated with infant and childhood blood pressure[9, 14]. In line with these studies, we did not find an association between maternal calcium intake in first trimester of pregnancy and offspring blood pressure. It should be noted that our population had a high dietary calcium intake. This could have affected our power to investigate the effects of low calcium intake on childhood blood pressure. Another explanation might be that mothers who used calcium supplements, also used other micronutrient supplements and the combined effect of these supplements might lead to a lower blood pressure in offspring[14].

Animal studies have shown that maternal iron restriction during pregnancy was associated with a higher blood pressure in the offspring[42-43]. In human subjects, Brion et al. observed in a prospective cohort study among 7,484 subjects that maternal iron supplement use and iron intake from food sources, assessed in third trimester of pregnancy, were not associated with offspring blood pressure[13]. Belfort et al. showed in a prospective longitudinal cohort study among 1,098 American children an association of maternal iron supplement use with a higher systolic blood pressure in children[10]. However, maternal iron dietary intake from food sources, assessed in first and second trimester of pregnancy, was not associated with offspring blood pressure at both time points[10]. We observed an association of higher maternal iron intake from food with lower blood pressure, although the effect size was small and borderline significant. We assessed dietary intake in first trimester of pregnancy. It might be that part of the differences in results between our and other studies can be explained by different timing of the exposure assessment. Animal studies have shown that offspring of iron restricted mothers have a higher blood pressure in later life, possibly mediated by cardiovascular adaptations in response to anaemia, which is in line with our findings[42, 44]. Previous studies in animals described the associations of maternal sodium intake with childhood blood pressure and suggested high sodium intake was associated with a higher blood pressure[15, 45]. In our study, maternal sodium intake was not associated with childhood blood pressure. However, sodium intake is poorly assessed by FFQ and, which could have led to an underestimation of the effect. Further experimental studies in animals and observational studies in humans are needed to assess the associations of maternal micronutrient intake with offspring blood pressure in later life, and investigate possible underlying mechanisms.

Maternal folate, homocysteine and vitamin B12 concentrations

Low folate and high homocysteine concentrations during pregnancy have been associated with lower birth weight and pregnancy complications, such as spontaneous abortion and pre-eclampsia[46-49]. Low folate and vitamin B12 levels and high homocysteine levels might affect vascular development and subsequently lead to endothelial dysfunction and higher blood pressure[18]. One previous study described the associations

of supplement intake, including iron, folic acid and vitamin B12 during pregnancy with a lower systolic blood pressure in children at the age of two years[50]. However, since the supplement contained a combination of these nutrients they could not infer which nutrient contributed to the effect.

We did not observe associations of maternal folate and homocysteine levels during first trimester of pregnancy with childhood blood pressure in their offspring. Since higher vitamin B12 levels are associated with lower homocysteine levels, and lower homocysteine levels are associated with lower blood pressure in adults, we expected that higher maternal vitamin B12 concentrations in the blood would be associated with a lower childhood blood pressure. However, our results show an opposite effect; higher maternal vitamin B12 levels were associated with a higher childhood diastolic blood pressure, although the effect size was small and borderline significant. However, after correction for multiple testing, this association was not statistically significant. To the best of our knowledge, this association has not been described before and underlying mechanisms are not known.

Conclusions

Our study shows within a population-based cohort, that normal variation in maternal intake of macronutrients and micronutrients during first trimester of pregnancy is not associated with childhood blood pressure. We found some indications that high maternal iron intake and low maternal vitamin B12 levels seemed to be associated with a lower blood pressure in children at the age of six years. However, we investigated multiple exposures, the effect sizes are small and the associations were not significant after correction for multiple testing. Further studies are needed to replicate these findings, to elucidate the underlying mechanisms and to assess whether these differences persist in later life.

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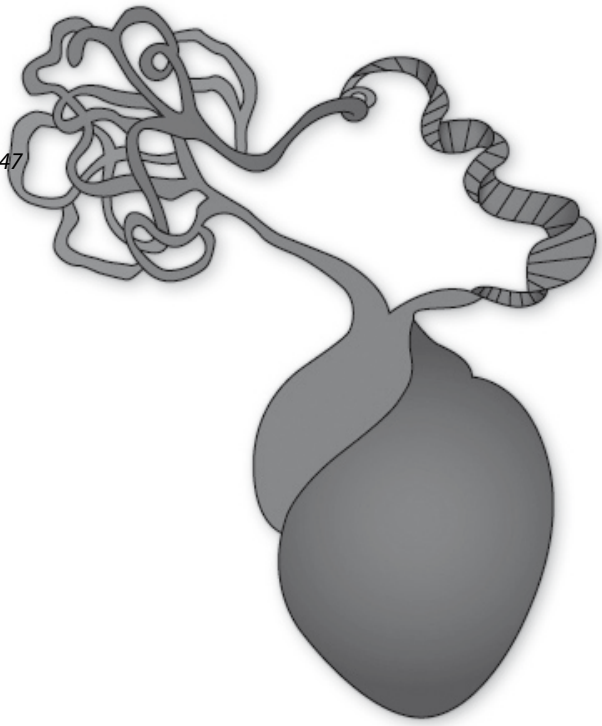
Chapter 3.5

Genome-wide profiling of blood pressure in adults and children

H Rob Taal
Germaine C Verwoert
Ayse Demirkan
A Cecile JW Janssens
Kenneth Rice
Georg Ehret
Albert V Smith
Ben FJ Verhaaren
Jacqueline CM Witteman
Albert Hofman
Meike W Vernooi
Andre G Uitterlinden
Fernando Rivadeneira
Arfan M Ikram
Daniel Levy
Albert J van der Heijden
Vincent WV Jaddoe
Cornelia M van Duijn

Adapted from Hypertension 2012;59:241-247



Abstract

Background: Hypertension is an important determinant of cardiovascular morbidity and mortality and has a substantial heritability, which is likely of polygenic origin. The aim of this study was to assess to what extent multiple common genetic variants contribute to blood pressure regulation in both adults and children, and to assess overlap in variants between different age groups, using genome wide profiling. **Methods:** SNP sets were defined based on a meta-analysis of genome-wide association studies on systolic (SBP) and diastolic blood pressure (DBP) performed by the Cohort for Heart and Aging Research in Genome Epidemiology (CHARGE, n=29,136), using different *P*-value thresholds for selecting single nucleotide polymorphisms (SNPs). Subsequently, genetic risk scores for SBP and DBP were calculated in an independent adult population (n=2,072) and a child population (n=1,034). The explained variance of the genetic risk scores was evaluated using linear regression models, including sex, age and body mass index.

Results: Genetic risk scores, including also many non-genome-wide significant SNPs explained more of the variance than scores based only on very significant SNPs in adults and children. Genetic risk scores significantly explained up to 1.2% (P=9.6*10^{-8}) of the variance in adult SBP and 0.8% (P=0.004) in children. For DBP, the variance explained was similar in adults and children (1.7% (P=8.9*10^{-10}) and 1.4% (P=3.3*10^{-5}) respectively).

Conclusion: These findings suggest the presence of many genetic loci with small effects on blood pressure regulation both in adults and children, indicating also a (partly) common polygenic regulation of blood pressure throughout different periods of life.

Introduction

Elevated blood pressure is an important risk factor for stroke and ischemic heart disease, and is estimated to contribute to half of the global risk for cardiovascular disease[1-2]. Antihypertensive treatment has been effective approach in reducing risks of cardiovascular events in people with hypertension[3].

The heritability of blood pressure levels is estimated to be 30-60%[4-5]. Several genes with large effects have been identified in familial forms of hypertension, including salt-sensitivity genes[6]. However, these explain a relatively small proportion of hypertension in the general population. Several common genetic variants associated with blood pressure have been identified through genome-wide association studies in adult populations, only explaining ~1% of the variance of blood pressure[7-8].

Despite the large size of the consortia used for gene discovery, many common variants with small effects on blood pressure remain unidentified. While their individual associations do not reach genome-wide significance, in combination these variants may nevertheless explain a substantial proportion of blood pressure. The extent to which unidentified common variants explain the missing heritability in blood pressure and other polygenic traits is an open and important question.

Recently, it has been shown that the presence of multiple variants affecting polygenic traits can be demonstrated by constructing genome wide prediction models (genetic risk scores) of common variants[9-10]. In a polygenic disease model, the more markers are used in the model, the better the disease is predicted. Such a model also implies that everybody in the population carries a substantial number of risk variants with small effects on the disease, but patients carry more of these variants than non-diseased people. This has been demonstrated in a recent study of schizophrenia, showing that one can predict disease using both genome-wide significant and non-significant SNPs, covering a large part of the genome[9].

This approach can also be used to evaluate the evidence of overlap in genes affecting a continuous outcome as blood pressure, in different age groups. In metabolic diseases, such as diabetes and blood pressure, there is increasing interest in the role of genetic determinants of blood pressure and other risk factors of cardiovascular disease in order to improve prevention of chronic disease and identify targets for therapeutic interventions.

One may argue that many genes regulating blood pressure maintenance are similar across age groups. We used genome wide profiling to evaluate to what extent multiple common genetic variants influence blood pressure in adults and secondly and to test whether there is overlap in genes contributing to blood pressure levels in children and adults, which might indicate whether there is a common polygenic regulation of blood pressure throughout different periods of life.

Methods

Genome-wide profiling

In genome-wide profiling, for a certain trait, genetic risk scores are constructed using data from a "discovery sample". Sets of common variants to calculate genetic risk scores consist of all SNPs achieving a certain significance threshold ($P_{discovery}$ threshold) in the discovery sample. In an independent "target sample", a subject's genetic risk score is computed across all SNPs with *P*-value lower than the $P_{discovery}$ threshold. The subject's genotype (coded 0/1/2) is multiplied by the regression coefficient for that SNP as estimated in the discovery sample, and divided by the total number of SNPs in that set. This risk score is calculated for all subjects in the independent target sample. Subsequently, an unbiased estimate of the variance explained by the genetic risk score is obtained by evaluating the increase in explained variance of the trait when adding the genetic risk score to a baseline model explaining that trait. The method has previously been described in detail[9]. In our study, we used systolic and diastolic blood pressure and hypertension as traits of interest. The discovery sample was the Cohort for Heart and Aging Research in Genome Epidemiology (CHARGE) consortium, with a total sample size of 29,136 participants[7]. We used two Dutch target samples; one adult sample (Rotterdam Study III) and one child sample (Generation R Study).

In the discovery sample, within each cohort of CHARGE, regression models were fitted for systolic blood pressure (SBP) and diastolic blood pressure (DBP) separately, and allele dosage, using an additive genetic model. The models were adjusted for sex, age, age squared and body mass index (BMI). Subsequently, the within-study associations were combined by prospective meta-analysis. The methods have been described in detail previously[7].

Next, SNPs were selected using the results from the meta-analyses of GWAS on SBP in the discovery sample, on the basis of their nominal *P*-value ($P_{discovery}$) for association with SBP, using different $P_{discovery}$ thresholds, ranging from 1.0×10^{-7} to 1.0. Subsequently, these sets of SNPs, with different $P_{discovery}$ thresholds, were used to calculate the genetic scores in the target samples. The same was done using the results from the meta-analysis of GWAS on DBP, creating separate genetic risk scores for DBP.

For each individual in the two independent target samples, the genetic risk scores were calculated by multiplying the number of risk alleles per SNP (0, 1 or 2) with the effect size of that SNP in the discovery meta-analysis (weighted approach), summed over all SNPs in the set and divided by the number of SNPs in the considered set. The calculations of individual scores for each set of SNPs were performed using the PLINK (v1.07) software, specifically by "profile scoring" option.

Subsequently, linear regression models were used to test the association between the individual genetic risk scores and SBP and DBP in the target samples. For subjects

using anti-hypertensive medication, we added 10 mmHg to the observed SBP values and 5 mmHg to the observed DBP values. Similarly to the discovery analysis the models were adjusted for sex, age BMI. For analyzing the explained variance for adult hypertension (SBP>=140 mmHg, DBP >=90 mmHg or use of antihypertensive medication) and childhood hypertension (SBP or DBP > p95 for age, gender and height[11]), we used logistic regression. We performed sensitivity analyses in the adults excluding subjects meeting the criteria for hypertension in the Rotterdam Study III. We have repeated the main analyses after removing SNPs in high linkage disequilibrium, using the LD-pruning option in the Plink software[12]. We pruned the data considering a window of 200 SNPs, removing one of a pair of SNPs if the LD is greater than 0.25, and shifting the window 5 SNPs forward to repeat the procedure.

For both the Rotterdam Study and the Generation R study, regression analyses were performed in SPSS 17.0 for Windows (SPSS Inc., Chicago IL, USA). The difference of the explained variance in the null (without genetic risk score) and alternative model (including a genetic risk score) was considered as the variance explained by the genetic score. A genetic risk score with a *P*-value <0.05 in the model was considered as significantly associated with the trait. We also assessed the difference between two subsequent models both including a genetic risk score using the Akaike Information Criterion (AIC)[13].

In the online supplement, we describe the discovery and target samples, their respective data collection procedures and quality control. The approaches for sensitivity analyses using unrelated traits as outcome and analysis using randomly selected SNP sets or a SNP set based on a biological pathway to calculate a genetic risk score are also presented in the online supplement (please see http://hyper.ahajournals.org).

Results

Demographic data of the CHARGE consortium have been published previously[7]. Table 1 shows the baseline characteristics for the Rotterdam Study III (RS-III) and the Generation R Study. The median age in RS-III was 56.0 years (95% range: 47.7 – 62.3), in the Generation R Study the median age was 6.0 years (95% range: 5.8 – 6.7).

Figures 1a and 1b show the increase in explained variance of SBP by the genetic risk scores for a range of different $P_{discovery}$ thresholds, in the adult and child target samples, respectively. When considering risk scores based on sets of SNPs with low *P*-values in the discovery sample (up to $P_{discovery}$ <1.0x10^{-4}), the risk scores significantly explained up to 0.3% of variance in SBP in adults (*P*=0.01). These scores were non-significant in children, increasing the explained variance by 0.1% (*P*=0.305). When adding more SNPs using greater $P_{discovery}$ thresholds, the variance explained by the genetic risk scores increased, in both adults and children. Adding genetic risk scores based on SNPs with a $P_{discovery}$ of

Table 1. Subject characteristics of the Generation R Study and the Rotterdam Study III

Characteristics	Rotterdam Study III (n = 2,078)	Generation R Study (n = 1,034)
Age (yrs)	56.0 (47.7 – 62.3)	6.0 (5.8 – 6.7)
Female (%)	56.1	52.2
BMI (weight(kg)/length(cm)^2)	26.9 (21.6 – 36.7)	15.9 (13.9 – 18.4)
Mean systolic blood pressure (mmHg)	132.7 (19.2)	102.5 (8.1)
Mean diastolic blood pressure –(mmHg)	82.6 (11.1)	60.5 (7.2)
Subjects with hypertension (%) *	47.3	
Subjects using anti-hypertensive medication (%)	20.8	
Children referred to nephrology for hypertension (%) †		0.5
Prevalent Type 2 Diabetes (%)	7.3	
Serum total cholesterol (mmol/l)	5.6 (1.1)	
HDL cholesterol (mmol/l)	1.5 (0.5)	

Values are means (sd) or medians (95%-range)

* Adults: Defined as SBP>140mmHg, DBP>90 mmHg or use of anti-hypertensive medication

Children: SBP or DBP >95th percentile for gender, age and height [12]

† Children were referred to pediatric nephrology department when the lowest blood pressure measurement was higher than the P99 for their gender, length and age [12].

<0.1, increased the explained variance of the model significantly with 1.2% in adults (P=9.6*10^{-8}). In 6 year old children, the risk score based on SNPs with $P_{discovery}$ of <0.3 significantly increased the explained variance of the model by 0.8% (P=0.004). The increase in explained variance in children was less pronounced compared to adults, but showed a similar pattern as the adult population.

Figures 2a and 2b show the proportion of explained variance of DBP for the various genetic risk scores. The difference between adults and children considering genetic risk scores based on sets of SNPs with a low P-value in the discovery sample (up to $P_{discovery}$ <1.0x10^{-4}), was more marked. The SNP sets explained up to 0.3% (P = 0.006) of the variance of DBP in the adult population but no variance in DBP was explained in children (P = 0.544). The genetic risk score based on SNPs with a $P_{discovery}$ <0.3 increased the explained variance significantly by 1.7% (P=8.9*10^{-10}) when the score was added to the baseline model. Again, in children, a similar pattern was observed. The same genetic risk score ($P_{discovery}$ threshold <0.3) significantly increased the explained variance of DBP by 1.4% (P=5.2*10^{-5}), almost a similar increase as in adults.

Genetic risk scores for systolic and diastolic blood pressure also were associated with hypertension in adults explaining up to 2.1% (P=2.0*10^{-9}, using $P_{discovery}$ <0.3) of the variance in hypertension in an adult population (Figure 3a and 3b). Adult based genetic risk scores for systolic and diastolic blood pressure did not significantly explain additional

Figure 1. Increase in explained variance in systolic blood pressure by genetic risk scores

Figure 1a

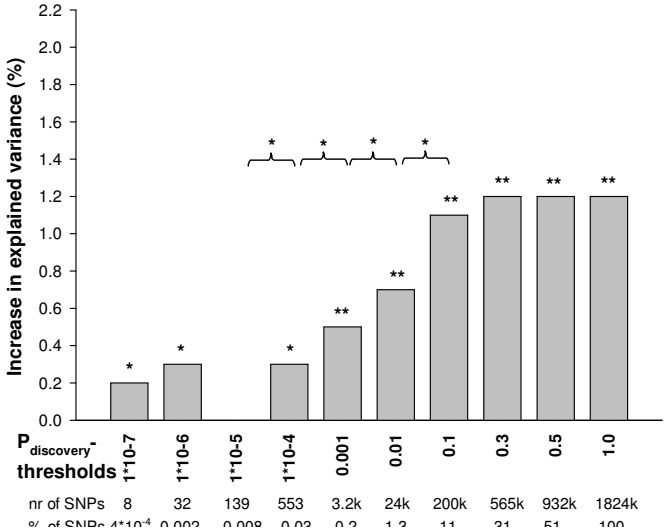
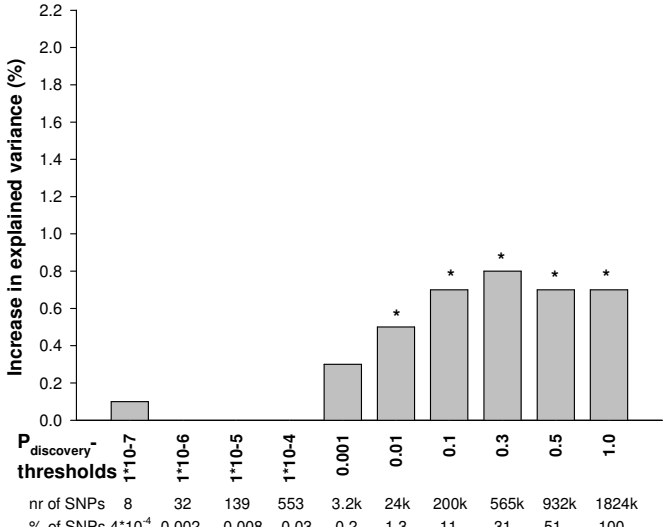


Figure 1b



Bars represent the increase in explained variance (%) of systolic blood pressure, when adding the genetic risk scores for different p-value thresholds, to the base line model for systolic blood pressure including BMI, sex and age as covariates. The baseline model explained 12.8% and 5.2 % of the variance in systolic blood pressure for adults and children respectively. We evaluated the difference in explained variance between two subsequent models by calculating the Akaike Information Criterion (AIC) of each model. The difference in AIC follows a χ^2 distribution with one degree of freedom, from which the P-value was derived.

* $p < 0.05$

** $p < 0.001$

1a. Rotterdam Study III

1b. Generation R Study

Figure 2. Increase in explained variance in diastolic blood pressure by genetic risk scores

Figure 2a

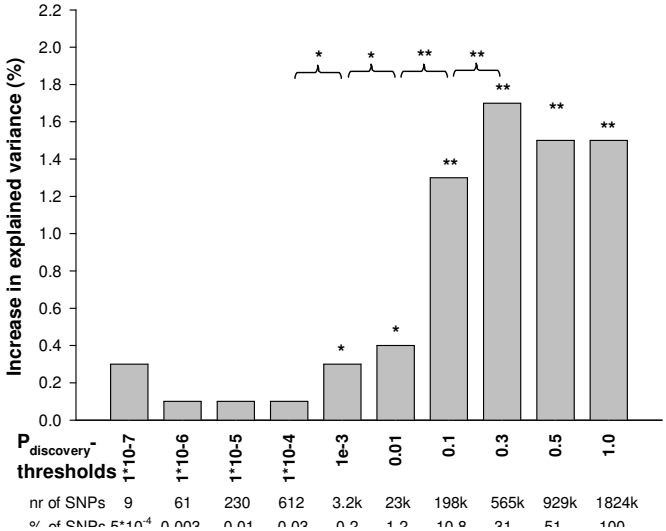
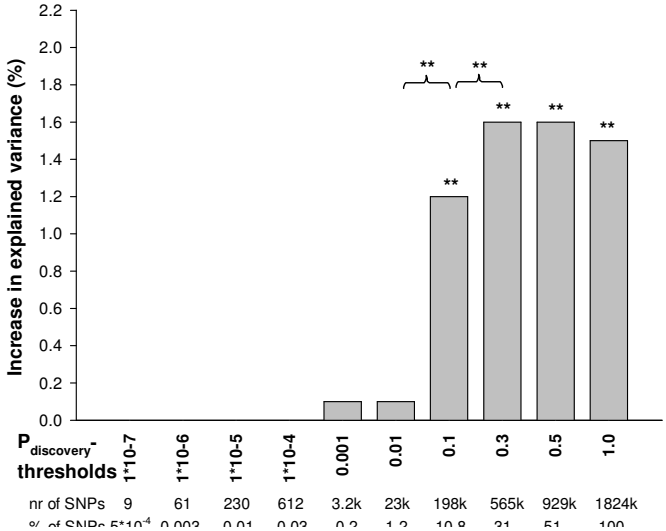


Figure 2b



Bars represent the increase in explained variance (%) of diastolic blood pressure, when adding the genetic risk scores for different p-value thresholds, to the base line model for diastolic blood pressure including BMI, sex and age as covariates. The baseline model explained 8.4% and 1.4 % of the variance in diastolic blood pressure for adults and children respectively. We evaluated the difference in explained variance between two subsequent models by calculating the Akaike Information Criterion (AIC) of each model. The difference in AIC follows a χ^2 distribution with one degree of freedom, from which the P-value was derived.

* $p < 0.05$

** $p < 0.001$

2a. Rotterdam Study III

2b. Generation R Study

Figure 3. Increase in explained variance in hypertension by genetic risk scores

Figure 3a

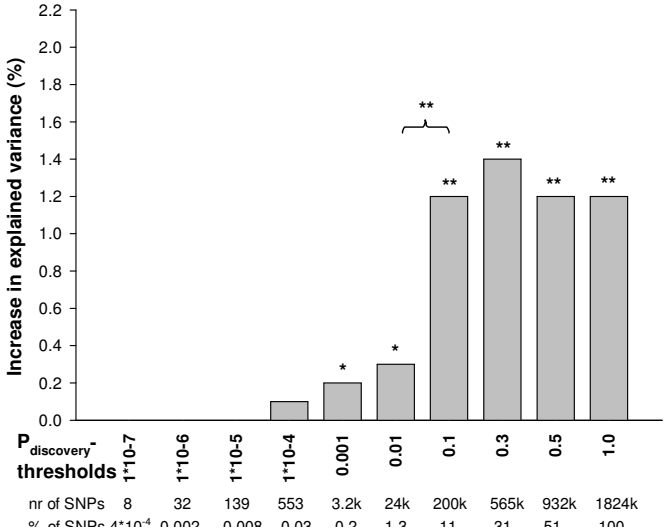
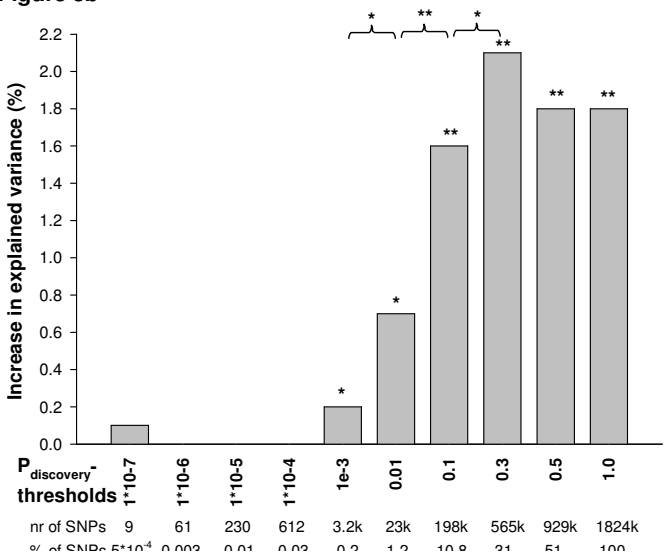


Figure 3b



Bars represent the increase in explained variance (%) of hypertension in adults (SBP>140, DBP>90 or the use of anti hypertensive medication), when adding systolic and diastolic genetic risk scores for different p-value thresholds, to the base line model for systolic blood pressure including BMI, sex and age as covariates. The baseline model explained 15.9% of the variance in hypertension in adults. We evaluated the difference in explained variance between two subsequent models by calculating the Akaike Information Criterion (AIC) of each model. The difference in AIC follows a χ^2 distribution with one degree of freedom, from which the P-value was derived.

* $p < 0.05$

** $p < 0.001$

3a. Systolic blood pressure scores

3b. Diastolic blood pressure scores

variance in childhood hypertension (see Supplement figure S1a and S1b, please see http://hyper.ahajournals.org).

To illustrate that these results were not due to chance, we tested whether the blood pressure based risk scores predicted also variation in intracranial volume (in adults) and head circumference (in children). Figures S2a/b and S3a/b show that the genetic risk scores for SBP and DBP did not explain variance of these unrelated traits significantly (please see http://hyper.ahajournals.org). Furthermore, we created random genetic risk scores of sufficient size (~565k SNPs, similar to the SNP set with $P_{discovery} < 0.3$) and evaluated the additional explained variance. The random genetic risk scores showed a significant increase in explained variance when added to the baseline model (see Table 2). The additional explained variance from the random genetic risk scores was 0.1-0.4% lower as compared to the original genetic risk scores (see Table 2). We also tested whether a set of SNPs from a biological pathway would lead to a higher increase in explained variance than SNP sets with a low P-discovery threshold. We used SNPs in the FGF (fibroblast growth factor) pathway as described by Tomaszewski et al[14]. This genetic risk score did not explain additional variance in all phenotypes except for adult hypertension based on the systolic blood pressure score (increase in explained variance 0.3, P=0.014, see Table 3).

In a strictly normotensive population the additional explained variance of the genetic risk score including the most significant SNPs ($P_{discovery}$-threshold $1.0x10^{-7}$) increased,

Table 2. Additional explained variance by a random genetic risk score versus original genetic risk score

	Additional explained variance		
Phenotype	**Random genetic risk score (%)**	**Original genetic risk score (%)**	**P-value for difference**
Adult systolic blood pressure (Rotterdam Study III)	1.0 †	1.2 †	0.07
Adult diastolic blood pressure (Rotterdam Study III)	1.3 †	1.7 †	0.01
Child systolic blood pressure (Generation R)	0.7 *	0.8 *	0.49
Child diastolic blood pressure (Generation R)	1.4 †	1.6 †	0.10
Hypertension – SBP scores (Rotterdam Study III)	1.1 †	1.4 †	0.02
Hypertension – DBP scores (Rotterdam Study III)	1.7 †	2.1 †	0.01

* P value < 0.05

† P value < 0.001

Random Genetic risk score – Genetic risk score calculated on SNP set containing ~565k SNPs which were randomly selected out of the discovery meta-analysis, irrespective of their P-value for association.

Original genetic risk score – Genetic risk score calculated on a SNP set with a $P_{discovery}$ threshold of 0.3. This SNP set contains ~565k SNPs and showed the largest increase in explained variance.

The P-value for difference between the models was obtained by calculating the difference in Akaike Information Criterion between the random model and the original model. This difference follows a χ^2 distribution with 1 degree of freedom

Table 3. Additional explained variance by a genetic risk score based on FGF-signaling pathway[14]

Phenotype	Additional explained variance by signaling pathway genetic risk score (%)
Adult systolic blood pressure (Rotterdam Study III)	0.1
Adult diastolic blood pressure (Rotterdam Study III)	0.0
Child systolic blood pressure (Generation R)	0.1
Child diastolic blood pressure (Generation R)	0.1
Hypertension – SBP scores (Rotterdam Study III)	0.3 *
Hypertension – DBP scores (Rotterdam Study III)	0.1

* P value < 0.05

while the risk scores with more liberal $P_{discovery}$-thresholds had a lower additive explained variance compared to the original analysis. Results of sensitivity analyses are shown in the supplement (figure S4a and S4b). We repeated the main analyses after removing SNPs in high linkage disequilibrium. The pattern was similar to the original analysis, but the explained variance was lower. Including more SNPs in the genetic risk score increased the variance explained by that score. The results of these analyses are shown in the supplement (Figure S5a/b, S6a/b and S7a/b, please see http://hyper.ahajournals.org).

Discussion

Our findings indicate that, in addition to the blood pressure variants now identified, large numbers of common genetic variants affecting blood pressure remain to be identified, and that these variants explain a significant part of the variance in blood pressure in adults and children. These non-significant, unidentified SNPs together explain a larger part of the variance than the genome wide significant SNPs only. We also showed that adult based genetic risk scores explained variance in blood pressure in children. This indicates not only that there is a polygenic effect on blood pressure in children, but more importantly, it indicates that there is overlap in variants involved with blood pressure maintenance in adults and children and that these variants act in throughout life.

In this study, we did not remove any SNPs that are in high linkage disequilibrium with each other. Pruned analyses, as presented in the Supplemental Material, do not change our conclusion that adding more non-genome wide significant SNPs to genetic risk scores increases the variance explained by these scores. However, as expected, removing SNPs by LD pruning results in a reduction of the variance explained. Since

SNPs in LD are removed randomly, in many cases informative SNPs are taken out of the analyses. Therefore a pruned analysis is expected to underestimate the true effect on the explained variance by the genetic risk scores.

The additive explained variance of genetic risk scores on blood pressure is maximizing around the $P_{discovery}$-threshold of 0.3 and does not increase with more liberal thresholds. This genetic risk score includes over 550k SNPs. This result suggests that SNPs with a $P_{discovery}$-value lower than 0.3 in the discovery sample add to the explained variance of blood pressure and that many common variants associated with blood pressure regulation have not been identified yet. SNPs with a $P_{discovery}$ higher than 0.3 are unlikely to be associated with blood pressure. Genetic risk scores of a similar size, consisting of randomly selected SNPs, still resulted in a significant increase in explained variance when added to the baseline model without a genetic risk score. The increase in explained variance based on the random genetic risk scores was lower than the increase based on the original genetic risk score models, although this difference was small and statistically significant in only half of the presented phenotypes. This result suggests that a sufficiently large number of SNPs tags many genes throughout the genome which influence blood pressure regulation. These findings are in line with our hypothesis that blood pressure is polygenic trait and that there are many more genes involved with blood pressure than are found so far in genome wide association studies. Currently, the advantage of using genetic risk scores based on SNPs selected on their P-value in a GWAS discovery sample, as compared to genetic risk scores based on a random set of SNPs, seems to be limited. Larger GWAS discovery samples with identification of new common and rare SNPs might lead to higher explained variance.

Although there have been several mutations described causing dominant, monogenic, forms of hypertension and more of such rare variants may still be undiscovered[6], our results support the hypothesis that hypertension is a polygenic disease, which is in part explained by a large number of genes regulating blood pressure. In our adult population, genetic risk scores including large numbers of SNPs, explain the largest proportion of variance in blood pressure, indicating the involvement of multiple genes in blood pressure regulation.

Risk scores containing highly significant SNPs, identified in large scale genome wide association meta-analyses in adults[7-8], were significantly associated with in blood pressure in adults, but not in children. There are several explanations possible for this finding, including a smaller number of subjects and lower power in the children cohort. However, this finding might also indicate that the genome wide significant SNPs found so far are related to late-onset pathology. It has been long hypothesized that there is a common polygenic regulation of blood pressure in adults and children. This is the first study showing evidence of such a mechanism.

It has been shown in literature that blood pressure tracks from childhood to adulthood[15]. This study indicates that genes are explaining a part of the blood pressure tracking over life. We show that the same set of genes, based on an adult discovery sample, explain part of the variation in blood pressure in both adults and children. We also showed that these SNP sets explain variation in hypertension in adults, indicating also a polygenic origin of hypertension. It has been shown that high blood pressure in childhood predisposes to hypertension in adulthood[16]. Adult based genetic risk scores do not explain variance in childhood hypertension in children significantly. This fits with the common view that causes of juvenile hypertension are different from adult hypertension[11].

The percentage of explained variance by genetic risk scores is still low, although the heritability has been shown to be substantial[4], yet compared to the variance explained by the genome wide significant SNPs on blood pressure found so far, there is a 4-5 fold increase in explained variance of blood pressure in our target samples.

In the coming years the variance explained by polygenic models may be improved further, using technology, such as whole genome sequencing, which can be used to identify low frequency variants. We used common variants only (MAF >0.01) to create genetic risk scores. Low frequency variants may add to the variance explained by these genetic risk scores. Also, we assumed an additive model, similar to the discovery analysis. We have to recognize that the biology of the genes involved in blood pressure regulation and possible interactions between these genes are unknown. Another possibility would be to construct genetic risk scores based on SNPs included in candidate biological pathways. A genetic risk score including SNPs from the FGF signaling pathway[14] seemed to explain a larger proportion explained variance in hypertension as compared to a genetic risk score including a similar number of SNPs, based on the previous top SNPs from the GWAS. This indicates that prior knowledge on biological models and underlying mechanisms might improve the explained variance by genetic risk scores. Alternative methods of constructing genetic risk scores may be better when further research reveals more of the underlying genetic biology of blood pressure regulation.

Specific common variants that are associated with blood pressure still need to be identified. Much research is still needed to identify more and specific genes associated with blood pressure regulation in adults. If these common variants overlap with blood pressure regulation in children, they could provide clues for early etiology of hypertension.

Conclusions

At this stage, individual prediction is not yet feasible. Without a doubt, the prediction of blood pressure will improve and might contribute to predicting high blood pressure in the future. Genetic profiling might be a way of identifying subgroups at genetically high risk for increased blood pressure at a population level[17], but whether it will be enough for personalised medicine and early treatment of people at risk for high blood pressure (and possibly also other risk factors for cardiovascular disease), remains to be determined. Our study shows that this may require the identification of many more common variants with small effects on blood pressure.

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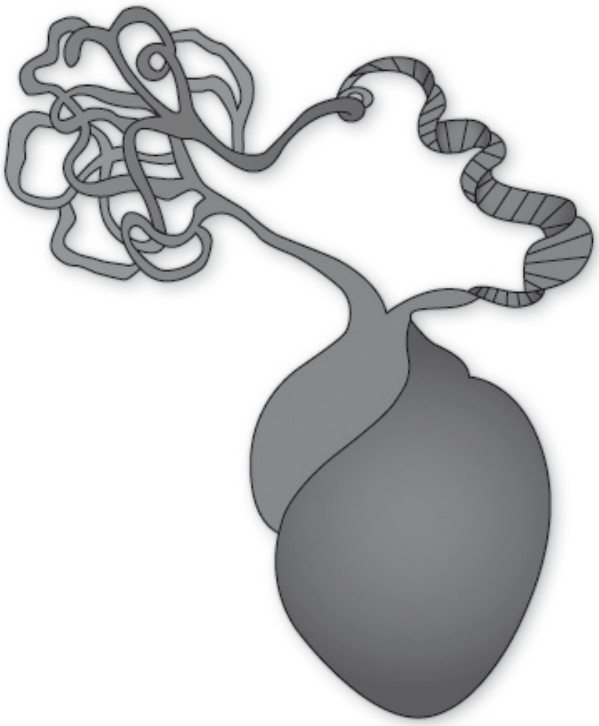
Chapter 4

Childhood kidney structure and function



Chapter 4.1

Normal kidney growth in fetal life and early childhood

JJ Miranda Geelhoed
H Rob Taal
Lidia Arends
Eric AP Steegers
Maarten Lequin
Albert Hofman
Albert J van der Heijden
Vincent WV Jaddoe

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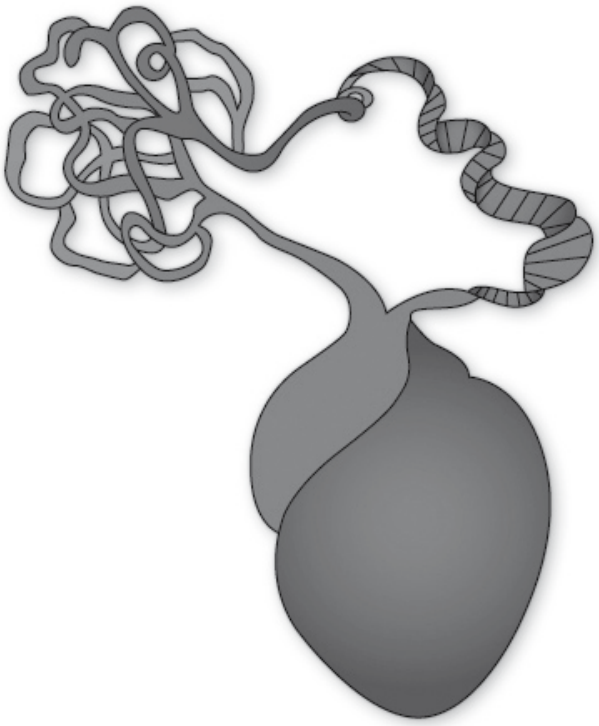


Abstract

Background: Assessing kidney growth and development in fetal life and early childhood is important. However, available information about growth of kidney structures in early life in healthy children is limited. We constructed reference curves for kidney growth from third trimester of pregnancy until early childhood using data of 1,158 healthy children.

Methods: This study was embedded in a population-based prospective cohort study from fetal life onwards. Kidney size, defined as length, width, depth and volume, was measured in third trimester of pregnancy (gestational age 30 weeks (total range: 27.1-35.1)) and at the postnatal ages of 6 (total range: 5.1-11.0) and 24 (total range: 21.6-31.6) months. Gender specific reference growth curves were constructed. Analyses were based on more than 5,000 kidney measurements.

Results: In third trimester of pregnancy and at the age of 6 months, all kidney measurements were larger in boys than in girls. At the age of 24 months, only kidney length was larger in boys. Trends were seen towards smaller left kidneys compared to right kidneys at all ages in both genders, except for kidney length. Gender specific reference curves based on (postconceptional) age were constructed for left and right kidney length, width, depth and volume.

Conclusion: Kidney size was significantly influenced by age and gender. Left kidney measurements tend to be smaller than right kidney measurements, except for kidney length. These reference curves can be used for assessing kidney structures by ultrasound in fetal life and early childhood.

Introduction

Assessing kidney size in children is important for clinical and epidemiological studies. Abnormal kidney development may have direct perinatal and neonatal treatment consequences[1]. Furthermore, smaller kidney size has been suggested to be related to hypertension and renal disease in adulthood. Kidney size can be measured non-invasively and efficiently with ultrasound. Few studies published reference ranges for kidney size in healthy children during fetal and neonatal life[2-3]. One study showed reference material on postnatal kidney growth from birth to 18 months of age[4]. These studies were based on data about postnatal kidney growth and mostly focused on kidney volume in relation to weight, height and body surface area. Recently, new reference centiles were generated to assess kidney size of children with 'single kidneys' to identify those patients with unfavourable course and relevant kidney impairment[5]. However, there are no studies available evaluating normal kidney growth from late fetal life until early childhood and thereby including both fetal and childhood kidney growth. This information may be of importance to identify abnormal kidney size and growth, with possible subsequent clinical consequences[6-7]. Therefore, the aim of this study was to construct reference curves for kidney structures including, kidney length, width, depth and volume in children from third trimester of pregnancy until the age of 24 months in a population-based cohort.

Methods

Study design

This study was embedded in the Generation R Study, a population-based, prospective cohort study from fetal life until young adulthood in Rotterdam, the Netherlands[8-9]. Detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of 1,232 mothers and their children[8-9]. In this subgroup, fetal kidney ultrasounds were performed in third trimester of pregnancy (gestational age of 30 weeks) and postnatal kidney ultrasounds were performed at the ages of 6 and 24 months. The study has been approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam. Written informed consent was obtained from all participants.

Population for analysis

In total, 1,232 women were enrolled in the Focus cohort. The present analysis was limited to singleton live births (n = 1,215) whose mothers participated in the third trimester measurements. Twin pregnancies (n = 15) and pregnancies leading to perinatal death (n = 2) were excluded from the analysis. Kidney ultrasounds were successfully performed in 95% (n = 1,158) of these subjects. Of the initial 1,215 singleton live births, 74% (n =

901) and 70% (n = 856) participated in the postnatal assessments at the ages of 6 and 24 months, respectively. Kidney ultrasounds were successfully performed in 83% (n = 747) and 80% (n = 683) of these infants, respectively. Missing values were mainly due to crying behaviour or unavailability of equipment or radiographer. Infants who had a postnatal kidney ultrasound at the ages of 6 and 24 months did not differ from the postnatal non-responders in fetal and birth characteristics. No kidney or ureterovesical anomalies other then mild pyelectasis over 10 mm (n = 3) were present in our study population. Analyses were based on more than 5,000 left and right kidney measurements.

Ultrasound measurements

Fetal left and right kidney size was measured in third trimester of pregnancy (gestational age 30 weeks (total range: 27.1-35.1). In a sagittal plane, the maximum longitudinal kidney length was measured placing the calipers on the outer edges of the caudal and cranial side. Antero-posterior (kidney width) and transverse kidney diameter (kidney depth) were measured perpendicular to each other, outer to outer, in an axial plane[10]. The cross-sectional area in which the kidney appeared symmetrically round and at its maximum width was used. The images were sufficiently magnified to ensure optimal measurements[10].

Postnatally, two-dimensional ultrasounds of the kidneys were performed using an ATL-Philips Model HDI 5000 (Seattle, Washington, USA) equipped with a 2.0 - 5.0 MHz curved array transducer in children at the ages of 6 and 24 months. The examination was carried out in a quiet room with the child quietly awake in a supine position. This position was standardised to prevent differences according to position[10-11]. The kidney was identified in the sagittal plane along its longitudinal axis. Measures of maximal bipolar kidney length, width and depth were obtained from both the left and right kidney. Kidney width and depth were measured at the level of the kidney hilum[10-11].

Mean length, width and depth were calculated as the average of three measurements and used for data analysis. Fetal and postnatal kidney volume were both calculated in cubic centimeters using the equation of an ellipsoid: volume (cm^3) = 0.523 x mean length (cm) x mean width (cm) x mean depth (cm)[11-12].

Intra and interobserver studies showed that for the fetal ultrasound measurements the intraclass correlation coefficient (ICC) was higher than 0.98 and the corresponding coefficient of variation (CV) lower than 6%. Bland and Altman plots to test agreement of measurements for fetal ultrasound, demonstrated 95% limits of agreement in proportions to be within 10% difference from the mean of measurements, indicating good reproducibility[13]. For the postnatal ultrasound measurements, the intraobserver ICCs ranged from 0.93 (left and right renal width and right renal thickness) to 0.99 (left renal length) and interobserver ICCs ranged from 0.64 (right renal thickness) to 0.90 (right re-

nal length). Limits of agreement in the Bland and Altman plots ranged from –8.0 to 9.2% (intraobserver left renal width) to the widest limit from –18.0 to 19.2% (interobserver left renal length)[14].

Data analysis

Differences of fetal and postnatal characteristics between boys and girls were assessed by t-tests and one-way ANOVA for independent samples. Furthermore, we tested differences of kidney measurements between both genders with t-tests for independent samples. Differences between left and right kidney were tested with paired sample t-tests.

Data were analyzed as recommended by Altman, Chitty and Royston[15-16]. For reference kidney growth curves, conceptional age was plotted against kidney length, width, depth and volume. From the original data, measurements more than two standard deviations (SDs) from the regression line, fitted on our data, were considered to be outliers and were therefore removed. The best fitting curve was determined using second-degree fractional polynominals[17]. The curve was fitted using repeated measurement analyses, taking into account the dependency in the data by specifying a constant covariance between measurements of the same subject.

Royston and Altman have shown how to apply a particular type of statistical model to longitudinal data to produce growth centiles and the same model may also be used to calculate valid size centiles[16, 18]. This multilevel modeling and was used in the Generation R study. The best fitting fractional polynominal curves were chosen by comparing the deviances and by visually checking the goodness of fit. The curves were fitted using repeated measurement analysis. Next, regression lines were fitted for the dependency of the residual SD on conceptional age[19]. Subsequently, plotting the SD scores against conceptional age was used to assess correctness of the model.

Finally centiles were derived and the curves were plotted on the data. Kidney growth reference curves were constructed for a conceptional age from 30 to 160 weeks. All statistical analyses were performed using the Statistical Package for the Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL, USA) and the Statistical Analysis System (SAS) for Windows, version 9.1.3.

Results

The percentage of boys was 52% (Table 1). The overall median age of infants at their 6 months visit was 6.3 months (total range: 5.1-11.0) and at their 24 months visit 25.1 months (total range: 21.6-31.6). Growth characteristics, including third trimester head circumference and postnatal weight and length at the ages of 6 and 24 months of age,

Table 1. Subject characteristics

	Boys	Girls	P-value
Third trimester fetal characteristics	**(n = 603)**	**(n = 555)**	
Gestational age (weeks)	30.4 (28.7-32.8)	30.3 (28.2-32.5)	0.05
Head circumference (cm)	28.8 (1.2)	28.3 (1.1)	<0.001
Abdominal circumference (cm)	26.7 (1.7)	26.5 (1.6)	0.2
Femur length (cm)	5.7 (0.3)	5.8 (0.3)	0.2
Estimated fetal weight (grams)	1632 (264)	1623 (252)	0.7
Characteristics at birth	**(n = 603)**	**(n = 555)**	
Gestational age at birth (weeks)	40.3 (36.2-42.4)	40.3 (36.1-42.4)	0.9
Gestational age < 37 weeks (%)	12 (3.4)	11 (3.3)	0.9
Birth weight (grams)	3557 (518)	3488 (506)	0.05
Birth weight < 2500 grams (%)	8	10	0.6
Small for gestational age (%)	6	9	0.4
Characteristics at 6 months	**(n = 379)**	**(n = 368)**	
Age at visit (months)	6.3 (5.4-8.0)	6.3 (5.4-8.2)	0.7
Weight at visit (grams)	8173 (837)	7640 (807)	<0.001
Length at visit (cm)	69.4 (2.4)	67.9 (2.4)	<0.001
Characteristics at 24 months	**(n = 333)**	**(n = 350)**	
Age at visit (months)	25.1 (23.6-28.0)	25.2 (23.4-28.3)	0.3
Weight at visit (grams)	12,890 (1,395)	12,429 (1,343)	<0.001
Length at visit (cm)	89.6 (3.2)	88.4 (3.3)	<0.001

Values are means (standard deviation), medians (95% range) or percentages.
Differences between boys and girls were compared using independent samples t-tests or ANOVA

were larger in boys than in girls (all p-values <0.001). No difference was found for gestational age at birth between boys and girls. In total, 15 children in our study group fulfilled this criterium for small for gestational age (< -2 SDS). Furthermore, only 18 children had a low birth weight (birth weight <2500 grams) and 23 children were born preterm (gestational age <37 weeks). Table 2 shows that in third trimester of pregnancy and at the age of 6 months all kidney measurements were larger in boys than in girls. At the age of 24 months, only kidney length was larger in boys than in girls. Furthermore, at all ages both genders showed trends towards smaller left kidney measurements compared to right kidney measurements, except for kidney length (Table 3).

Available data for constructing the curves for kidney growth are shown in Table 4. Formulas for growth reference curves describing the mean with the corresponding standard deviation are given in Table 5. Standard deviation increased linearly with gestational age. Reference kidney growth curves of individual measurements and fitted centiles are given in Figure 1. Reference values for kidney length, width, depth and volume are given in the Appendix.

Table 2. Differences in boys and girls stratified for left and right kidney structures

	Left kidney			Right kidney		
Kidney measurement	**Boys**	**Girls**	**P-value**	**Boys**	**Girls**	**P-value**
Gestational age 30 weeks	**(n = 604)**	**(n = 553)**		**(n = 603)**	**(n = 556)**	
Length (mm)	39.5 (32.0-47.0)	38.4 (32.7-45.0)	<0.001	39.6 (32.0-46.6)	38.5 (32.2-45.3)	<0.001
Width (mm)	21.6 (17.0-28.0)	21.1 (16.5-26.0)	0.001	22.4 (17.0-28.0)	22.0 (17.0-27.6)	0.02
Depth (mm)	22.7 (17.0-29.0)	22.1 (17.9-27.8)	0.001	23.2 (17.8-30.0)	22.9 (18.0-29.0)	0.03
Volume (cm^3)	10.3 (5.4-17.9)	9.5 (5.4-15.0)	<0.001	11.0 (5.8-18.5)	10.3 (5.8-16.8)	<0.001
Age 6 months	**(n = 375)**	**(n = 358)**		**(n = 379)**	**(n = 368)**	
Length (mm)	60.1 (48.6-70.4)	58.6 (50.0-67.4)	<0.001	58.8 (49.7-69.3)	57.5 (49.5-67.6)	<0.001
Width (mm)	28.2 (22.3-36.4)	27.6 (21.6-35.7)	0.01	28.0 (21.8-35.4)	27.5 (21.4-36.5)	0.04
Depth (mm)	26.5 (20.1-33.0)	25.5 (20.2-32.2)	<0.001	27.8 (21.3-34.9)	27.0 (20.9-34.0)	0.001
Volume (cm^3)	23.8 (14.0-36.6)	21.9 (13.9-33.4)	<0.001	24.0 (15.3-35.6)	22.4 (14.2-34.6)	<0.001
Age 24 months	**(n = 330)**	**(n = 318)**		**(n = 347)**	**(n = 336)**	
Length (mm)	66.8 (56.1-80.3)	65.7 (53.7-78.0)	0.03	65.2 (54.8-76.6)	64.3 (53.2-76.5)	0.04
Width (mm)	30.8 (25.6-37.1)	30.1 (23.5-37.8)	0.01	30.7 (25.6-38.0)	30.6 (25.2-37.1)	0.61
Depth (mm)	30.9 (24.8-38.9)	30.1 (24.2-38.1)	0.002	32.0 (25.5-39.4)	31.9 (25.9-39.6)	0.79
Volume (cm^3)	33.6 (22.8-51.6)	31.8 (19.3-52.2)	0.004	33.8 (22.9-53.3)	33.1 (22.1-49.2)	0.19

Values are means (95% range).

Differences between boys and girls were compared using independent samples t-tests.

Table 3. Differences in left and right kidney structures stratified for gender

	Boys			Girls		
Kidney measurement	**Left kidney**	**Right kidney**	**P-value**	**Left kidney**	**Right kidney**	**P-value**
Gestational age 30 weeks	**(n = 604)**	**(n = 603)**		**(n = 553)**	**(n = 556)**	
Length (mm)	39.5 (32.0-47.0)	39.6 (32.0-46.6)	0.79	38.4 (32.7-45.0)	38.5 (32.2-45.3)	0.45
Width (mm)	21.6 (17.0-28.0)	22.4 (17.0-28.0)	<0.001	21.1 (16.5-26.0)	22.0 (17.0-27.6)	<0.001
Depth (mm)	22.7 (17.0-29.0)	23.2 (17.8-30.0)	<0.001	22.1 (17.9-27.8)	22.9 (18.0-29.0)	<0.001
Volume (cm^3)	10.3 (5.4-17.9)	11.0 (5.8-18.5)	<0.001	9.5 (5.4-15.0)	10.3 (5.8-16.8)	<0.001
Age 6 months	**(n = 375)**	**(n = 379)**		**(n = 358)**	**(n = 368)**	
Length (mm)	60.1 (48.6-70.4)	58.8 (49.7-69.3)	<0.001	58.6 (50.0-67.4)	57.5 (49.5-67.6)	<0.001
Width (mm)	28.2 (22.3-36.4)	28.0 (21.8-35.4)	0.50	27.6 (21.6-35.7)	27.5 (21.4-36.5)	0.72
Depth (mm)	26.5 (20.1-33.0)	27.8 (21.3-34.9)	<0.001	25.5 (20.2-32.2)	27.0 (20.9-34.0)	<0.001
Volume (cm^3)	23.8 (14.0-36.6)	24.0 (15.3-35.6)	0.35	21.9 (13.9-33.4)	22.4 (14.2-34.6)	0.06
Age 24 months	**(n = 330)**	**(n = 347)**		**(n = 318)**	**(n = 336)**	
Length (mm)	66.8 (56.1-80.3)	65.2 (54.8-76.6)	<0.001	65.7 (53.7-78.0)	64.3 (53.2-76.5)	<0.001
Width (mm)	30.8 (25.6-37.1)	30.7 (25.6-38.0)	0.68	30.1 (23.5-37.8)	30.6 (25.2-37.1)	0.02
Depth (mm)	30.9 (24.8-38.9)	32.0 (25.5-39.4)	<0.001	30.1 (24.2-38.1)	31.9 (25.9-39.6)	<0.001
Volume (cm^3)	33.6 (22.8-51.6)	33.8 (22.9-53.3)	0.30	31.8 (19.3-52.2)	33.1 (22.1-49.2)	<0.001

Values are means (95% range).

Differences between left and right kidney structures were compared using paired samples t-tests.

Table 4. Number of successful measurements per variable, according to age

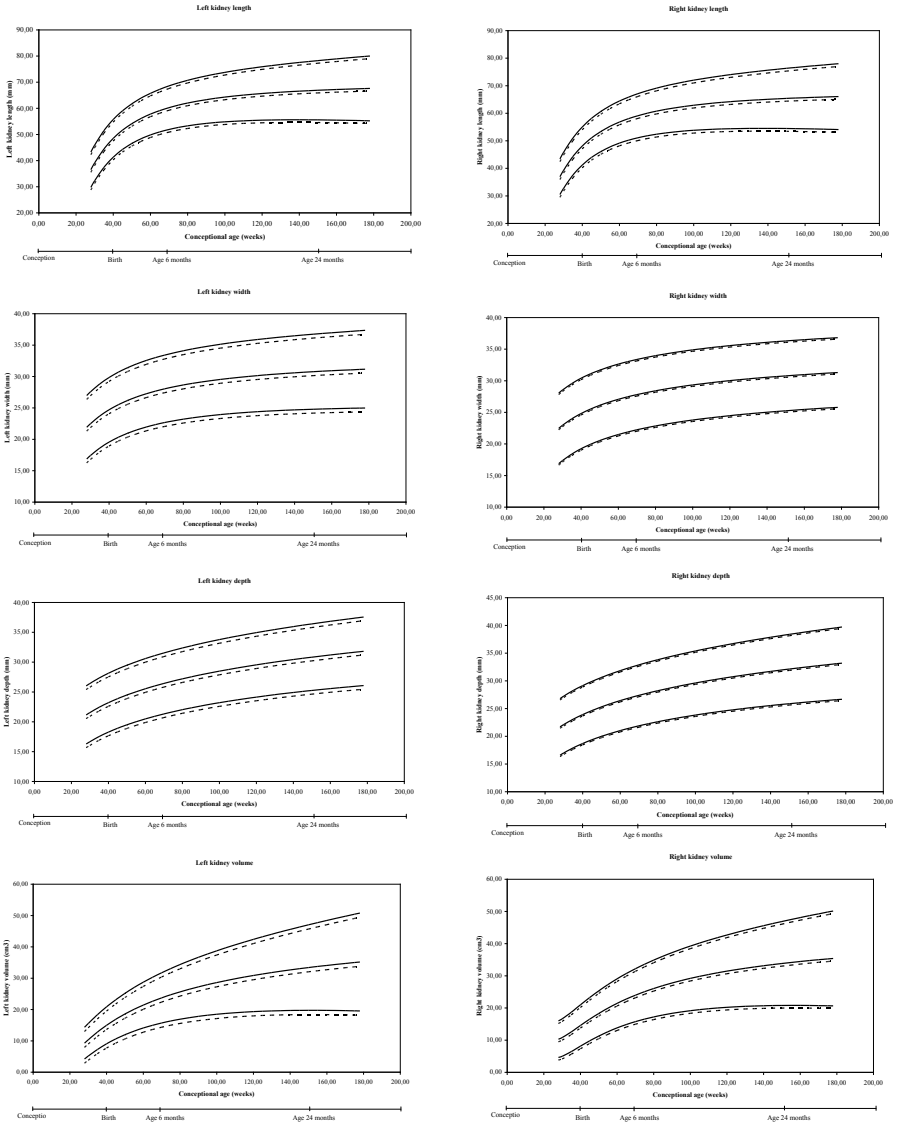
	Successful measurements (% (n))		
Kidney measurement	**Gestational age 30 weeks (n = 1,215)**	**Age 6 months (n = 901)**	**Age 24 months (n = 856)**
Left kidney			
Length (mm)	94.9 (1153)	81.2 (732)	78.2 (669)
Width (mm)	95.2 (1157)	77.2 (696)	72.8 (623)
Depth (mm)	95.1 (1155)	76.8 (692)	76.9 (624)
Volume (cm^3)	94.4 (1147)	76.6 (690)	72.9 (615)
Right kidney			
Length (mm)	94.7 (1151)	82.9 (747)	81.9 (701)
Width (mm)	95.4 (1159)	82.4 (742)	80.6 (690)
Depth (mm)	95.4 (1159)	82.2 (741)	80.6 (690)
Volume (cm^3)	94.5 (1148)	82.1 (740)	80.4 (688)

Table 5. Reference curves for kidney structures: equations for the mean and SD of each measurement based on age in exact weeks

Kidney measurement	Measurement	Regression equation
Left kidney		
Length (mm)	Mean	$70.7392 + 48231 \times (1/Age^2) - 22290 \times (1/Age^2)*\ln(Age) - 1.0112 \times Gender$
	SD	$3.033 + 0.020 \times Age$
Width (mm)	Mean	$33.9989 + 2465.42 \times (1/Age^2) - 408.99 \times (1/Age) - 0.6208 \times Gender$
	SD	$2.577 + 0.004 \times Age$
Depth (mm)	Mean	$2.5301 + 5.7694 \times \ln(Age) - 0.6145 \times Gender$
	SD	$2.502 + 0.0031 \times Age$
Volume (cm^3)	Mean	$56.8477 + 4222.85 \times (1/Age^2) - 273.13 \times (1/\sqrt{Age}) - 1.3538 \times Gender$
	SD	$1.639 + 0.0375 \times Age$
Right kidney		
Length (mm)	Mean	$69.05 + 46279 \times (1/Age^2) - 21206 \times (1/Age^2) \times \ln(Age) - 1.0561 \times Gender$
	SD	$2.899 + 0.0194 \times Age$
Width (mm)	Mean	$37.4342 + 122.84 \times (1/Age^2) - 78.8602 * (1/\sqrt{Age}) - 0.2290 \times Gender$
	SD	$2.977 - 0.00025 \times Age$
Depth (mm)	Mean	$1.2261 + 6.2168 \times \ln(Age) - 0.2474 \times Gender$
	SD	$2.576 + 0.005 \times Age$
Volume (cm^3)	Mean	$45.4105+22573 \times (1/Age^2) - 1766.41 \times (1/Age) - 0.8152 \times Gender$
	SD	$2.128 + 0.0321 \times Age$

Gender = 0 for boys and gender = 1 for girls. Age is defined as postconceptional age (weeks). SD, standard deviation.

Figure 1. Gender-differentiated growth curves for left and right kidney length, with, depth and volume measurements in relation to conceptional age with 3rd and 97th fitted centiles. The straight lines represent boys and the dotted lines represent girls.



Discussion

We constructed gender-specific reference growth curves for kidney length, width, depth and volume using measurements from a large population-based prospective cohort study of healthy children followed from fetal life until early childhood. Left and right

kidney length was at all ages larger in boys than in girls. Left kidney measurements tend to be smaller than right kidney measurements, except for kidney length.

The major strength of our study is its prospective design from early fetal life and the size of the population-based cohort. Our reference curves were based on more than 5,000 kidney measurements. To our knowledge, no previous studies focused on kidney size in early life were based on such large numbers. Of these kidney measurements, all fetal ultrasounds were carried out by two sonographers and 86% of all postnatal ultrasounds were performed by one trained sonographer[20]. A limitation could be that of all children participating in the Generation R measurements at the ages of 6 and 24 months, kidney measurements were successfully performed in 83% and 80% of these infants, respectively. Missing values were mainly due to crying behavior or unavailability of equipment or radiographer. The effect estimates would be biased if the subject characteristics differ between those included and not included in the present analyses. This seems unlikely. Another limitation could be that the current study was performed in a healthy, population based cohort study. The selection towards a healthy population in our cohort may lead to a limited generalizibility to preterm children or children with a small size for gestational age at birth children. However, these numbers were too small to be assessed in further detail.

In third trimester of pregnancy and at the age of 6 months, all kidney measurements were larger in boys than in girls. At the age of 24 months, only kidney length was larger in boys than in girls. Several studies in healthy neonates and adults have also shown that males have larger kidneys than females[21-22]. One explanation for this finding may be a growth stimulating effect of androgens or Y-chromosome related genes. Another explanation could be due to the idea that testosterone levels are significantly higher during fetal life in males compared to females[23-24]. Most previous studies assessing kidney size beyond the neonatal period did not report a difference between boys and girls[25-26]. The differences we found were small, which may explain why they were missed in previous studies.

Previously published data on kidney size showed conflicting results concerning differences between left and right side. Some studies found no difference in kidney measurements between left and right side[25, 27]. However, others have found the left kidney to be the largest[28-29]. Concerning kidney length, there has been no disagreement about the left being the longest[4, 21-22, 26, 28]. In the present study, the left kidney was the longest in both genders at the postnatal ages of 6 and 24 months. In fetal life we found no significant difference in kidney length between left and right side.

Kidney growth is fastest during the first few weeks of life and the rate of increase gradually slows through the remainder of the first year of life and finally stabilizes[30]. To overcome the problem of non-linear infant kidney growth, some sonographic standards, including means and standard deviations, for kidney size in relation to age have been

published[30-32]. A few other studies created linear or non-linear polynominal regression equations for kidney size during the first year of life[25-26]. One study created reference materials for kidney size in healthy children beyond the neonatal period[4]. They only focused on kidney volume in relation to weight, height and body surface area and did not report data about prenatal kidney growth. To our knowledge, the present study is the first to provide prospective longitudinal reference material on kidney size covering the whole period from fetal life until infancy in a healthy population.

Conclusions

kidney size differed between boys and girls from the age of 30 weeks of pregnancy until 24 months of age. Left kidney measurements tend to be smaller than right kidney measurements. Gender-differentiated reference growth curves for both left and right kidneys were constructed for kidney length, width, depth and volume. These reference curves may be of importance to identify abnormal kidney size and growth, with possible subsequent clinical consequences.

Note: Supplementary information is available on the Pediatric Nephrology website: www.springer.com/medicine/pediatrics/journal/467

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Chapter 4.2

Maternal smoking during pregnancy and kidney volume in the offspring

H Rob Taal
JJ Miranda Geelhoed
Eric AP Steegers
Albert Hofman
Henriette A Moll
Maarten Lequin
Albert J van der Heijden
Vincent WV Jaddoe

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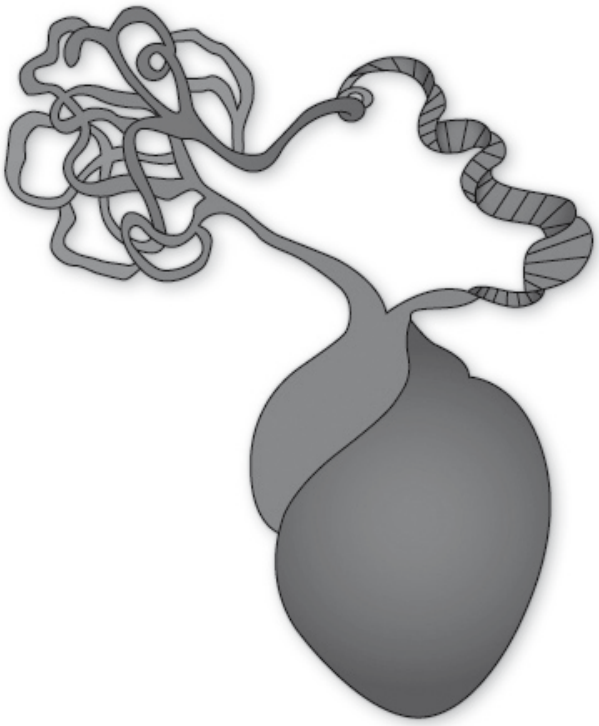


Abstract

Background: An adverse fetal environment leads to smaller kidneys, with fewer nephrons, which might predispose an individual to the development of kidney disease and hypertension in adult life.

Methods: In a prospective cohort study among 1072 children followed from early fetal life onwards, we examined whether maternal smoking during pregnancy, as important adverse fetal exposure is associated with fetal (third trimester of pregnancy, n=1,031) and infant kidney volume (2 years of age, n=538) measured by ultrasound. Analyses were adjusted for various potential confounders.

Results: Among continued smoking mothers, we observed dose-dependent associations between the number of cigarettes smoked during pregnancy with kidney volume in fetal life. Smoking less than 5 cigarettes per day was associated with larger fetal combined kidney volume, while smoking more than 10 cigarettes per day tended to be associated with smaller fetal combined kidney volume (p for trend: 0.002). This pattern was not significant for kidney volume at the age of 2 years.

Conclusion: Our results suggest that smoking during pregnancy affects kidney development in fetal life with a dose-dependent relationship. Further studies are needed to assess the underlying mechanisms and whether these differences in fetal kidney volume have postnatal consequences for kidney function and blood pressure.

Introduction

The developmental plasticity hypothesis suggests that various adverse intra-uterine exposures lead to persistent fetal developmental adaptations. These adaptations may be beneficial on short term but may have adverse consequences in postnatal life[1]. Also, they may lead to smaller kidneys with a reduced number of nephrons, which in turn leads to glomerular hyperfiltration and sclerosis, predisposing the individual to renal damage and subsequent development of higher blood pressure, impaired kidney function and end stage kidney disease in adulthood[2]. This hypothesis is supported by many studies showing associations of low birth weight with cardiovascular disease and chronic renal failure[3-5]. Low birth weight is also associated with impaired renal growth, raised blood pressure, and impaired renal function[5-9]. Thus far, the specific adverse fetal exposures and mechanisms underlying these associations are not known. Maternal smoking is a very important modifiable adverse fetal exposure in Western countries and leads to a decrease of 150 to 200 grams in offspring birth weight[10]. Maternal smoking during pregnancy may also have both direct and indirect adverse effects on fetal kidney development. Several studies suggested that maternal smoking during pregnancy is also associated with higher blood pressure in the offspring, independent of birth weight[11], which might be explained by an adverse kidney development[12].

For the present study, we hypothesized that maternal smoking during pregnancy, as specific adverse fetal exposure, affects early kidney growth. In a population-based prospective cohort study, among 1,072 mothers and children, we evaluated the associations of maternal smoking during pregnancy with kidney volume in fetal life and infancy, both of those mothers who smoked during first trimester only and those who continued smoking throughout pregnancy.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood in Rotterdam, The Netherlands[13-14]. Detailed assessments were conducted in a subgroup of Dutch children and their parents. Mothers, who were already participating during pregnancy, were asked to participate in a sub cohort for additional detailed renal and cardiovascular measurements. These women were all enrolled before a gestational age of 24 weeks. In total 80% of these mothers, were willing to participate in the sub cohort. Data on smoking during pregnancy was available from prospectively collected questionnaires. For the present study, kidney measurements were performed at a gestational age of 30 weeks

and at the postnatal age of 24 months. Written informed consent was obtained from all participants. The Medical Ethics Committee of the Erasmus Medical Center Rotterdam has approved the study.

Population for analysis

In total 1,232 women were enrolled in the subgroup study. Twin pregnancies (n=15) and pregnancies leading to perinatal death (n=2) were excluded from the analysis, leading to 1,215 singleton live births. No renal or uterovesical anomalies other then mild pyelectasis over 10 mm (n=3) were present in our study. Information about smoking during pregnancy was not available in 110 subjects. Of the remaining 1,105 mothers, fetal kidney characteristics were successfully measured in 1,031 subjects (93 %). In total, 67 % (n= 740) of the mothers and children with information about smoking during pregnancy participated in the follow-up study at the age of 2 years, with successful kidney measurements in 73% of these children (n=538). Missing data were mostly due to crying and oppositional behaviour. In total 1,072 (97%) had at least one complete kidney measurement.

Maternal smoking during pregnancy

Information about active smoking was obtained by postal questionnaires in the first, second and third trimester of pregnancy. Response rates for these questionnaires were 90%, 93% and 92%, respectively[15]. Active maternal smoking at enrolment was assessed in the first questionnaire by asking whether mother smoked in pregnancy (no, first trimester only, continued smoking during first trimester). In the second and third-trimester questionnaires, mothers were asked whether they smoked in the past 2 months (no, yes) respectively. Among the smoking mothers, the number of cigarettes was classified into different categories: less than five cigarettes per day; five to ten cigarettes per day; and more than ten cigarettes per day. Dose response analyses for first trimester only and continued smoking mothers were based on the first and third trimester questionnaires, respectively.

Kidney measurements

All ultrasound exams were performed using an ATL-Philips Model HDI 5000 (Seattle, WA, USA) equipped with a 5.0 MHz, high frequency curved array transducer. Ultrasound examinations were carried out in a dedicated and well-equipped research center in third trimester of pregnancy. Fetal left and right kidney biometrics were assessed. In a sagittal plane, the maximum longitudinal kidney length was measured placing the callipers on the outer edges of the caudal and cranial side. Antero-posterior and transverse kidney diameter were measured perpendicular to each other, from the one outer edge to the other, in an axial plane. The cross-sectional area in which the kidney appeared

symmetrically round at its maximum width was used. The images were sufficiently magnified to ensure optimal measurements[16]. Postnatally, two-dimensional ultrasounds of the kidneys were performed in children at the age of 24 months. The examination was carried out in a quiet room with the child quietly awake in a supine position. This position was standardised to prevent differences according to position[16-17]. The kidney was identified in the sagittal plane along its longitudinal axis. Measures of maximal bipolar kidney length, width and depth were obtained from both the left and right kidney. Kidney width and depth were measured at the level of the kidney hilum[16-17]. Both fetal and postnatal kidney volume were calculated using the equation of an ellipsoid: volume (cm^3) = 0.523 x length (mm) x width (mm) x depth (mm). Left and right kidney volumes were added for the combined kidney volume (cm^3)[18].

Intra- and inter-observer reproducibility. Two well-trained, experienced sonographers performed all measurements. Quality checks were frequently carried out and feedback was provided to minimize interoperator differences. We have previously shown that kidney characteristics can be reliably measured in children[19]. The intraobserver interclass correlation coefficients (ICC) ranged from 0.93 (left and right kidney width and right renal thickness) to 0.99 (left kidney length) and interobserver ICC ranged from 0.64 (right kidney thickness) to 0.90 (right kidney length), indicating good reproducibility.

Covariates

Maternal height and was measured at enrolment. Information on maternal pre-pregnancy weight and maternal educational level was assessed using self reported questionnaires. We defined maternal weight gain as the difference between weight at enrolment and weight in third trimester of pregnancy as described before[20]. Educational level of the father and family income were also assessed using questionnaires at enrolment. Total daily energy intake was established using semiquantitive food frequency questionnaires in first trimester of pregnancy. Exact gestational age was established using fetal biometry measured in first trimester of pregnancy[21]. Fetal biometrics including head circumference (HC), abdominal circumference (AC), and femur length (FL) were measured using standardized ultrasound procedures in third trimester of pregnancy and estimated fetal weight (EFW) was calculated using the formula by Hadlock: EFW (gr) = 10 x (1.326 – 0.00326 x AC x FL + 0.0107 x HC + 0.0438 x AC + 0.158 x FL)[22]. Fetal biometrics were measured at the same visit as the fetal kidney measurement. Date of birth, birth weight and sex were obtained from midwife and hospital registries. Postnatal height and weight were measured at the same visit as the postnatal kidney measurement.

Statistical Methods

Differences in maternal and offspring characteristics between the non-smoking, first trimester only and continued smokers were assessed using t-tests and nonparametric

tests for independent samples. Associations of maternal smoking, both first trimester only and continued smoking, with kidney volume were assessed using multiple linear regression models. We adjusted for covariates that changed the effect estimate of smoking during pregnancy on kidney volume more than 10%, when added to the baseline model including gender, age and estimated fetal weight. All regression models were adjusted for fetal sex, maternal height and weight before pregnancy, maternal weight gain during pregnancy, total daily caloric intake, maternal and paternal educational level and household income. We further adjusted the analyses focused on fetal kidney volume for estimated fetal weight in third trimester of pregnancy (30 weeks) and gestational age at visit. The models focused on postnatal kidney volume were additionally adjusted for age, weight and height at visit. Systolic and diastolic blood pressure at intake, parity, maternal alcohol use during pregnancy and folic acid supplement use did not materially change the effect estimate.

Subsequently, using similar models we examined the associations of the number of cigarettes smoked with kidney volumes. Missing values in covariates ranged from 0 to 10%, except for paternal educational level (13.9%) and daily total energy intake (14.7%), and were imputed as mean for continuous variables and separate category for categorical variables. Since, no differences in main results were observed between analyses on complete and imputed analyses, we only present data on imputed analyses. Tests for trends were performed using multiple non-linear regression analyses, including a squared term of the smoking during pregnancy categories, using the non-smoking group as reference group. All measures of association are presented with their 95% confidence intervals (95% CI). We considered a P-value lower than 0.05 as statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics are presented in Table 1. The percentage of boys was 52.1%. The overall median maternal age was 31.8 (95% range: 23.5 - 37.7) years. The study group comprised 31 children who were born with a small size for gestational age (birth weight less than -2 SDS for gestational age), 37 children with a birth weight less than 2,500 grams and 41 children who were born preterm. Table 2 shows all kidney characteristics measured in third trimester of pregnancy and at the postnatal age of 2 years.

Table 3 gives the adjusted associations of maternal smoking during pregnancy with fetal and postnatal combined volume. The results of the unadjusted model are presented in Table S1 in the supplement. Overall, compared to non smoking, no significant associations of first trimester only smoking or continued smoking during pregnancy with fetal

Table 1. Subject characteristics (n=1,072)

	Smoking during pregnancy (n=1,072)		
	Non (n=805)	**First trimester only** (n=101)	**Continued** (n=166)
Maternal characteristics			
Age (yrs)	31.7 (24.9-37.7)	30.8 (21.8-38.4)	30.4 (21.3-37.2) *
Height (cm)	171.2 (6.3)	170.7 (6.7)	169.6 (6.2) **
Pre pregnancy weight (kg)	69.5 (12.9)	66.5 (10.4) **	67.7 (13.3)
Weight gain during pregnancy(kg)	8.0 (2.0-14.0)	9.0 (3.0-16.1) **	8.0 (1.0-14.7)
Systolic blood pressure (mmHg)	118.7 (12.8)	118.4 (13.8)	118.0 (12.4)
Diastolic blood pressure (mmHg)	69.9 (10.1)	68.2 (9.2)	67.6 (9.3) **
Parity ≥ 1 (%)	39.4	27.7 *	44.6
Highest education (%)			
Primary school	1.2	5.0 **	6.2 **
Secondary school	30.7	37.6 **	56.8 **
Higher education	68.1	57.4 **	37.0 **
Household income (%)			
<1200 euro	1.5	1.2	12.9 **
1200-1600 euro	4.5	4.7	9.3 **
>1600 euro	94.0	94.1	77.8 **
Alcohol use during pregnancy (%)	51.8	54.9	52.2
Total daily energy intake (kcal)	2109 (496)	2147 (526)	2244 (492) **
Folic acid supplement use (%)	93.1	89.0	78.2 **
Paternal characteristics			
Age biological father	33.6 (26.9-42.8)	32.8 (25.5-41.1)	33.1 (23.0-41.8) *
Highest education (%)			
Primary school	2.7	1.2	10.4 **
Secondary school	31.2	32.5	59.7 **
Higher education	66.1	66.3	29.9 **
Third trimester fetal characteristics			
Gestational age at assessment (weeks)	30.4 (28.8-32.2)	30.3 (28.8-31.9)	30.2 (28.6-31.7)
Head circumference (mm)	287 (266-307)	288 (267-306)	283 (263-305) **
Abdominal circumference (mm)	266 (239-293)	267 (237-299)	262 (233-295) *
Femur length (mm)	57 (53-63)	57 (52-62)	56 (51-61) **
Estimated fetal weight (g)	1619 (1264-2118)	1643 (1181-2119)	1527 (1167-2094) **
Birth characteristics			
Gestational age (weeks)	40.3 (37.1-42.1)	39.9 (36.2-42.0)	40.1 (36.8-42.1)
Male (%)	51.9	44.1	57.8
Birth weight (g)	3580 (2660-4350)	3570 (2487-4643)	3412 (2342-4243) **
Low birth weight (% <2,500 g)	3.0	4.9	6.1 *
Small size for gestational age (% < -2 SD)	2.2	2.9	6.6 *
Preterm birth (%)	4.1	5.9	4.9

* = p<0.05

** = p<0.01

Values are means (sd) or medians (95% range)

Table 2. Fetal and postnatal kidney characteristics

	30 weeks of gestation (n = 1,031)	2 years (n = 538)
Age at assessment (weeks)	30.2 (28.8-32.1) [#]	109 (103-120) [##]
Left kidney structures		
Length (mm)	39.0 (33.0-45.2)	66.3 (57.3-76.7)
Width (mm)	21.0 (17.0-26.0)	30.2 (25.6-36.3)
Depth (mm)	22.0 (18.0-27.0)	30.3 (25.5-36.4)
Volume (cm^3)	9.6 (6.0-14.9)	31.7 (22.7-46.3)
Right kidney structures		
Length (mm)	39.0 (33.0-45.0)	64.7 (55.9-75.3)
Width (mm)	22.0 (18.0-27.0)	30.6 (26.3-35.7)
Depth (mm)	23.0 (18.9-28.0)	31.7 (26.9-38.2)
Volume (cm^3)	10.2 (6.4-16.4)	32.2 (23.6-47.0)
Combined kidney volume (cm^3)	19.9 (12.6-30.7)	64.0 (48.5-93.6)

Values are medians (95% range)

[#] Gestational age during pregnancy at assessment

[##] Postnatal age at assessment

Table 3. Maternal smoking during pregnancy and fetal and postnatal kidney volume

		Combined kidney volume	
Smoking during pregnancy		**30 weeks of gestation (n = 1,031)**	**2 years (n = 538)**
Non		*Reference* (n = 769)	*Reference (n = 415)*
First-trimester only *(overall)*		-0.06 (-1.12 , 0.99) (n = 98)	3.92 (0.24 – 7.61) * (n = 49)
Continued *(overall)*		0.25 (-0.65, 1.15) (n = 164)	0.18 (-3.08 , 3.43) (n= 74)
Number of cigarettes smoked per day	*<5/day*	1.71 (0.51 , 2.92) ** (n = 76)	0.49 (-3.81 , 4.78) (n= 32)
	5-10/day	-0.55 (-1.99 , -0.89) (n = 55)	-0.48 (-5.66 , 4.71) (n = 24)
	>10/day	-1.95 (-3.73 , -0.16) * (n = 32)	-2.33 (-8.65 , 4.00) (n = 15)
P-value for trend		***0.002***	***0.331***

* p<0.05, ** p<0.01

Values are regression coefficients (95% CI) and reflect the difference in kidney volume for different categories of maternal smoking during pregnancy. All regression models were adjusted for fetal sex, maternal height and weight before pregnancy, maternal weight gain during pregnancy, total daily caloric intake, maternal and paternal educational level and household income. The models for fetal kidney size were also adjusted for estimated fetal weight, and gestational age at visit. The postnatal kidney analyses were additionally adjusted for age, weight and height at visit.

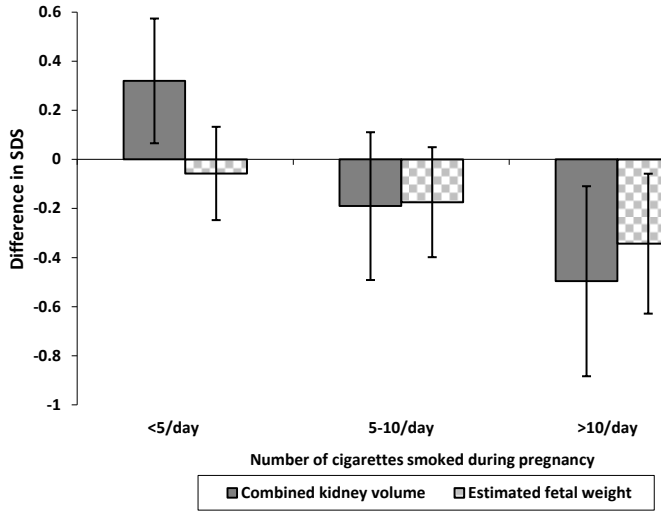
Tests for trend were calculated using non-linear regression models including a squared term of the number of cigarettes smoked during pregnancy categories.

kidney volume were observed. Smoking during first trimester only, but not continued smoking, was positively associated with combined kidney volume at the age of 2 years. Among first trimester only smokers, we did not observe consistent dose response associations between the number of cigarettes smoked and combined kidney volume in fetal life or at the age of 2 years (data not shown).

Among mothers who continued smoking during pregnancy, we observed a dose-dependent association, between the number of cigarettes smoked during third trimester with fetal combined kidney volume (Table 3). Compared to non-smoking, smoking less than five cigarettes per day was associated with larger fetal combined kidney volume. Smoking more than ten cigarettes per day tended to be associated with smaller combined kidney volume. We observed non-significant associations between the number of cigarettes smoked during pregnancy and kidney volumes at the age of 2 years.

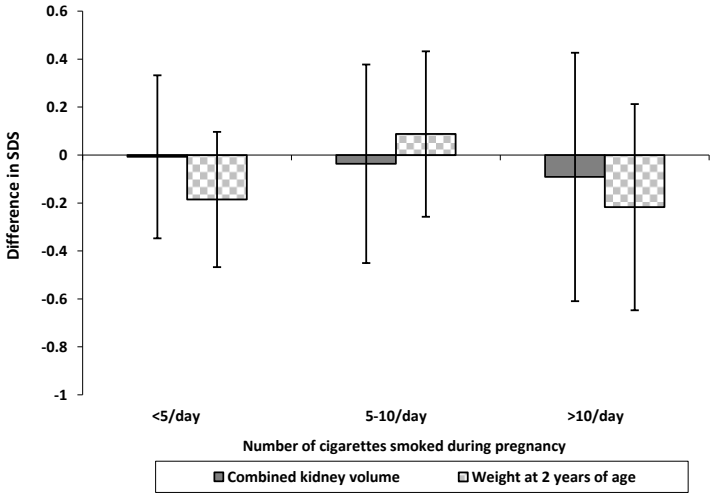
Figure 1 shows the regression coefficients of multiple linear regressions on combined kidney volume and estimated fetal weight are affected separately by smoking throughout pregnancy. Estimated fetal weight proportionally decreased with the number of cigarettes smoked per day (p-value for trend: 0.01). Combined kidney volume however, was larger in offspring of mothers who smoked less than 5 cigarettes per day,

Figure 1



Bars represent regression coefficients (95% CI) and reflect the difference in SD scores of fetal kidney volume and estimated fetal weight for different numbers of cigarettes smoked continuously throughout pregnancy. All regression models were adjusted for fetal sex, gestational age at visit, maternal height and weight before pregnancy, maternal weight gain during pregnancy, systolic and diastolic blood pressure at intake, parity, maternal alcohol use during pregnancy, folic acid supplement use, total daily caloric intake, maternal and paternal educational level and household income. The model for estimated fetal weight was adjusted for the same covariates.

Figure 2



Bars represent regression coefficients (95% CI) and reflect the difference in SD scores of kidney volume at two years of age and weight at two years of age for different numbers of cigarettes smoked continuously throughout pregnancy. All regression models were adjusted for sex, age at visit, maternal height and weight before pregnancy, maternal weight gain during pregnancy, systolic and diastolic blood pressure at intake, parity, maternal alcohol use during pregnancy, folic acid supplement use, total daily caloric intake, maternal and paternal educational level and household income. The model for weight at two years of age was adjusted for the same covariates.

and became smaller with increasing numbers of cigarettes smoked per day (p-value for trend: 0.003). Figure 2 shows the associations of continued maternal smoking during pregnancy with postnatal combined kidney volume and body weight. No significant associations were observed.

Discussion

Results from this population-based prospective cohort study from early fetal life onwards suggest that first trimester only smoking is not consistently associated with kidney volume in fetal life and infancy, whereas continued smoking during pregnancy affects kidney volume in fetal life. The effect size and direction depends on the number of cigarettes smoked. Smoking less than five cigarettes per day was associated with larger fetal combined kidney volume. This association was not significant at the age of 2 years. Smoking more than ten cigarettes per day tended to be associated with smaller combined kidney volume in both fetal and postnatal life.

Maternal smoking during pregnancy is an important modifiable adverse fetal exposure leading to various pregnancy complications in Western countries[10, 23]. The recently

demonstrated associations between maternal smoking during pregnancy with high blood pressure in the offspring suggest that fetal exposure to smoking has permanent renal and cardiovascular consequences[24]. We showed associations of continued smoking during pregnancy with fetal kidney volume but not with postnatal kidney volume. The effect of continued smoking during pregnancy was dependent on the number of cigarettes smoked. This suggests a differential effect of maternal smoking during pregnancy, depending on the specific period of exposure and number of cigarettes smoked.

The main strength of our study is the prospective design from early fetal life onwards and the size of the population-based cohort. Our analyses were based on more than 1,500 complete kidney ultrasounds. The size of the cohort enabled us to assess the associations between the number of cigarettes smoked during pregnancy and kidney volume. The ultrasound measurements were carried out by two sonographers with good reproducibility.

Of all children with available data on maternal smoking during pregnancy, 67% participated in the follow measurements at the age of 2 years. Subjects who did not visit the research centre in postnatal life did not differ in fetal kidney volume from the original sample. However, more mothers in this group continued smoking during pregnancy and mothers were less well educated than the original sample. This selective loss to follow-up, might have led to loss of power in the postnatal analyses, and possibly selection bias. Although we performed a meticulous adjustment, especially regarding socioeconomic status, residual confounding might still be an issue because of the observational design of the study.

Another reason for the lacking associations in postnatal life might be that kidney measurements are more difficult at the age of two years, introducing random error, which leads to a reduction in statistical power. One might also assume that some hypertrophy of nephrons is already ongoing in kidneys with lower nephron numbers (i.e. smaller fetal kidney volume) due to heavy smoking mothers, decreasing the difference in kidney volume over time. The increased kidney volume in mothers who smoked less than 5 cigarettes per day, might have disappeared due to return to the normal kidney growth curve in postnatal life, while the reduced body size catches up.

Using questionnaires for collecting data on smoking behaviour might have resulted in underestimation of the number of cigarettes smoked per day, since some individuals might not report their smoking behaviour accurately. We did not measure cotinine levels to check smoking status in the mothers. Although assessing maternal smoking during pregnancy seems to be a valid method, misclassification may occur[25-26]. Another limitation might be that the present study was performed in a healthy population based cohort. The absolute numbers of subjects with low birth weight and preterm birth were small. Therefore, generalizability of our results to preterm or low birth weight children is limited.

One other study among 34 subjects showed that maternal smoking during pregnancy leads to a different fetal kidney growth pattern compared to non-smoke exposed subjects. This study suggested that reduction in kidney growth is present in third trimester of pregnancy resulting in relatively thinner kidneys[12]. This is in line with our finding that smoking more than 10 cigarettes in third trimester negatively affects in fetal and infant kidney volume. To our knowledge, no other studies examined the associations between maternal smoking during pregnancy and kidney size.

The mechanisms underlying the associations between smoking during pregnancy and fetal and infant kidney volume found in this study are not known. Animal studies showed that nicotine has both vasodilatatory and vasoconstrictive effect on vasculature, depending on the dose[27-29]. Higher dosage of nicotine were associated with vasoconstriction and might partly explain our findings of smaller kidney size when smoking large numbers of cigarettes during pregnancy[29]. The larger kidneys among subjects exposed to less than five cigarettes per day could be explained by vasodilatory effects of nicotine on renal vasculature. Animal studies showed vasodilatory effects of low dose nicotine in the kidney in pre-constricted kidney vasculature[30-31]. Maternal smoking during pregnancy affects fetal growth in different trimesters of pregnancy, with the largest effect in the third trimester. Results suggest that smoking during pregnancy preferentially affects peripheral tissues[25]. The vasodilatory effect of nicotine on kidney vasculature might lead to increased kidney volume despite the negative effect on estimated fetal weight. However, further studies are needed to elucidate the vasoactive effects of nicotine in growth restricted subjects.

Another possible mechanism could be the role of the renin-angiotensin system (RAS). It has been shown that an intact RAS is needed for normal kidney development and disruption is associated with lower glomerular number, decreased renal function and increased adult blood pressure[32-33]. It also has been shown that smoking during pregnancy alters AT1/AT2 receptor ratio in renal tissue[34]. This could partly explain our findings that smoking more than ten cigarettes per day is associated with smaller fetal kidney volume. Further studies are needed to evaluate the effect of maternal smoking during pregnancy on RAS in humans.

The associations of low birth weight with hypertension and impaired renal function and end stage renal disease in adulthood are well established[3, 6, 9, 35-36]. Smaller kidneys with a reduced number of nephrons in low birth weight children might lead to hyperfiltration resulting in glomerular sclerosis[2, 37-40]. This may predispose the individual to renal damage and development of higher blood pressure, impaired kidney function and end stage kidney disease in adulthood. This hypothesis is supported by animal studies, which have shown that various adverse intrauterine environmental exposures, such as low protein intake, lead to fetal growth retardation and smaller kidney size with a lower

nephron number[41-43]. Post mortem studies in humans showed that a lower nephron number is associated with both low birth weight and hypertension[37, 39, 44-45].

Newborn kidney volume has been shown to be a surrogate of total nephron number. This was shown in 15 infants who died before 3 months of age, in whom an ultrasound was made in the first 2 days of life. There was a strong and direct relationship between kidney mass and nephron number[46]. Since nephron number varies between 250,000 and 2,000,000 per kidney and nephron development ceases after birth[44, 47], these findings suggest that early kidney development may be an underlying mechanism for the associations between maternal smoking during pregnancy and increased blood pressure in later life. Whether the differences in kidney volume due to smoking during pregnancy in this study also correlate with nephron number needs to be studied.

Conclusions

Our results suggest that maternal smoking during pregnancy is associated with an altered kidney volume in late pregnancy but not in infancy. The direction and size of the effect depends on the number of cigarettes smoked. These results should be considered as hypothesis generating. Further studies are needed to identify the underlying mechanisms and to assess whether these changes in kidney size in early life are related to renal function and blood pressure development in later life.

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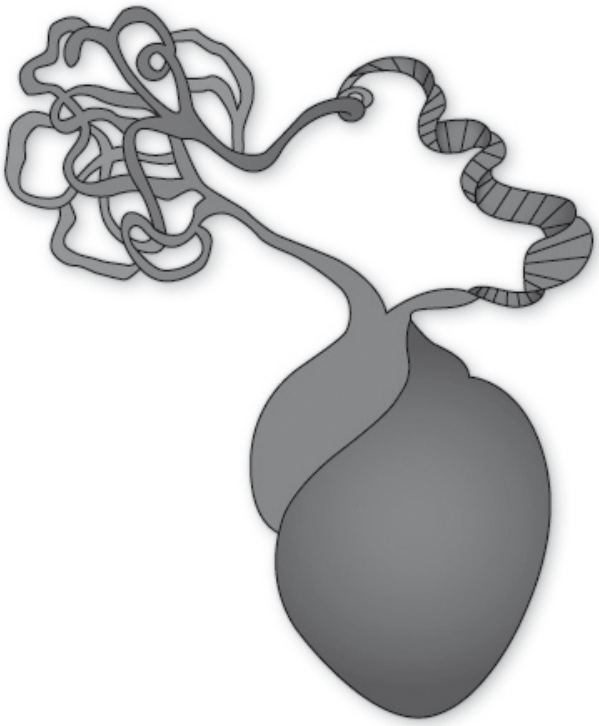
Chapter 4.3

Environmental exposures during pregnancy and kidney growth and function in childhood

Hanneke Bakker
H Rob Taal
Layla L de Jonge
Albert Hofman
Albert J van der Heijden
Vincent WV Jaddoe

Submitted



Abstract

Background: Low birth weight is associated with end-stage renal and cardiovascular disease. This association may be explained by persistent smaller kidneys with a reduced number of nephrons in children with low birth weight, and subsequent renal dysfunction and high blood pressure in later life. We examined whether kidney dimensions in childhood are influenced by maternal life style characteristics during pregnancy and birth outcomes and whether kidney dimensions are related with kidney function and blood pressure in school age children.

Methods: This study was embedded in a population-based prospective cohort study from fetal life onwards among 6,368 children and their parents. Information about maternal and fetal exposures was available. At the age of 6 years, we measured kidney volume by ultrasound, kidney function by creatinine and cystatin C levels, and blood pressure.

Results: The glomerular filtration was estimated. Maternal body mass index and calorie intake were positively associated with combined childhood kidney volume (p-values <0.05). Continued maternal smoking during pregnancy was associated with smaller combined kidney volume and lower glomerular filtration rate in childhood (p-values <0.05). As compared to term born children, preterm born children had a smaller combined kidney volume at the age of 6 years (difference -6.81 mm^3 (95% confidence interval -9.73 to -3.89)). As compared to children born with an appropriate size for gestational age at birth, those born with a small size for gestational age at birth had a smaller childhood kidney volume (difference -7.97 mm^3 (95% confidence interval -10.58 to -5.36)). Size at birth also was associated with glomerular filtration rate and systolic and diastolic blood pressure (p-values < 0.05). Children with a smaller combined kidney volume had a lower estimated glomerular filtration rate and lower blood pressure in childhood (-4.82 ml/min per 1.73m^2 per SD change in combined kidney volume, and -0.44 mmHg per SD, respectively).

Conclusion: Our results suggest that childhood kidney growth and function is influenced by several maternal and fetal characteristics. Smaller kidney size in childhood is associated with lower glomerular filtration rate and lower blood pressure.

Introduction

Low birth weight is consistently associated with higher risks of end stage renal disease and hypertension in later life[1-3]. Brenner et al. hypothesised that the mechanisms underlying these associations include fetal renal developmental adaptations in response to various adverse early exposures[4]. These fetal adaptations may lead to smaller kidneys with a reduced number of nephrons, and subsequently to glomerular hyperfiltration and sclerosis, which predisposes to development of impaired kidney function and eventually end stage kidney disease in adulthood[5-7]. This hypothesis is supported by both animal and human studies showing that neonatal kidney volume and nephron number is reduced in fetal growth restricted subjects[8-9]. Also, a post mortem study showed that patients with hypertension had significantly fewer but greater glomeruli per kidney than nonhypertensive subjects[10]. Kidney size is correlated with the number of glomeruli and can be used in epidemiological studies as measure of kidney development[11]. We have previously shown that maternal anthropometrics, fetal growth and blood flow patterns correlate with third trimester fetal kidney volume. Also, maternal smoking during pregnancy influences kidney volume in infancy[12-13]. Follow up studies suggested that maternal undernutrition or obesity, and fetal growth restriction affect renal function in adulthood, but results are inconsistent[9, 14-16]. Studies in children focused on early influences and consequences of kidney growth are important since small differences at young age seem to track into adulthood[17], and may be associated with the risks of renal disease and hypertension in later life. Identification of early determinants of childhood kidney volume and function could be important for early prevention of renal disease in adulthood.

In a population-based prospective cohort study from fetal life onwards among 6,368 children and their parents, we examined whether kidney dimensions in childhood are influenced by maternal life style characteristics during pregnancy and birth outcomes and whether kidney dimensions are related with kidney function and blood pressure in school age children.

Methods

Design and study population

This study was embedded in the Generation R Study, a population-based, prospective cohort study from fetal life until young adulthood in Rotterdam, the Netherlands[18]. The vast majority of mothers were enrolled during pregnancy, but enrolment was allowed until childbirth. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. All participants or their parents gave written

consent to the study. In total 9,778 mothers were enrolled in the study, and 8,305 of their children participated in the follow up measurements in children aged 6 years. In total, 6,690 (81%) of these children visited the research center for renal follow up measurements. We excluded children with echocardiographic evidence of congenital heart disease (n=28) or kidney abnormalities (n=12). Kidney volume or blood pressure measurements were successfully performed in 6,368 (95%) children (Figure 1). In 4,272 (67.1%) of those children blood samples for kidney function measurements were available.

Main exposure variables

Maternal life style related characteristics during pregnancy

Maternal height was measured at enrolment. Information on maternal parity, and pre-pregnancy weight was assessed using self reported questionnaires. Pre-pregnancy body mass index (BMI, kg/m^2) was calculated[19]. Maternal blood pressure was measured at intake with the validated Omron 907 automated digital oscillometric sphygmanometer (OMRON Healthcare B.V. Hoofddorp, the Netherlands)[20]. We assessed maternal smoking during pregnancy by questionnaire (no smoking; first trimester only; continued smoking during pregnancy). We assessed maternal dietary intake at enrolment in the study in Dutch mothers using a modified version of a previously validated semi quantitative food frequency questionnaire (FFQ)[21]. The FFQ considered food intake over the prior three months, thereby covering the dietary intake in first trimester of pregnancy. We used the Dutch food composition table for calculating daily intake of nutritional values[22]. Total caloric intake was calculated. Information on folic acid supplement use (0.4-0.5 mg) and the initiation of supplementation was obtained by questionnaires at enrolment of the study.

Birth characteristics

Gestational age in early pregnancy was established using first trimester fetal ultrasound[23]. Information on gestational age at birth and birth weight was obtained from community midwife and hospital registries. Preterm birth was defined as birth < 37.0 weeks of gestation. Gestational age adjusted SDS were constructed for birth weight, using reference growth charts[24]. We defined small size for gestational age at birth (SGA) as being <5th sex specific percentile for weight and large size for gestational age at birth (LGA) as being >95th sex specific percentile for weight.

Main outcome variables

Kidney dimensions

Left and right kidney biometrics were assessed with an ATL-Philips HDI 5000 instrument (Seattle, WA, USA) equipped with a 2.0–5.0 MHz curved array transducer until September 2010 or with a General Electric Logiq E9 (Milwaukee, WI, USA) equipped with a 2.0-7.0 MHz curved array transducer after September 2010. During the examination the child was awake in a quiet room and calm in a standardized prone position. We identified the left and right kidney in the sagittal plane along its longitudinal axis. We performed measurements of maximal bipolar kidney length, width and depth. At the level of the hilum kidney width and depth were measured. The cross-sectional area in which the kidney appeared symmetrically round at its maximum width was used. Kidney volume was calculated using the equation of an ellipsoid: volume (cm^3) = 0.523 x length (mm) x width (mm) x depth (mm)[25]. Combined kidney volume was calculated by summing right and left kidney volume. Quality checks were frequently carried out and feedback was provided to minimise inter-operator differences. Good reproducibility was pursued with intraobserver interclass correlation coefficients (ICC) ranged from 0.93 (left and right kidney width and right renal thickness) to 0.99 (left kidney length) and interobserver ICC ranged from 0.64 (right kidney thickness) to 0.90 (right kidney length)[26].

Kidney function

Serum creatinine was measured with the enzymatic method, on a Cobas c 502 analyzer (Roche Diagnostic, Germany). We additionally measured serum cystatin C by a particle enhanced immunoturbidimetric assay on Cobas c 702 analyzer (Roche Diagnostic, Germany). Estimated glomerular filtration rate (eGFR) was calculated according to the revised Schwartz formula from 2009[27]; eGFR = 36.5 * (height (cm)/creatinine (µmol/l). This formula is validated in children with different characteristics[28].

Blood pressure

Systolic and diastolic blood pressure was measured at the right brachial artery, four times with one minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus ™ (Paramus, NJ, USA). The measurements were conducted while the child was quietly lying in supine position. A cuff was selected with a cuff width approximately 40% of the arm circumference and long enough to cover 90% of the arm circumference.

Covariates

Maternal age was registered at enrolment. Maternal ethnicity was classified by the countries of birth of the parents, according to the Dutch standard classification criteria of Statistics Netherlands[18]. Maternal education was defined as the highest completed

education according to the classification of Statistics Netherlands and was categorized in primary, secondary or higher[18, 29]. Maternal alcohol consumption during pregnancy was assessed using questionnaires (no alcohol pregnancy, alcohol consumption until pregnancy was acknowledged or continued use during pregnancy) in different trimesters of pregnancy. Breastfeeding (yes/no) was assessed using questionnaires. At the age of 6 years, child height and weight were measured without shoes and heavy clothing, and body mass index (kg/m^2) was calculated.

Statistical analysis

First, we performed multiple linear regression models to explore the associations of maternal characteristics with childhood kidney volumes, kidney function and blood pressure. These models were first adjusted for sex and age (crude model), second for potential confounders (confounder model) and third for birth outcomes (intermediate model). Since we measured blood pressure four times, we applied linear mixed models[30], which fit the four blood pressure measurements within the same child as repeated outcome measures. One of the advantages of this approach is that subjects with the maximum number of blood pressure measurements available and the least individual variability in their blood pressure measurements are assigned the highest weight in the analysis[31]. For comparison of effect estimates we present the result as difference in outcome per standard deviation scores for continuous variables and change per category for categorical variables. Second, we assessed the associations of birth outcomes (gestational age at birth, birth weight, gestational age adjusted birth weight) with left, right and combined kidney volume, kidney function and blood pressure. Finally, we used similar multiple linear regression models to assess the associations of kidney dimensions with kidney function, blood pressure at age 6 years. To reduce the possibility of potential bias associated with missing data (ranging from 0 to 30%), missing values in maternal, fetal and child covariates were multiple imputed. Five imputed datasets were made and effect sizes and standard errors for each dataset were calculated. Finally, results from all five datasets were pooled and presented in this manuscript[32]. Regression coefficients for systolic and diastolic blood pressure were calculated on repeated measurements in a mixed model using SAS (SAS Institute Inc., Cary, NC, USA). All other statistical analyses were performed using the Statistical Package for the Social Sciences version 20.0 for Windows (SPSS, Chicago, IL, USA).

Results

Subject characteristics

Maternal and child characteristics are shown in Table 1. At the age of 6 years (95 % range 5.6 - 7.9 years), mean (SD) combined left and right kidney volume was 120.3 (23.5) mm^3, systolic and diastolic blood pressure were 102.7 (8.2) mmHg, and 60.7 (6.9) mmHg respectively. The mean glomerular filtration rate was 118.8 (16.4) ml/min per 1.73m^2. Observed data before multiple imputation is presented in Supplementary material (Table S1).

Table 1. Subject characteristics (n = 6,368)

	Participants
Maternal characteristics	
Age, yr	30.5 (5.2)
Height, cm	167.5 (7.4)
Pre-pregnancy weight, kg	66.4 (12.5)
Pre-pregnancy body mass index, kg/m2	24.0 (6.1)
Weight gain during pregnancy, kg	7.4 (3.8)
Systolic blood pressure, mmHg	115.6 (12.2)
Diastolic blood pressure, mmHg	68.1 (9.5)
Parity ≥1, %	43.9
Ethnicity, %	
European	61.1
Non -European	38.9
Highest education, %	
Primary or secondary school	54.7
Higher education	45.3
Smoking, %	
Non-smoking	74.9
First trimester only	8.6
Continued smoking	16.4
Total daily energy intake, kcal	2046 (557)
Daily fat intake, %	36.2 (5.0)
Daily protein intake, %	14.9 (2.6)
Daily carbohydrate intake, %	48.6 (5.8)
Folic acid supplement use, %	
No	27.0
Preconceptional	41.8
Postconceptional	31.2
Infant characteristics	
Gestational age, weeks	39.8 (1.8)
Birth weight, g	3427.7 (554.4)
Gender, %	
Male	50.4
Female	49.6
Preterm birth, %	6.0

Table 1. Subject characteristics (n = 6,368) (continued)

	Participants
Small size for gestational age, % <5th percentile	6.0
Large size for gestational age, % >95th percentile	4.4
Breastfeeding, %	
No	8.0
Yes	92.0
Child characteristics	
Age, years	6.1 (0.5)
Height, cm	119.5 (6.1)
Weight, kg	23.3 (4.3)
Body mass index, kg/m^2	16.2 (1.9)
Kidney volume left, mm^3	61.3 (13.3)
Kidney volume right, mm^3	59.0 (12.2)
Kidney volume combined, mm^3	120.3 (23.5)
Systolic blood pressure, mmHg	102.7 (8.2)
Diastolic blood pressure, mmHg	60.7 (6.9)
Creatinine, µmol/l	37.2 (5.6)
Cystatin C, µg/l	780 (8.0)
GFR, ml/min per 1.73m^2	118.8 (16.4)

Values are means (standard deviation) or number (%), GFR, glomerular filtration rate.

Maternal characteristics and kidney volume, kidney function and blood pressure

Table 2 shows that in the fully adjusted regression model, maternal anthropometrics are positively associated with kidney volume and glomerular filtration rate. Each SD increase in maternal height was associated with an increase in combined kidney volume of 2.63 mm^3 and an increase of 0.92 ml/min per 1.73m^2 in glomerular filtration rate in childhood, respectively. Similarly maternal systolic blood pressure was positively associated with combined kidney volume and childhood systolic and diastolic blood pressure.

Not first trimester only, but continued maternal smoking during pregnancy was inversely associated with combined kidney volume (difference -1.98 mm^3 (95% CI -3.80 to –0.17)) and inversely associated with glomerular filtration rate (difference -1.82 ml/min per 1.73m^2 (95% CI -3.45 to -0.19)). We did not observe a significant association of continued maternal smoking and blood pressure at the age of 6 years. Folic acid supplement use was not associated with childhood kidney size, kidney function and childhood blood pressure. Results from the crude model and model adjusted for only confounders are shown in Supplementary material (Tables S2 and S3).

Table 2. Associations of maternal life style characteristics with childhood kidney volume, kidney function and blood pressure

	Difference (95%) in kidney size			Difference (95%) kidney function			Difference (95%) blood pressure	
Maternal characteristics	**Left kidney volume (mm^3)**	**Right kidney volume (mm^3)**	**Combined kidney volume (mm^3)**	**Creatinine (µmol/l)**	**Cystatin C (mg/l)**	**GFR (ml/min per 1.73m^2)**	**Systolic blood pressure (mmHg)**	**Diastolic blood pressure (mmHg)**
Height	1.42**	1.20**	2.63**	0.36**	1.0	0.92**	0.22	-0.01
N = 5758 (1SD = 7.4)	(1.04 to1.80)	(01.54)	(1.97 to 3.28)	(0.16 to 0.56)	(-2.0 to 4.0)	(0.31 to 1.52)	(-0.02 to 0.47)	(-0.22 to 0.20)
Pre-pregnancy weight	0.51**	0.28	0.80*	0.07	1.0	0.56	0.32*	0.04
N = 4768 (1SD = 12.6)	(0.13 to 0.88)	(-0.06 to 0.63)	(0.15 to 1.46)	(-0.13 to 0.27)	(-2.0 to 4.0)	(-0.04 to 1.16)	(0.07 to 0.56)	(-0.17 to 0.25)
Systolic blood pressure	0.54**	0.34	0.89**	0.12	0.0	-0.19	0.90**	0.45**
N = 5724 (1SD = 12.2)	(0.16 to 0.92)	(-0.01 to 0.69)	(0.23 to 1.55)	(-0.08 to 0.32)	(-3.0 to 4.0)	(-0.78 to 0.41)	(0.65 to 1.14)	(0.24 to 0.66)
Diastolic blood pressure	-0.03	0.09	0.09	0.05	1.0	-0.02	0.87**	0.66**
N = 5724 (1SD = 9.5)	(-0.41 to 0.36)	(-0.26 to 0.44)	(-0.58 to 0.75)	(-0.15 to 0.25)	(-2.0 to 4.0)	(-0.62 to 0.58)	(0.63 to 1.12)	(0.45 to 0.88)
Total daily calorie intake	0.38	0.33	0.73*	0.07	0.0	-0.16	0.03	0.04
N = 4445 (1SD = 557.4)	(-0.04 to 0.80)	(-0.06 to 0.71)	(0.00 to 1.45)	(-0.14 to 0.29)	(-4.0 to 3.0)	(-0.81 to 0.48)	(-0.23 to 0.30)	(-0.19 to 0.27)
Smoking								
N = 5524								
Non	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
First trimester	-0.25	-0.35	-0.63	0.24	-3.0	0.06	-0.10	0.30
	(-1.54 to 1.05)	(-1.53 to 0.83)	(-2.86 to 1.60)	(-0.11 to 0.58)	(-14.0 to 7.0)	(-1.98 to 2.10)	(-0.93 to 0.73)	(-0.41 to 1.02)
Continued	-0.92	-1.02*	-1.98*	0.43	10.0*	-1.82*	-0.34	0.38
	(-1.97 to 0.13)	(-1,98 to -0.07)	(-3.80 to -0.17)	(-0.12 to 0.97)	(0.002 to 0.018)	(-3.45 to -0.19)	(-1.01 to 0.34)	(-0.20 to 0.96)
Folic acid supplement use								
N = 4493								
Non	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Preconceptional	1.05	0.85	1.95	0.27	0.005	-0.86	-0.69	-0.25
	(-0.12 to 2.21)	(-0.21 to 1.91)	(-0.06 to 3.96)	(-0.32 to 0.87)	(-0.004 to 0.015)	(-2.66 to 0.95)	(-1.44 to -0.06)	(-0.90 to 0.39)
Postconceptional	-0.22	-0.02	-0.21	0.33	0.002	-0.99	-0.86*	-0.84**
	(-1.36 to 0.92)	(-1.06 to1.01)	(-2.17 to 1.76)	(-0.25 to 0.92)	(0.007 to 0.011)	(-2.6 to 0.79)	(-1.59 to 0.13)	(1.47 to -0.21)

Values reflect the difference (95% confidence interval) in outcome values based on multiple regression models. All models included maternal age, BMI, parity, ethnicity educational level, smoking, total daily calorie intake and alcohol consumption during pregnancy, and child birth weight, gestational age, sex, breastfeeding and current age and body mass index.
*P<0.05, **P<0.01.

Birth outcomes and kidney volume, kidney function and blood pressure

Table 3 shows that as compared to term born children, preterm born children had a smaller combined kidney volume at the age of 6 years (difference 6.81 mm^3 (95% confidence interval 9.73 to 3.89). The linear trend analyses showed that a 1 SD longer duration of gestational age was associated with a larger combined kidney volume (1.27 mm^3 (95% CI 0.60 to 1.93), higher glomerular filtration rate (0.70 ml/min per 1.73m^2 (95% CI 0.09 1.30)), and lower systolic blood pressure (0.36 mmHg (95% CI 0.60 to 0.12)). Birth weight was positively associated with combined kidney volume and glomerular filtration rate, but inversely associated with systolic and diastolic blood pressure in childhood (p-values for trend <0.05). As compared to children born with an appropriate size for gestational age at birth, those born with a small size for gestational age at birth had a smaller childhood kidney size (difference -7.97 mm^3 (95% CI -10.58 to -5.36)). Size at birth also was associated with glomerular filtration rate and blood pressure in childhood (p-values < 0.05). Results from models only adjusted for sex and age are given in the Supplementary material (Table S4).

Kidney dimensions and kidney function and blood pressure

Overall, all kidney dimensions are inversely associated with creatinine and cystatin C levels and positively associated with glomerular filtration rate. Glomerular filtration rate increased by 4.82 ml/min per 1.73m (95%CI 4.20 to 5.44) per 1 SD increase in combined kidney volume. Also, combined kidney volume was positively associated with a higher systolic blood pressure (increase per 1 SD 0.44 mmHg (95% CI 0.17 to 0.70).

Discussion

Results from this population-based prospective cohort study from early fetal life onwards showed that several maternal characteristics, preterm birth and small size for gestational age at birth are associated with smaller kidneys, and lower glomerular filtration rate at the age of six years. Smaller kidney size is associated with lower glomerular filtration and lower blood pressure in childhood.

Methodological considerations

A major strength of our study is its prospective design from fetal life onwards within a large population-based cohort. Our analyses were based on more than 6,000 children with kidney growth, kidney function and blood pressure measurements available. Of the children who participated in the Generation R Study at age 6 years, more than 75% did participate in the kidney follow up studies. Overall, children and their mothers who did not visit the research center for follow-up measurements did more frequently

Table 3. Associations of birth outcomes with kidney volume, kidney function and blood pressure

	Difference (95%) in kidney size			Difference (95%) kidney function			Difference (95%) blood pressure	
Birth characteristics	**Left kidney volume (mm^3)**	**Right kidney volume (mm^3)**	**Combined kidney volume (mm^3)**	**Creatinine (µmol/l)**	**Cystatin C (mg/l)**	**GFR (ml/min per 1.73m^2)**	**Systolic blood pressure (mmHg)**	**Diastolic blood pressure (mmHg)**
Gestational age N = 6,368								
<37.0 weeks	-3.40**	-3.71**	-6.81**	-0.13	-7.0	-0.80	0.20	0.17
N = 357	(-5.09 to -1.71)	(-5.25 to -2.18)	(-9.73 to -3.89)	(-1.01 to 0.74)	(-21.0 to 6.0)	(-3.44 to 1.84)	(-0.87 to 1.27)	(-0.76 to 1.10)
37.0-41.9 weeks	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
N = 5,577								
>=42.0 weeks	0.06	0.29	0.44	-0.95*	0.0	2.70*	-0.74	-0.47
N = 434	(-1.34 to 1.46)	(-1.00 to 1.57)	(-1.99 to 2.86)	(-1.68 to -0.22)	(-11.0 to 11.0)	(0.50 to 4.90)	(-1.63 to 0.15)	(-1.24 to 0.31)
Trend (SDS)	0.59	0.71	1.27	-0.15	0.0	0.70	-0.36	-0.11
	(0.21 to 0.98)	(0.36 to 1.06)	(0.60 to 1.93)	(-0.35 to 0.06)	(-3.0 to 3.0)	(0.09 to 1.30)	(-0.60 to -0.12)	(-0.32 to 0.10)
P value	0.002	< 0.001	< 0.001	0.15	0.84	0.02	0.004	0.29
Birth weight N = 6,368								
<2,000 grams	-5.69**	-4.20*	-9.88**	2.27*	-9.0	-8.23*	2.50*	1.65
N = 77	(-9.32 to -2.06)	(-7.52 to -0.88)	(-16.15 to -3.61)	(0.16 to 4.37)	(-41.0 to 24.0)	(-14.42 to -2.04)	(1.52 to 2.03)	(-0.34 to 3.64)
2,000-2,499 grams	-3.85**	-3.70**	-7.43**	-1.07	-6.0	0.74	-0.79	-0.09
N = 206	(-5.93 to -1.77)	(-5.60 to -1.80)	(-11.02 to 3.83)	(-2.16 to 0.03)	(-23.0 to 11.0)	(-2.55 to 4.03)	(-2.14 to 0.56)	(-1.25 to 1.08)
2,500-2,999 grams	-2.44**	-1.93**	-4.34**	-0.00	-3.0	-1.58	0.04	0.37
N = 930	(-3.55 to -1.33)	(-2.95 to -0.91)	(-6.27 to -2.41)	(-0.60 to 0.59)	(-12.0 to 6.0)	(-3.37 to 0.21)	(-0.68 to 0.77)	(-0.25 to 1.00)
3,000-3,499 grams	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Referenc*
N = 2,203								
3,500-3,999 grams	0.84	1.34**	2.13**	-0.17	-7.0*	1.09	-0.88**	-0.57*
N = 2,046	(-0.04 to 1.72)	(0.53 to 2.14)	(0.61 to 3.64)	(-0.63 to 0.28)	(-14.0 to 0.0)	(-0.28 to 2.46)	(-1.44 to -0.31)	(-1.06 to -0.09)
4,000-4,499 grams	2.03**	2.59**	4.49**	0.16	-9.0	1.35	-0.89*	-0.53
N = 745	(0.81 to 3.25)	(1.48 to 3.71)	(2.38 to 6.60)	(-0.47 to 0.79)	(-18.0 to 1.0)	(-0.53 to 3.23)	(-1.67 to -0.11)	(-1.21 to 0.14)
≥4,500 grams	3.91**	5.94**	9.44**	-0.02	-11.0	2.38	-1.75*	-0.99
N = 161	(1.45 to 6.38)	(3.67 to 8.22)	(5.12 to 13.76)	(-1.35 to 1.31)	(-31.0 to 9.0)	(-1.61 to 6.38)	(-3.34 to -0.15)	(-2.36 to 0.39)

Table 3. Associations of birth outcomes with kidney volume, kidney function and blood pressure (continued)

	Difference (95%) in kidney size			Difference (95%) kidney function			Difference (95%) blood pressure	
Birth characteristics	**Left kidney volume (mm^3)**	**Right kidney volume (mm^3)**	**Combined kidney volume (mm^3)**	**Creatinine (µmol/l)**	**Cystatin C (mg/l)**	**GFR (ml/min per 1.73m^2)**	**Systolic blood pressure (mmHg)**	**Diastolic blood pressure (mmHg)**
Trend (SDS)	1.64	1.86	3.43	0.02	-3.0	1.04	-0.42	-0.34
P value	(1.25 to 2.02)	(1.51 to 2.22)	(2.76 to 4.09)	(-0.18 to 0.23)	(-6.0 to 0.0)	(0.42 to 1.65)	(-0.67 to -0.17)	(-0.55 to -0.12)
	< 0.001	< 0.001	< 0.001	0.83	0.08	0.001	0.001	0.002
Birth weight for gestational age N = 6,368								
Small for gestational age N = 385	-3.99** (-5.49 to -2.48)	-4.04** (-5.42 to -2.66)	-7.97** (-10.58 to -5.36)	-0.27 (-1.09 to 0.55)	-1.0 (-14.0 to 11.0)	-1.85 (-4.32 to 0.61)	0.65 (-0.32 to 1.62)	0.83 (-0.01 to 1.67)
Normal for gestational age N = 5,641	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Large for gestational age N = 282	0.19 (-1.20 to 1.59)	0.45 (-0.83 to 1.73)	0.71 (-1.70 to 3.13)	-0.95* (-1.68 to -0.22)	0.0 (-11.0 to 11.0)	2.74* (0.54 to 4.93)	-0.74 (-1.63 to 0.15)	-0.47 (-1.24 to 0.30)
Trend (SDS)	1.54	…1.72	3.20	0.11	-3.0	0.80	…0.31	…-0.34
P value	(1.16 to 1.92)	(1.37 to 2.06)	(2.54 to 3.85)	(-0.09 to 0.31)	(-6.0 to 0.0)	(0.21 to 1.40)	(-0.55 to 0.06)	(-0.55 to -0.13)
	< 0.001	< 0.001	< 0.001	0.27…	0.05	0.008	0.014	0.001
						…		

Values are regression coefficients (95% CI) based on multiple regression models and reflect the difference for each outcome for the birth weight or gestational age group, as compared to the reference group. Models are adjusted for maternal age, BMI, parity, ethnicity, educational level, smoking, total daily calorie intake and alcohol consumption during pregnancy, and child birthweight, gestational age, sex, breastfeeding and current age and body mass index.

*P<0.05

**P<0.01.

smoke during pregnancy and were less educated than the total sample. Of all children who visited the research center at age of six years, 95% provided useful blood pressure or kidney measurements. Selective follow up might have led to selection bias if the investigated associations would be different in those included and not included in the analysis. This seems unlikely but cannot be excluded. In total, 67% of all children provided useful blood samples for measurements of creatinine and cystatin C levels, since not all participants in the study gave consent for collecting blood samples. There were no differences in maternal and birth characteristics between children with and without blood samples. However, children without blood samples had smaller kidney dimensions and higher blood pressure at the age of six years. These differences might have led to and underestimation of the associations between kidney dimensions and kidney function. Furthermore, statistical power might have been reduced due to the missing data. We used well described and validated measurements to measure kidney size and function. Currently, no precise measurement of nephron number is possible in vivo. We used kidney size as measure of kidney development. Kidney size seems to be correlated with the number of glomeruli and can be used in epidemiological studies as measure of kidney development. Postmortem studies in humans showed that a lower nephron number is associated with low birth weight and hypertension[10, 33]. A recent study showed an association between newborn kidney volume and nephron number in fifteen infants, who died before three months of age, in whom an ultrasound was performed in the first two days of life. There was a strong relationship between kidney mass and nephron number[11]. This association is supported by study by Hinchliffe et al. demonstrating a strong correlation between renal volume and glomerular number up to 40 weeks of gestation, in eleven spontaneously aborted fetuses[34]. Several other post-mortem studies in humans, who died in the perinatal period, showed consistent associations between renal size and glomerular number[35-36]. Therefore, kidney volume seems to be a good surrogate for nephron number. However, it could be that glomerular enlargement due to glomerulosclerosis attenuates the differences in kidney volume and therefore might lead to an underestimation of the associations. Furthermore, differences might be smaller and therefore more difficult to detect. This could have affected the power of our study to establish associations. Finally, although we had information about a large number of potential confounders, residual confounding might still be an issue due to the observational design of the study.

Early life and kidney growth and function.

Several studies showed associations of low birth weight with renal disease and hypertension in later life[1-3]. The underlying mechanisms for these associations are not exactly known. It has been hypothesized that early life adverse exposures impact fetal programming and increases the risk of chronic disease in adult life[4, 37]. More specifically, adverse

fetal exposures in early life may lead to reduced congenital nephron number. This reduction may lead to glomerular hyperfiltration and subsequent glomerulosclerosis, which on long term might lead to impaired renal function and hypertension[5-6]. This hypothesis is supported by animal and human studies showing associations between low birth weight and nephron number[36, 38-40]. Also, a post mortem study showed that patients with hypertension had significantly fewer but greater glomeruli per kidney than non-hypertensive subjects[10]. The associations of low birth weight with kidney function and blood pressure are well-established but the effect estimates seem small[1, 3].

Maternal life style related characteristics might lead to adverse fetal environment and subsequently to renal developmental adaptations, which might have long term consequences. We observed that maternal height and pre-pregnancy weight, daily calorie intake and maternal folic acid use during pregnancy were associated with larger kidney size and lower glomerular filtration rate. We also observed that maternal continued smoking during pregnancy was associated with smaller kidney volume and lower kidney function. In the models adjusted for potential confounders, maternal smoking during pregnancy was not associated with diastolic blood pressure. Previous studies indicated an association between smoking and kidney volume and blood pressure with inconsistent results[12, 41-43]. Recently, large cohort study in young Swedish men showed a small significant increase in systolic and diastolic blood pressure in subjects exposed to maternal smoking during pregnancy[44]. Further research is needed to provide insight in the consequences of maternal smoking on blood pressure and kidney function in later life.

A systematic overview in 2009 indicated that individuals born with low birth weight have a 70% increased risk of getting chronic kidney disease in later life[1]. In our study, birth weight was positively associated with kidney estimated glomerular filtration rate. Especially children with a birth weight lower than 2,000 grams had significantly lower kidney function compared to children with a normal weight. Rather than birth weight or gestational age per se, size at birth for gestational age may be a better reflection of intra-uterine growth restriction. A Norwegian study showed that young adults born with a small size for gestational age had an increased risk of low-normal creatinine clearance compared with children with appropriate birth weight for gestational age[15]. This is in line with our findings showing that children born with a small size for gestational age children had smaller kidneys and impaired kidney function compared to children born appropriate size for gestational age. A Dutch study in severely growth restricted children did not show associations of birth weight with renal function in young adults[9].

Not much is known about the associations of kidney volume with kidney function. A recent study, which included 257 healthy control subjects, indicated that renal mass was negatively associated with creatinine levels in serum[45]. In line with this study, we observed strong negative associations of kidney dimensions with creatinine, cystatin C

and positive associations with glomerular filtration rate, which is in line with the hyperfiltration hypothesis.

We hypothesized that smaller kidneys are associated with higher blood pressure, but observed a positive association. Di Zazzo et al found no consistent association between kidney size and systolic and diastolic blood pressure. It might be that our association might be explained by residual confounding ofchild body composition.

The associations between adverse fetal exposures and blood pressure in later life might partly be explained by adaptations of kidney development. However, also other pathways and mechanism may underlie these associations, which may include the renin-angiotensin-system, influences on arterial stiffness and fetal myocyte development. Future studies are needed to investigate possible underlying mechanisms and identify possible targets for intervention.

Conclusions

The present study suggests that maternal life style related characteristics influence childhood kidney volume and impaired kidney function. Also, birth weight, gestational age and size for gestational age at birth are associated with kidney growth, kidney function and blood pressure at the age of six years. Furthermore, kidney volume, as a marker of congenital nephron endowment, is associated with kidney function, which is in line with the hyperfiltration hypothesis. Surprisingly, we found a positive association of kidney volume with systolic blood pressure. Further studies focused on the underlying mechanisms of these associations and the influence on risk of developing hypertension and kidney disease are needed. The associations between kidney volume and kidney function in the present study, implicate that kidney volume might be a predictor of kidney function in later life. Future studies should focus on determinants, for example genetic and epigenetic influences, of development of kidney dimensions.

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Supplements

Table S1. Subject characteristics.

	Observed data	Imputed data
Maternal characteristics		
Age (years)	30.5 (5.2)	30.5 (5.2)
Height (cm)	167.5 (7.4)	167.5 (7.4)
Pre-pregnancy weight (kg)	66.5 (12.6)	67.4 (17.4)
Pre-pregnancy body mass index (kg/m2)	23.6 (4.2)	24.0 (6.1)
Weight gain during pregnancy (kg)	7.4 (3.9)	7.4 (3.8)
Systolic blood pressure (mmHg)	115.6 (12.2)	115.6 (12.2)
Diastolic blood pressure (mmHg)	68.1 (9.5)	68.1 (9.5)
Parity ≥1 (%)	42.2	43.9
Missing	3.3	
Ethnicity (%)		
European	60.0	61.1
Non -European	37.4	38.9
Missing	2.4	
Highest education (%)		
Primary or secondary school	48.5	54.7
Higher education	42.5	45.3
Missing	9.1	
Smoking (%)		
Non-smoking	64.8	74.9
First trimester only	7.7	8.6
Continued smoking	14.2	16.4
Missing	13.3	
Alcohol use during pregnancy (%)		
Non	36.6	46.4
First trimester only	10.9	13.3
Continued	32.1	40.3
Missing	20.4	
Total daily energy intake (kcal)	2046.7 (557.4)	2027.3 (561.9)
Daily fat intake (%)	36.2 (5.7)	36.2 (5.0)
Daily protein intake (%)	14.9 (2.6)	14.9 (2.6)
Daily carbohydrate intake (%)	48.7 (6.5)	48.6 (5.8)
Folic acid supplement use (%)		
No	17.4	27.0
Preconceptional	29.7	41.8
Postconceptional	21.9	31.2
Missing	31.0	
Infant characteristics		
Gestational age (weeks)	39.8 (1.8)	39.8 (1.8)
Birth weight (g)	3427.9 (553.8)	3427.7 (554.4)
Gender (%)		
Male	50.4	50.4
Female	49.6	49.6
Missing	0.0	

Preterm birth (%)	6.0		6.0
Missing	1.5		
Small size for gestational age, % <5th percentile		6.0	6.0
Large size for gestational age, % >95th percentile		4.4	4.4
No	5.9		8.0
Yes	71.9		92.0
Missing	21.2		
Child characteristics			
Age (years)	6.1 (0.5)		6.1 (0.5)
Height (cm)	119.5 (6.1)		119.5 (6.1)
Weight (kg)	23.3 (4.3)		23.3 (4.3)
Body mass index (kg/m2)	16.2 (1.9)		16.2 (1.9)
Kidney volume left (mm3)	61.3 (13.3)		
Kidney volume right (mm3)	59.0 (12.2)		
Kidney volume combined (mm3)	120.3 (23.5)		
Systolic blood pressure (mmHg)	102.7 (8.2)		
Diastolic blood pressure (mmHg)	60.7 (6.9)		
Creatinine (µmol/l)	37.5 (6.1)		
Cystatin C (mg/l)	78.0 (8.0)		
GFR (ml/min per 1.73m^2)	118.80 (16.41)		

Values are means (standard deviation)

Missing values for continuous variables are for maternal characteristics: age (n = 0), height (n = 610), pre-pregnancy weight (n = 1600), pre-pregnancy body mass index (n = 1609), weight gain during pregnancy (n = 834), systolic blood pressure (n = 644), diastolic blood pressure (n = 644), total daily energy intake (n = 1923), daily fat intake (n = 1923), daily carbohydrate intake (n = 1923), daily protein intake (n = 1923), for infant characteristics: gestational age (n = 46), birth weight (n = 12), for child characteristics: age (n = 0), height (n = 8), weight (n = 8), body mass index (n = 8)

Table S2. Associations of maternal life style characteristics with childhood kidney volume, kidney function and blood pressure according to the confounded model (adjusted for sex and current age)

	Difference (95%) in kidney size			Difference (95%) kidney function			Difference (95%) blood pressure	
Maternal characteristics	**Left kidney volume (mm^3)**	**Right kidney volume (mm^3)**	**Combined kidney volume (mm^3)**	**Creatinine (µmol/l)**	**Cystatin C (µg/l)**	**GFR (ml/min per 1.73m^2)**	**Systolic blood pressure (mmHg)**	**Diastolic blood pressure (mmHg)**
Height	1.49**	1.44**	2.93**	0.31**	1.0	0.97**	-0.18	-0.37**
N = 5758 (1SD = 7.4)	(1.51 to 1.84)	(1.13 to 1.75)	(2.33 to 3.54)	(0.14 to 0.48)	(-2.0 to 3.0)	(0.46 to 1.48)	(--0.39 to 0.04)	(-0.55 to -0.19)
Pre-pregnancy weight	1.58**	1.46**	3.03**	0.22*	2.0	0.51	0.67**	0.07
N = 4768 (1SD = 12.6)	(1.21 to 1.96)	(1.11 to 1.80)	(2.37 to 3.69)	(0.03 to 0.40)	(-1.0 to 5.0)	(-0.05 to 1.07)	(0.43 to 0.90)	(-0.13 to 0.26)
Systolic blood pressure	0.87**	0.58**	1.47**	0.21*	2.0	-0.27	0.99**	0.43**
N = 5724 (1SD = 12.2)	(0.53 to 1.22)	(0.27 to 0.90)	(0.87 to 2.08)	(0.04 to 0.38)	(-1.0 to 4.0)	(-0.79 to 0.24)	(0.78 to 1.20)	(0.25 to 0.61)
Diastolic blood pressure	0.23	0.18	0.43	0.02	1.0	0.13	0.94**	0.63**
N = 5724 (1SD = 9.5)	(-0.12 to 0.57)	(-0.14 to 0.49)	(-0.18 to 1.04)	(-0.15 to 0.19)	(-1.0 to 4.0)	(-0.38 to 0.65)	(0.73 to 1.15)	(0.45 to 0.81)
Total daily calorie intake	0.54**	0.48*	1.04**	0.12	0.0	-0.17	-0.13	-0.13
N = 4445 (1SD = 557.4)	(0.15 to 0.94)	(0.11 to 0.84)	(0.35 to 1.74)	(-0.07 to 0.31)	(-3.0 to 3.0)	(-0.75 to 0.41)	(-0.37 to 0.12)	(-0.33 to 0.08)
Smoking								
N = 5524								
Non	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
First trimester	-0.03 (-1.61 to 0.31)	-0.19 (-1.34 to 0.95)	-0.29 (-2.48 to 1.90)	0.24 (-0.11 to 0.58)	-3.0 (-12.0 to 7.0)	-0.21 (-2.07 to 1.64)	0.04 (-0.73 to 0.81	0.18 (-0.47 to 0.82)
Continued	-0.65 (-1.61 to 0.31)	-1.15* (-2.03 to -0.27)	-1.87* (-3.66 to -0.18)	0.75** (0.27 to 1.22)	12.0** (5.0 to 19.0)	-2.76** (-4.20 to -1.32)	0.77* (0.17 to 1.36)	0.87** (0.37 to 1.37)
Folic acid supplement use								
N = 4493								
Non	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Preconceptional	0.29 (-0.72 to 1.30)	0.18 (-0.79 to 1.14)	1.03 (-0.74 to 2.80)	-0.05 (-0.54 to 0.44)	5.0 (-3.0 to 13.0)	0.36 (-1.14 to 1.85)	-1.44** (-2.07 to -0.82)	-0.93** (-1.46 to -0.40)
Postconceptional	-0.33 (-1.38 to 0.73)	0.64 (-0.28 to 1.56)	-0.06 (-1.91 to 1.80)	0.09 (-0.43 to 0.60)	0.0 (-8.0 to 8.0)	0.00 (-1.57 to 1.57)	-1.18** (-1.84 to -0.53)	-1.11** (-1.66 to -0.55)

Values are regression coefficients (95% CI) based on multiple regression models and reflect the difference for each outcome for the birth weight or gestational age group, as compared to the reference group. Models are adjusted for sex and current age.

*P<0.05

**P<0.01

Table S3. Associations of maternal life style characteristics with childhood kidney volume, kidney function and blood pressure according to the crude model (adjusted for sex, current age and covariates)

	Difference (95%) in kidney size			Difference (95%) kidney function			Difference (95%) blood pressure	
Maternal characteristics	**Left kidney volume (mm^3)**	**Right kidney volume (mm^3)**	**Combined kidney volume (mm^3)**	**Creatinine (µmol/l)**	**Cystatin C (µg/l)**	**GFR (ml/min per 1.73m^2)**	**Systolic blood pressure (mmHg)**	**Diastolic blood pressure (mmHg)**
Height	1.76**	1.59**	3.33**	0.34**	0.0	1.16**	0.11	-0.10
N = 5758 (1SD = 7.4)	(1.38 to 2.13)	(1.25 to 1.92)	(2.69 to 3.97)	(0.15 to 0.53)	(-3.0 to 3.0)	(0.58 to 1.75)	(-0.13 to 0.35)	(-0.30 to 0.11)
Pre-pregnancy weight	0.78**	0.60**	1.37**	0.08	0.0	0.72*	0.25*	-0.02
N = 4768 (1SD = 12.6)	(0.40 to 1.15)	(0.26 to 0.95)	(0.72 to 2.02)	9-0.12 to 0.28)	(-3.0 to 3.0)	(0.13 to 1.30)	(0.01 to 0.49)	(-0.23 to 0.19)
Systolic blood pressure	0.50*	0.29	0.80*	0.14	1.0	-0.24	0.92**	0.45**
N = 5724 (1SD = 12.2)	(0.12 to 0.89)	(-0.06 to 0.64)	(0.46 to 1.14)	(-0.06 to 0.34)	(-2.0 to 4.0)	(-0.83 to 0.36)	(0.68 to 1.16)	(0.24 to 0.66)
Diastolic blood pressure	-0.13	-0.02	-0.12	0.06	1.0	-0.10	0.90**	0.68**
N = 5724 (1SD = 9.5)	(-0.51 to 0.26)	(-0.38 to 0.34)	(-0.79 to 0.55)	(-0.14 to 0.26)	(-2.0 to 4.0)	(-0.70 to 0.50)	(0.66 to 1.15)	(0.47 to 0.89)
Total daily calorie intake	0.46*	0.41*	0.89*	0.09	0.0	-0.15	0.03	0.02
N = 4445 (1SD = 557.4)	(0.04 to 0.88)	(0.02 to 0.79)	(0.16 to 1.62)	(-0.12 to 0.30)	(-4.0 to 3.0)	(-0.79 to 0.50)	(-0.24 to 0.29)	(-0.21 to 0.25)
Smoking								
N = 5524								
Non	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
First trimester	-0.21	-0.32	-0.55	0.27	-4.0	0.01	-0.08	0.29
	(-1.51 to 1.10)	(-1.51 to 0.88)	(-2.81 to 1.70)	(-0.07 to 0.62)	(-14.0 to 7.0)	(-2.03 to 2.06)	(-0.91 to 0.75)	(-0.42 to 1.00)
Continued	-1.45**	-1.63**	-3.11**	0.41	11.0**	-2.14*	-0.21	0.50
	(-2.50 to-0.40)	(-2.59 to -0.67)	(-4.93 to -1.29)	(-0.13 to 0.96)	(3.0 to 19.0)	(-3.76 to -0.52)	(-0.88 to 0.46)	(-0.07 to 1.08)
Folic acid supplement use								
N = 4493								
Non	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Preconceptional	1.43*	1.30*	2.78**	0.27	5.0	-0.66	-0.76*	-0.33
	(0.26 to 2.60)	(0.23 to 2.36(	(0.76 to 4.80)	(-0.32 to 0.86)	(-4.0 to 14.0)	(-2.46 to 1.14)	(-1.50 to -0.01)	(-0.97 to 0.31)
Postconceptional	0.09	0.35	0.48	0.33	1.0	-0.82	-0.92*	-0.90**
	(-1.05 to 1.24)	(-0.69 to 1.40)	(-1.50 to 2.46)	(-0.26 to 0.91)	(-8.0 to 10.0)	(-2.59 to 0.95)	(-1.65 to -0.19)	(-1.53 to -0.27)

Values are regression coefficients (95% CI) based on multiple regression models and reflect the difference for each outcome for the birth weight or gestational age group, as compared to the reference group. Models are adjusted for maternal age, BMI, parity, ethnicity, educational level, smoking, total daily calorie intake and alcohol consumption during pregnancy, and child sex, breastfeeding and current age and body mass index.

*P<0.05, **P<0.01

Table S4. Associations of birth outcomes with kidney volume, kidney function and blood pressure

	Difference (95%) in kidney size			Difference (95%) kidney function			Difference (95%) blood pressure	
Birth characteristics	**Left kidney volume (mm^3)**	**Right kidney volume (mm^3)**	**Combined kidney volume (mm^3)**	**Creatinine (µmol/l)**	**Cystatin C (µg/l)**	**GFR (ml/min per 1.73m^2)**	**Systolic blood pressure (mmHg)**	**Diastolic blood pressure (mmHg)**
Gestational age								
N = 6,368								
<37.0 weeks	-3.16**	-3.44**	-6.35**	-0.11	-1.0	-1.08	0.59	0.24
N = 357	(-4.59 to -1.74)	(-4.75 to-2.13)	(-8.86 to -3.83)	(-0.81 to 0.60)	(-12.0 to 10.0)	(-3.23 to 1.06)	(-0.31 to 1.48)	(-0.50 to 0.98)
37.0-41.9 weeks	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
N = 5,577								
>=42.0 weeks	0.21	0.44	0.70	-0.88**	0.0	2.86**	-0.53	-0.46
N = 434	(-1.08 to 1.51)	(-0.75 to 1.63)	(-1.58 to 2.97)	(-1.52 to -0.24)	(-10.0 to 10.0)	(0.90 to 4.82)	(-1.34 to 0.27)	(-1.14 to 0.22
Trend (SDS)	0.78	0.86	1.63	-0.18	-1.0	0.88	-0.39	-0.17
	(0.46 to 1.11)	(0.56 to 1.16)	(1.05 to 2.20)	(-0.34 to -0.14)	(-4.0 to 1.0)	(0.38 to 1.38)	(-0.59 to -0.18)	(-0.34 to 0.00)
P value	< 0.001	< 0.001	< 0.001	0.03	0.27	0.001	< 0.001	0.05
Birth weight N = 6,368								
<2,000 grams	-6.00**	-5.12**	-10.89**	2.04*	11.0	-7.67**	1.81	1.05
N = 77	(-7.52 to -4.47)	(-7.78 to -2.47)	(-15.78 to -6.01)	(0.49 to 3.60)	(-13.0 to 34.0)	(-12.44 to -2.90)	(0.01 to 3.60)	(-0.44 to 2.53)
2,000-2,499 grams	-4.37**	-4.53**	-8.81**	-0.99*	1.0	0.29	-0.45	-0.23
N = 206	(-6.22 to 2.52)	(-6.22 to -2.83)	(-12.06 to -5.57)	(-1.93 to -0.05)	(-14.0 to 16.0)	(-2.60 to 3.17)	(-1.62 to 0.73)	(-1.22 to 0.76)
2,500-2,999 grams	-2.45**	-2.13**	-4.58**	-0.03	0.0	-1.66*	-0.09	0.31
N = 930	(-3.45 to -1.46)	(-3.05 to -1.22)	(-6.32 to -2.83)	(-0.54 to 0.47)	(-8.0 to 8.0)	(-3.21 to -0.12)	(-0.72 to 0.55)	(-0.22 to 0.84)
3,000-3,499 grams	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
N = 2,203								
3,500-3,999 grams	1.86**	2.27**	4.09**	-0.15	-5.0	1.28*	-0.67**	-0.73**
N = 2,046	(1.07 to 2.64)	(1.54 to 2.99)	(2.71 to 5.47)	(-0.55 to 0.24)	(-11.0 to 1.0)	(0.08 to 2.48)	(1.16 to -0.17)	(-1.15 to -0.31)

Table S4. Associations of birth outcomes with kidney volume, kidney function and blood pressure (continued)

	Difference (95%) in kidney size			Difference (95%) kidney function			Difference (95%) blood pressure	
Birth characteristics	**Left kidney volume (mm^3)**	**Right kidney volume (mm^3)**	**Combined kidney volume (mm^3)**	**Creatinine (µmol/l)**	**Cystatin C (µg/l)**	**GFR (ml/min per 1.73m^2)**	**Systolic blood pressure (mmHg)**	**Diastolic blood pressure (mmHg)**
4,000-4,499 grams	3.49**	3.97**	7.40**	0.16	-5.0	1.83*	-0.77*	-0.92**
N = 745	(2.40 to 4.58)	(2.97 to 4.97)	(5.89 to 9.31)	(-0.37 to 0.69)	(-13.0 to 4.0)	(0.20 to3.45)	(1.45 to -0.09)	(-1.50 to -0.35)
≥4,500 grams	5.82**	6.27**	11.71**	0.16	-7.0	2.10	-0.63	-0.96
N = 161	(3.65 to 7.98)	(4.26 to 8.27)	(7.88 to 15.54)	(-0.93 to 1.25)	(-24.0 to 10.0)	(-1.24 to 5.44)	(-2.00 to 0.74)	(-2.12 to 0.19)
Trend (SDS)	2.27		4.62	0.02	-3.0	1.20	-0.29	-0.40
	(1.94 to 2.59)	2.41	(4.05 to 5.19)	(-0.14 to 0.19)	(-5.0 to 0.0)	(0.70 to 1.70)	(-0.50 to -0.09)	(-0.57 to -0.23)
P value	< 0.001	(2.11 to 2.70)	< 0.001	0.77	0.02	< 0.001	0.005	< 0.001
		< 0.001						
Birth weight for gestational age								
N = 6,368								
Small for gestational age	-4.93**	-5.29**	-10.16**	-1.63	5.0	-2.80*	0.81	1.09**
N = 385	(-6.28 to -3.58)	(-6.53 to -4.05)	(-12.53 to -7.78)	(-0.86 to 0.54)	(-5.0 to 16.0)	(-4.94 to -0.66)	(-0.04 to 1.66)	(0.38 to 1.80)
Normal for gestational age	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
N = 5,641								
Large for gestational age	0.30	0.55	0.88	-0.92**	0.0	2.97**	-0.56	-0.49
N = 282	(-0.99 to 1.59)	(-0.64 to 1.73)	(-1.39 to 3.14)	(-1.56 to -0.28)	(-10.0 to 9.0)	(1.02 to 4.92)	(-1.37 to 0.24)	(1.17 to 0.19)
Trend (SDS)	2.34	2.21	4.51	0.13	-2.0	0.92	-0.11	-0.35
	(2.05 to 2.64)	(1.89 to 2.53)	(3.95 to 5.07)	(-0.03 to 0.30)	(-5.0 to 0.0)	(0.43 to 1.42)	(-0.31 to 0.10)	(-0.52 to -0.18)
P value	< 0.001	< 0.001	< 0.001	0.11	0.07	< 0.001	0.30	< 0.001

Values are regression coefficients (95% CI) based on multiple regression models and reflect the difference for each outcome for the birth weight or gestational age group, as compared to the reference group. Models are adjusted for child sex and current age.

*P<0.05

**P<0.01.

Chapter 4.4

Genetic variants associated with adult blood pressure and their relation with fetal kidney volume

H Rob Taal
Leontine CL van den Hil
Albert Hofman
Albert J van der Heijden
Vincent WV Jaddoe

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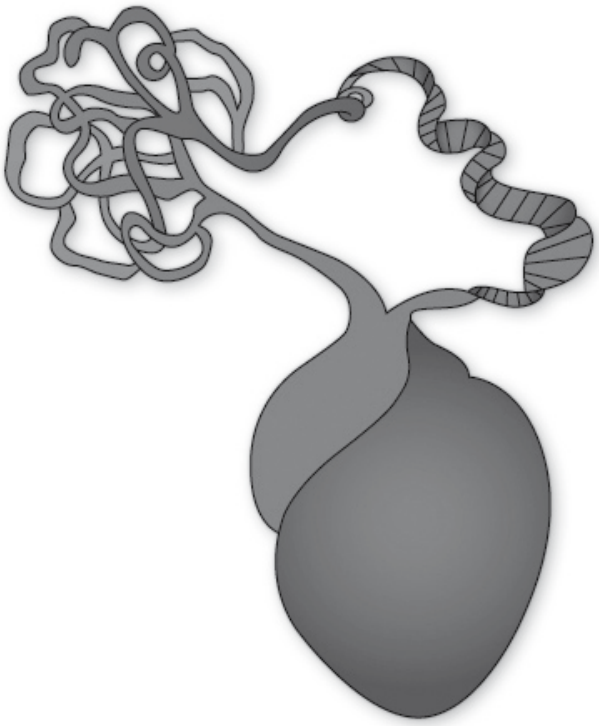

Abstract

Background: Smaller kidneys with reduced number of nephrons in early life lead to impaired kidney function and risk for hypertension and chronic kidney disease. These associations might be partly explained by common genetic variation. We aimed to assess the associations between common genetic variants, which have recently shown to be associated with blood pressure or kidney function, with fetal kidney volume.

Methods: In a prospective population based cohort study in Rotterdam, the Netherlands, we investigated among 855 children, followed from early fetal life onwards (born 2003 -2005), whether common genetic variants previously associated with blood pressure or kidney function, were associated with combined third trimester fetal kidney volume.

Results: After taking into account multiple testing, only rs12940887 (near *ZNF652*) was significantly associated with fetal kidney volume (β: 0.88 (95%CI: 0.40; 1.37) cm^3 per minor allele, p-value<0.001), but the effect showed the opposite direction as expected. The remaining common genetic variants were not associated with fetal kidney volume. We also did not find associations of genetic variants previously shown to affect newborn kidney volume, with third trimester fetal kidney volume.

Conclusions: Our results suggest that common genetic variants, associated with kidney function or disease and blood pressure, do not affect the third trimester fetal kidney volume. Further studies are needed to elucidate the mechanisms underlying the associations between small kidney size and increased risks of hypertension and impaired kidney function in adulthood.

Introduction

Many studies have shown associations of low birth weight with cardiovascular disease and chronic renal failure[1-2]. Low birth weight is also associated with impaired renal growth, raised blood pressure and impaired kidney function in later life[1-3]. The hyperfiltration hypothesis suggests that smaller kidneys with lower numbers of nephrons lead to hyperfiltration in the remnant nephrons, eventually resulting in glomerular sclerosis[4-5]. This may predispose the individual to renal damage and development of higher blood pressure, impaired kidney function and end stage kidney disease in adulthood[4]. A previous study showed associations of kidney size and low nephron number with hypertension[6]. Nephron number has been shown to vary widely between individuals, ranging from 250,000 to 2,000,000 nephrons per kidney[7-8]. A strong relationship between newborn kidney volume and nephron number was shown in fifteen infants who died before three months of age, in whom an ultrasound was performed in the first two days of life[9]. Several other post-mortem studies in humans, who died in the perinatal period, showed consistent associations between renal size and glomerular number[8, 10]. Therefore, kidney volume seems to be a valid marker for nephron number. Kidney growth and development is complex and influenced by many genetic and environmental factors[11-12]. Multiple genes are involved in kidney development, e.g. in regulating the branching process of the ureteric bud. Mutations in these genes are known to cause agenesis or dysgenesis of the kidney[11]. It seems likely that also common genetic variants account for part of the normal variation in nephron endowment. Thus far, only a few genetic variants have been shown to affect kidney volume[9, 13-14]. It might be that common genetic variants, previously associated with blood pressure or kidney function, are also associated with kidney volume. These common genetic variants are identified in genome wide association studies conducted in thousands of individuals and explain ~1-2% of the variation in these phenotypes[15-21].

We hypothesized that common genetic variants underlie part of the associations of smaller kidney size in early life, as marker for a lower nephron number, with higher blood pressure and impaired kidney function in later life. Therefore we assessed in a population-based cohort study among 855 subjects, the associations of 58 common genetic variants, previously shown to be related to blood pressure or kidney function in adult life, with fetal kidney volume. We expected that a blood pressure increasing allele or kidney function decreasing risk allele would be associated with a smaller kidney volume in early life. Also, we attempted to replicate the associations of four common genetic variants and one haplotype with fetal kidney volume[9, 13-14].

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood in Rotterdam, The Netherlands[22-23]. Detailed assessments of fetal and postnatal growth and development have been conducted in a randomly selected subgroup of Dutch children and their parents. Mothers, who were already participating during pregnancy, were asked to participate in additional detailed renal and cardiovascular measurements. These women were all enrolled before a gestational age of 24 weeks. In total 80% of the approached mothers were willing to participate in these additional studies. Fetal kidney ultrasounds were performed in the third trimester of pregnancy (median age: 30.4 weeks of gestation (90% range 28.8–32.1 weeks)). In total 1,232 women were enrolled in the subgroup cohort. Twin pregnancies (n=15) and pregnancies leading to perinatal death (n=2) were excluded from the analysis, leading to 1,215 singleton live births. No renal or ureterovesical anomalies other then mild pyelectasis over 10mm (n=3) were present in our study. Kidney ultrasounds were successfully performed in 95% (n=1,158) of these subjects. DNA was available in 855 (74%) of these subjects. The study was approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam. Written informed consent was obtained from all participants[22-23].

Genotyping

Cord blood for DNA isolation was available in 74% of all live-born participating children. Sex-mismatch rate between genome based sex and midwife-record based sex was low (<0.5%), indicating that possible contamination of maternal DNA was extremely low. Missing cord blood samples were mainly due to logistical constraints at the delivery. Individual genotype data were extracted from the genome-wide Illumina 610 Quad Array. If SNPs were not directly genotyped, we used MACH (version 1.0.15) software to impute genotypes using the HapMap II CEU (release 22) as reference set.

Selection of common genetic variants

The PubMed database was searched with 'genome wide association study', combined with 'blood pressure' or 'kidney function' as search criteria. We selected genome wide association studies, since these provide robust evidence of association, conducted in samples of European ancestry. Seven genome wide association studies were found; four on blood pressure and hypertension, three on kidney function and kidney disease[15-21]. If identical study populations were used in subsequent genome wide association studies, we selected common genetic variants from the genome wide association study with the largest sample size. Furthermore, we selected SNPs to assess in our study if the P-value

of the association was $<5.0x10^{-8}$. If SNPs were in high linkage disequilibrium (R^2 HapMap CEU ≥ 0.5) with each other, we selected the SNP with the strongest association reported, unless these SNPs were associated with different phenotypes. In total, 30 SNPs related to blood pressure and 28 SNPs related to kidney function or disease, were selected for this study. Next, the Pubmed database was searched with 'common variant' and 'kidney size' or 'kidney volume' as search criteria, to identify the studies which assessed associations between common genetic variants and kidney size or volume. We identified four studies investigating genes in the branching pathway; the PAX-gene, the RET-gene, the ALDH1A2-gene and the GDNF-gene. We selected SNPs in our study if the P-value of the association in previous studies was < 0.05[9, 13-14, 24].

Kidney measurements

Fetal left and right kidneys were measured in the third trimester of pregnancy with an ATL-Philips HDI 5000 instrument (Seattle, WA, USA) equipped with a 2.0–5.0 MHz curved array transducer. In a sagittal plane the maximum longitudinal kidney length was measured, with the callipers placed on the outer edges of the caudal and cranial sides. Antero-posterior (kidney width) and transverse (kidney depth diameters were measured perpendicular to each other, from the one outer edge to the other, in an axial plane. Values of maximum bipolar kidney length, width and depth were obtained from both the left and right kidney. Kidney width and depth were measured at the level of the kidney hilum. Fetal kidney volume was calculated, using the equation of an ellipsoid: volume (cm^3) = (0,523 x length (mm) x width (mm) x depth (mm))/1000. Left and right kidney volumes were added for the combined kidney volume (cm^3)[25-26]. Fetal growth characteristics (head circumference (HC), abdomen circumference (AC) and femur length were measured at the same visit, and fetal weight was estimated[27]. Two well-trained, experienced sonographers performed all measurements. Quality checks were frequently carried out and feedback was provided to minimize interoperator differences. Fetal growth measurements were shown to be measured reliably. The intra- and interobserver interclass correlation coefficients were all higher than 0.98, indicating good reproducibility[28]. The estimated fetal weight (EFW) is calculated by the formula: EFW (grams) =10 * (1.326-0.00326 *AC* FL+0.0107 *HC+0.0438 *AC+0.158 * FL)[29].

Statistical methods

Associations of common genetic variants and fetal kidney volume were assessed using linear regression, assuming an additive model. The model was adjusted for sex, gestational age at measurement and estimated fetal weight. We adjusted for these variables because of their relation with kidney volume[30]. The results did not differ materially between analyses with and without adjustment. In order to assess the combined effects of the common genetic variants, we calculated a risk allele score, by summing the risk

alleles (common genetic variants previously associated with higher adult blood pressure or impaired kidney function) per individual, and analyzed the association of the number of risk alleles with fetal kidney volume. To take into account multiple testing, we applied a Bonferroni correction and considered a P-value lower than $8.6x10^{-4}$ (0.05/58) as statistically significant. Controlling for the false discovery rate, a less conservative approach[31-32], did not change the results materially. All statistical analyses were performed using the Statistical Package for the Social Science version 17.0.2 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Table 1 presents the maternal and fetal subject characteristics, including all measured kidney characteristics. The combined kidney volume, as well as the relative kidney volume was higher in boys than in girls ($p<0.01$).

Table 1. Population characteristics

Maternal characteristics (n=855)			
Age			31.9 (21.9-39.4)
Height			171.1 (6.4)
Weight			71.4 (12.8)
Body mass index			23.4 (18.9-34.6)
Parity (%)	≥ 1		40.6
Fetal characteristics (n=855)			
sex (males %)			53.3
Gestational age at measurement			30.4 (28.5-32.7)
Estimated fetal weight (g)			1639 (268)
Right kidney structures			
		Length (mm)	39.0 (32.1-46.0)
		Width (mm)	23.0 (18.0-29.7)
		Dept (mm)	22.0 (17.0-28.0)
		Volume (cm^3)	10.3 (5.8-17.9)
Left kidney structures			
		Length (mm)	39.0 (32.7-47.0)
		Width (mm)	22.0 (17.0-28.0)
		Dept (mm)	21.0 (16.9-26.9)
		Volume (cm^3)	9.6 (5.4-16.1)
Combined kidney volume (cm^3)			20.6 (5.6)
Kidney volume/EFW (cm^3/kg)			12.7 (3.1)

Values are means (sd) or medians (95% range)

Estimated fetal weight is the estimated weight at measurement

Table 2. Common genetic variants known to be associated with blood pressure or kidney function and their association with fetal kidney volume

SNP	Chr.	Position	Minor Allele	Minor allele frequency	Gene (in/near)	Previously reported effect on blood pressure	Effect estimate for fetal kidney volume (cm^3)	P-value	Direction as expected
Blood pressure									
rs2932538	1	113018066	A	0.26	MOV10	SBP ↓; DBP ↓[21]	-0.38 (-0.89; 0.13)	0.15	No
rs17367504	1	11785365	G	0.15	MTHFR-NPPB	SBP ↓; DBP ↓; hypertension ↓[21]	0.12 (-0.52; 0.76)	0.71	Yes
rs13082711	3	27512913	C	0.23	SLC4A7	DBP ↑[21]	-0.52 (-1.08; 0.04)	0.07	Yes
rs3774372	3	41852418	C	0.15	ULK4	DBP ↑ [21]	-0.17 (-0.84; 0.50)	0.63	Yes
rs419076	3	170583580	T	0.47	MECOM	SBP ↑; DBP ↑[21]	-0.35 (-0.82; 0.11)	0.14	Yes
rs1458038	4	81383747	A	0.30	FGF5	SBP ↑ ; DBP ↑ [21]	0.43 (-0.07; 0.93)	0.09	No
rs13107325	4	103407732	T	0.05	SLC39A8	SBP ↓; DBP ↓[21]	0.79 (-0.47; 2.04)	0.22	Yes
rs13139571	4	156864963	A	0.22	GUCY1A3-GUCY1B3	DBP ↓[21]	0.36 (-0.28; 0.94)	0.22	Yes
rs1173771	5	32850785	A	0.41	NPR3-C5orf23	SBP ↓; DBP ↓; hypertension ↓[21]	-0.19 (-0.66; 0.27)	0.41	No
rs11953630	5	157777980	T	0.37	EBF1	SBP ↓; DBP ↓[21]	0.30 (-0.20; 0.79)	0.24	Yes
rs1799945	6	26199158	G	0.14	HFE	SBP ↑; DBP ↑; hypertension ↑[21]	-0.14 (-0.80; 0.53)	0.69	Yes
rs805303	6	31724345	A	0.38	BAT2-BAT5	SBP ↓; DBP ↓; hypertension ↓ [21]	0.10 (-0.38; 0.59)	0.68	Yes
rs4373814	10	18459978	C	0.42	CACNB2 (5')	SBP ↑; DBP ↑; hypertension ↑[21]	0.00 (-0.47; 0.47)	1.00	-
rs1813353	10	18747454	C	0.31	CACNB2 (3')	SBP ↓; DBP ↓; hypertension ↓[21]	-0.59 (-1.11; -0.07)	0.03	No
rs4590817	10	63137559	C	0.15	C10orf107	SBP ↓; DBP ↓; hypertension ↓[21]	0.20 (-0.45; 0.84)	0.55	Yes
rs932764	10	95885930	G	0.44	PLCE1	SBP ↑; hypertension ↑[21]	-0.35 (-0.82; 0.12)	0.15	Yes
rs11191548	10	104836168	C	0.08	CYP17A1-NT5C2	SBP ↓; DBP ↓[21]	0.47 (-0.44; 1.39)	0.31	Yes
rs381815	11	16858844	T	0.24	PLEKHA7	SBP ↑; DBP ↑ [21]	-0.34 (-0.88; 0.20)	0.22	Yes
rs7129220	11	10307114	A	0.09	ADM	SBP ↑; DBP ↑[21]	-0.59 (-1.39; 0.20)	0.14	Yes
rs633185	11	100098748	G	0.28	FLJ32810-TMEM133	SBP ↓; DBP ↓; hypertension ↓[21]	-0.03 (-0.52; 0.47)	0.99	No
rs3184504	12	110368991	T	0.47	SH2B3	SBP ↑; DBP ↑ [17, 21]	-0.19 (-0.66; 0.29)	0.44	Yes
rs10850411	12	113872179	C	0.31	TBX5-TBX3	SBP ↓; DBP ↓[21]	-0.10 (-0.59; 0.39)	0.68	No
rs17249754	12	88584717	A	0.16	ATP2B1	SBP ↓; DBP ↓; hypertension ↓[21]	-0.64 (-1.28; 0.00)	0.05	No
rs1378942	15	72864420	C	0.35	CYP1A1-ULK3	SBP ↑; DBP ↑ [21]	0.15 (-0.34; 0.64)	0.54	No
rs2521501	15	89238392	T	0.31	FURIN-FES	SBP ↑; DBP ↑[21]	-0.44 (-0.94; 0.05)	0.08	Yes
rs13333226	16	20273155	G	0.18	UMOD	Hypertension ↓[15]	-0.03 (-0.66; 0.59)	0.92	No
rs17608766	17	42368270	C	0.15	GOSR2	SBP ↑[21]	-0.20 (-0.84; 0.45)	0.55	Yes

Table 2. Common genetic variants known to be associated with blood pressure or kidney function and their association with fetal kidney volume (continued)

SNP	Chr.	Position	Minor Allele	Minor allele frequency	Gene (in/near)	Previously reported effect on blood pressure	Effect estimate for fetal kidney volume (cm^3)	P-value	Direction as expected
Blood pressure									
rs12940887	17	44757806	T	0.38	ZNF652	SBP ↑; DBP ↑ [21]	0.88 (0.40; 1.37)	<0.001	No
rs1327235	20	10917030	G	0.46	JAG1	DBP ↑ [21]	0.13 (-0.34; 0.60)	0.58	No
rs6015450	20	57184512	G	0.12	GNAS-EDN3	SBP ↑; DBP ↑; hypertension ↑ [21]	-0.51 (-1.18; 0.17)	0.14	Yes
Kidney function									
rs267734	1	149218101	C	0.21	ANXA9	eGFRcrea ↑ [19]	0.29 (-0.29; 0.86)	0.33	Yes
rs7422339	2	211248752	A	0.32	CPS1	eGFRcrea ↓ [19]	0.41 (-0.08; 0.90)	0.10	No
rs1260326	2	27584444	T	0.39	GCKR	eGFRcrea ↑ [19]	0.17 (-0.31; 0.64)	0.50	Yes
rs13538	2	73721836	G	0.21	NAT8-ALMS1	eGFRcrea ↑ [19]	0.17 (-0.41; 0.75)	0.56	Yes
rs10206899	2	73754408	C	0.21	NAT8-ALMS1	serum creat ↑ [16]	0.22 (-0.36; 0.80)	0.46	No
rs347685	3	143289827	C	0.28	TFDP2	eGFRcrea ↑ [19]	0.01 (-0.53; 0.53)	0.99	Yes
rs17319721	4	77587871	A	0.45	SHROOM3	eGFRcrea ↓ [19]	0.11 (-0.37; 0.58)	0.66	No
rs6420094	5	176750242	G	0.31	SLC34A1	eGFRcrea ↓ [19]	-0.48 (-0.97; 0.01)	0.06	Yes
rs11959928	5	39432889	A	0.44	DAB2	eGFRcrea ↓ [19]	-0.50 (-0.97; -0.04)	0.03	Yes
rs2279463	6	160588379	G	0.12	SLC22A2	eGFRcrea ↓ [19]	0.17 (-0.56; 0.91)	0.64	No
rs3127573	6	160601383	G	0.12	SLC22A2	serum creat ↑ [16]	0.06 (-0.66; 0.79)	0.87	No
rs881858	6	43914587	G	0.29	CVEGFA	eGFRcrea ↑ [19]	0.03 (-0.48; 0.54)	0.90	Yes
rs7805747	7	151038734	A	0.26	PRKAG2	eGFRcrea ↓ [19]	0.05 (-0.47; 0.57)	0.85	No
rs6465825	7	77254375	C	0.39	TMEM60	eGFRcrea ↓ [19]	-0.16 (-0.63; 0.31)	0.50	Yes
rs10109414	8	23807096	T	0.42	STC1	eGFRcrea↓ [19]	-0.65 (-1.11; -0.18)	<0.01	No
rs4744712	9	70624527	A	0.41	PIP5K1B-FAM122A	eGFRcrea ↓ [19]	-0.12 (-0.60; 0.37)	0.64	Yes
rs10774021	12	219559	C	0.35	SLC6A13	eGFRcrea ↑ [19]	-0.27 (-0.77; 0.22)	0.28	No
rs653178	12	110492139	C	0.47	ATXN2	eGFRcys ↑ [19]	-0.18 (-0.65; 0.30)	0.47	Yes
rs626277	13	71245697	C	0.41	DACH1	eGFRcrea ↑ [19]	-0.03 (-0.50; 0.43)	0.89	No
rs2453533	15	43428517	A	0.38	GATM, SPATA5L1	eGFRcrea ↓ [19]	0.14 (-0.35; 0.64)	0.57	Yes
rs491567	15	51733885	C	0.24	WDR72	eGFRcrea ↑ [19]	-0.15 (-0.70; 0.41)	0.61	No
rs1394125	15	73946038	A	0.35	UBE2Q2-FBXO22	eGFRcrea ↓ [19]	0.16 (-0.32; 0.65)	0.51	No
rs12917707	16	20275191	T	0.18	UMOD	eGFRcrea ↑; CKD ↓ [19]	-0.05 (-0.69; 0.59)	0.88	No

Table 2. Common genetic variants known to be associated with blood pressure or kidney function and their association with fetal kidney volume (continued)

SNP	Chr.	Position	Minor Allele	Minor allele frequency	Gene (in/near)	Previously reported effect on blood pressure	Effect estimate for fetal kidney volume (cm^3)	P-value	Direction as expected
Blood pressure									
rs9895661	17	56811371	C	0.19	TBX2-BCAS3	eGFRcrea ↓ [19]	-0.37 (-1.01; 0.26)	0.25	Yes
rs8068318	17	56838548	C	0.28	TBX2-BCAS3	Serum creat ↑ [16]	0.17 (-0.37; 0.70)	0.54	No
rs12460876	19	38048731	C	0.37	SLC7A9	eGFRcrea ↑ [19]	-0.59 (-1.07; -0.12)	0.02	No
rs4805834	19	38145499	T	0.14	SLC7A9	Serum creat ↑ [16]	-0.99 (-1.68; -0.31)	<0.01	Yes
rs911119	20	23560737	C	0.21	CST3-CST4, CST9	eGFRcys↑[19]	0.38 (-0.21; 0.97)	0.21	No

Effect estimates are regression coefficients (95% CI) and reflect the difference in kidney volume per minor allele. All regression models were adjusted for fetal sex, gestational age at measurement and estimated fetal weight. We assumed an additive model.

Chr, chromosome; SBP, systolic blood pressure; DBP, diastolic blood pressure

Expected direction: We expected that a blood pressure increasing risk allele or a risk allele, known to be associated with impaired kidney function is associated with decreased kidney volume.

Table 2 gives the associations of the selected common genetic variants, known to be associated with blood pressure or kidney function, with fetal kidney volume. Out of 30 genetic variants known to be associated with blood pressure, only rs12940887 (near *ZNF652*) was significantly associated with fetal kidney volume (β: 0.88 (95%CI: 0.40; 1.37) cm3 per minor allele, P<0.001), but the effect of this variant did not show the expected direction. Overall, eighteen common variants (60%) previously associated with blood pressure, showed the expected direction of the association with fetal kidney volume, but these associations were not significant. Of the common variants known to be associated with kidney function or kidney disease, four (rs11959928 (in *DAB2*), rs10109414 (in *STC1*), rs12460876 (in *SLC7A9*) and rs4805834 (near *SLC7A9*)) out of 28 variants (14.3%) showed evidence of association with fetal kidney volume (P<0.05), though they did not reach the significance threshold after adjustment for multiple testing. The expected direction of the associations was found in two (rs11959928 and 4805834) of these common variants. Overall, thirteen (46.4%) common genetic variants showed the expected direction of the association with fetal kidney volume. A risk allele score summing all risk alleles of the common genetic variants associated with adult blood pressure and kidney function, was not associated with fetal kidney volume (β -0.04 (95% CI: -0.11 , 0.03) cm^3 per risk allele, P=0.25).

We did not find evidence of associations between common genetic variants in *PAX2* (rs4244341 and rs11592735), *RET* (rs1800860) and *ALDH1A2* (rs7169289) and fetal kidney volume (Table 3). The previously described AAA-haplotype in *PAX2* (rs11190688, rs11190702 and 11599825)[13] was not significantly associated with fetal kidney volume.

Table 3. Common genetic variants and their association with fetal kidney volume

SNP	Chr.	Position	Minor Allele	Minor allele frequency	Gene	Previously reported effect on kidney volume	Effect estimate for fetal kidney volume (cm^3)	P-value	Direction as expected
rs11190688 rs11190702 rs11599825	10	102514460 102532515 102510165	Haplotype: AAA		PAX2	↓[13]	0.19 (-0.52; 0.89)	0.60	No
rs4244341	10	102498567	T	0.22	PAX2	↓[13]	-0.37 (-0.94; 0.20)	0.21	Yes
rs11592735	10	102518253	A	0.04	PAX2	↓[13]	-0.43 (-1.73; 0.87)	0.52	Yes
rs1800860	10	42926693	A	0.30	RET	↓[9]	0.31 (-0.21; 0.82)	0.25	No
rs7169289	15	56030975	G	0.17	ALDH1A2	↑[14]	0.30 (-0.38; 0.98)	0.39	Yes

Effect estimates are regression coefficients (95% CI) and reflect the difference in kidney volume per minor allele. All regression models were adjusted for fetal sex, gestational age at measurement and estimated fetal weight. We assumed an additive model.

Chr, chromosome

Expected direction: We expected that the common genetic variants, known to be associated with kidney volume, showed the same direction as described before in literature.

Discussion

Results from this population-based prospective cohort study suggest that common genetic variants, which have previously shown to be associated with blood pressure or kidney function[15-20], not explain the associations of smaller kidneys with higher blood pressure and impaired kidney function in later life. Also, previously found associations of common genetic variants involved in the branching pathway with neonatal kidney size[9, 13-14], were not confirmed in this study.

Previous studies showed the association of low birth weight with smaller kidneys and a lower number of nephrons[7-8, 10]. Smaller kidneys, with a reduced nephron number, may lead to hyperfiltration in the remaining nephrons, resulting in glomerular sclerosis. Subsequently this may predispose an individual to the development of higher blood pressure, impaired kidney function and chronic kidney disease[4, 10, 33-35]. Postmortem studies in humans showed that a lower nephron number is associated with low birth weight and hypertension[6, 36]. A recent study showed an association between newborn kidney volume and nephron number in fifteen infants, who died before three months of age, in whom an ultrasound was performed in the first two days of life. There was a strong relationship between kidney mass and nephron number[9]. This association is supported by study by Hinchliffe et al demonstrating a strong correlation between renal volume and glomerular number up to 40 weeks of gestation, in eleven spontaneously aborted fetuses[37]. Several other post-mortem studies in humans, who died in the perinatal period, showed consistent associations between renal size and glomerular number[8, 10]. Low birth weight has also been shown to be associated with low nephron number[8, 10, 33, 36]. Ultrasound studies in fetuses and children have shown that low birth weight is associated with smaller kidney size[35, 38-40]. Previously we have shown that kidney characteristics track from third trimester of pregnancy to the postnatal age of two years[30]. Therefore, fetal kidney volume also seems to be a good surrogate for nephron number. However, differences in fetal kidney volume might be smaller and therefore more difficult to detect. This could have affected the power of our study to establish associations.

The nephron number varies widely between individuals ranging from 250,000 to 2,000,000 nephrons per kidney[7-8]. The fetal kidney develops forms two components, the metanephric mesenchyme and the Wolffian duct. The ureteric bud forms as an outgrowth of the Wolffian duct and reciprocal induction between the metanephric mesenchyme and the ureteric bud results in branching of the uretiric bud. Through various complicated processes, metanephric tubules and glomerular components are formed. Kidney and nephron growth and development are influenced by genetic and various environmental factors[11-12, 41-44]. Many molecular mechanisms are required for different aspects of nephrogenesis, such as the ureteric bud outgrowth and branching[11-12]. Ureteric branching is a very important part of nephrogenesis and thought to be a major

determinant of nephron endowment[11-12]. Several genes, such as PAX2, RET and GDNF, have been suggested to be involved in these steps in nephrogenesis. Mutations in these genes seem to cause kidney agenesis or dysgenesis and fewer nephrons in animals and humans[11, 45]. Adverse fetal environmental exposures could also affect kidney development, although information on specific adverse fetal exposures affecting kidney development is limited. It has been shown that continued smoking during pregnancy of more than ten cigarettes per day is associated with smaller kidneys in fetal life[41] and higher blood pressure in childhood[46]. Also, other environmental exposures, such as nutrition, folic acid supplementation, and placental dysfunction might affect kidney development. Several animal studies have shown that low maternal protein intake and vitamin A deficiency during pregnancy lead to smaller kidneys and increased blood pressure in the offspring[42-44].

It seems likely that genetic factors partly explain variation of nephron number, and thus kidney size, in the general population[11]. There have been no heritability studies performed on kidney volume in healthy populations, but a study in families with polycystic kidney disease showed that kidney volume has a heritability of 0.42[47]. The association between smaller kidneys and higher blood pressure, impaired kidney function and kidney disease in adulthood, might be partly explained by common genetic variation, resulting in lower nephron endowment in individuals. In our study, we only found rs12946454, near *ZNF652*, to be significantly associated with a larger kidney volume. *ZNF652* has been implicated in tumor genesis[48]. Another gene in this region, *PHB*, is shown to be involved in angiogenesis[49]. It might be that this variant influences angiogenesis which may subsequently lead to a larger kidney volume. Further research is needed to identify underlying mechanisms and to evaluate whether carrying kidney volume increasing alleles of this variant also leads to better kidney function and lower blood pressure.

We did not observe any associations in the expected direction of previously identified common variants affecting blood pressure and kidney function in adulthood[15-21], with fetal kidney volume. We expected that a blood pressure increasing allele or a kidney function decreasing allele would be associated with a smaller kidney volume in fetal life. This suggests that these variants do not underlie the association between smaller kidneys and increased blood pressure and impaired kidney function in later life. It might be that we cannot identify associations, because of the small differences in kidney size in fetal life. It also could indicate that there might be other genes underlying this association. Combining the information of all common genetic variants, by calculating a risk allele count, also did not show evidence for association of these variants with fetal kidney volume. Genome wide association studies including much larger sample sizes than published so far, might result in common genetic variants that do affect kidney volume. These studies might be powerful enough to detect small differences in blood

pressure or kidney function possibly caused by slightly smaller or larger kidneys and could provide evidence for a genetic basis of the hyperfiltration hypothesis.

Other interesting genes could be genes involved in the kidney branching morphogenesis. Mutations in genes such as *PAX2* and *RET* lead to hypoplasia or agenesis of the kidney[9, 11, 13]. Both *PAX2* and *RET* play a role in the outgrowth of the ureteric bud and are mainly involved in first phase kidney development[11].To determine whether common genetic variants in these genes also reduce kidney volume, several studies were conducted in a Canadian newborn population[9, 13-14]. Variants in *PAX2*, *RET* and *ALDH1A2* have been shown to affect kidney volume[9, 13-14]. Subjects carrying a specific *PAX2*-haplotype or the adverse allele of rs1800860 in the *RET* gene had a 10% smaller kidney volume than subjects not carrying these variants[9, 13]. Also rs4244341 and rs11592735 in the *PAX2* gene were associated with a smaller kidney volume[13]. Homozygosity for the minor allele of rs7169289 in *ALDH1A2* was associated with a 22% larger newborn kidney volume[14]. In our study, we did not find any association of these variants with fetal kidney volume. The absolute effects on fetal kidney volume might be smaller and more difficult to establish, as compared to a newborn population. This study (n=855) includes a larger number of subjects, as compared to the previous studies[9, 13-14]. However, it could also be that these variants exert their effect later in pregnancy. Also, the variants in *PAX2*, *RET* and *ALDH1A2* have not been associated with increased blood pressure or impaired kidney function in adulthood as far as we know. These findings do not support the hypothesis that genetic factors partly underlie the association between smaller kidneys and increased blood pressure or impaired kidney function.

The lack of evidence for a genetic basis of the hyperfiltration hypothesis could indicate that an adverse fetal environment, such as fetal nicotine exposure, is more important than genetic factors in explaining this hypothesis. It seems likely that genetic factors only cause small differences in kidney volume, which could be difficult to detect and might be of little clinical interest, but combined could add to the hyperfiltration hypothesis. Further research is necessary to identify specific adverse fetal exposures and genetic factors that underlie the association of kidney volume with blood pressure and kidney function.

Conclusions

Our results suggest that common genetic variants, previously associated with adult blood pressure and kidney function, do not underlie the associations of smaller kidneys with a reduced nephron endowment in early life with higher blood pressure or impaired kidney function in later life.

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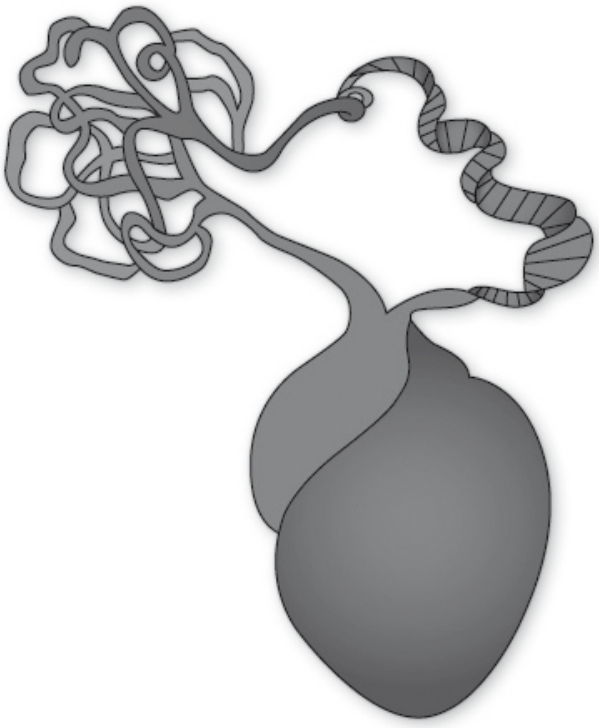
Chapter 5

General Discussion



Introduction

Many epidemiological studies suggest that early life events have an important role for the susceptibility to develop adult diseases. Low birth weight and subsequent childhood growth have been associated with a wide range of adult diseases, such as metabolic, cardiovascular and renal diseases[1-6]. These observations have resulted in the "Developmental Origins of Health and Disease" Hypothesis, which proposes that an organism may develop in different ways, depending on the environment it is exposed to[7]. The aim of this study was to identify specific fetal exposures and genetic determinants underlying the associations of early growth with obesity, and measures of cardiovascular and renal structures and function. In this chapter, we give an overview of the main findings of the studies in this thesis, what they add to the existing body of literature and possible clinical implications of these studies. Furthermore, we consider methodological issues and give suggestions for future research.

Main findings

Early growth and body composition

The relationship between birth weight and obesity is complex. Both low and high birth weight are associated with an increased risk of obesity in later life[8]. Also, early childhood growth seems to be an important determinant of body composition in later life. Birth weight and early childhood growth are not independent of each other. Catch-up growth in infancy has been proposed to be one of the underlying mechanisms of the associations of low birth weight with adult diseases[4]. Children born small size for gestational age (SGA) frequently show catch-up growth in early postnatal life[9-10]. Many studies have shown that catch-up growth in children born SGA is associated with metabolic diseases in later life[11]. Children born large size for gestational age (LGA), also show compensatory catch-down growth in early life, but the effect of catch-down growth in children born LGA has less frequently been studied. In this thesis, we showed that children born small size and large size for gestational age display catch-up and catch-down growth respectively in the first months of life. Despite this compensatory growth these children still have persistent differences in height, weight and head circumference until the age of 4 years. We also demonstrated that catch-up and catch-down growth in children born SGA and LGA modifies the association of size at birth with subcutaneous fat mass, body mass index and the risk of being overweight or obese at the age of four years (this thesis). Furthermore, effect modification of the association of birth weight with childhood overweight by postnatal catch-up and catch-down growth was also noted in children born

appropriate size for gestational age (AGA). Children born AGA with catch-up growth and children born LGA without catch-down growth have a higher subcutaneous fat mass and body mass index in childhood, and are at increased risk for childhood overweight. Results from our study suggest that these groups could be potential targets for early prevention of childhood obesity.

Genetics of early growth

Heritability studies show that growth in early life is partly determined by genetic factors (D.O. Mook-Kanamori, in press [12]). Therefore, common genetic variants are likely to affect growth in early life and might partly explain the association between early growth and adult disease. In a population based cohort from the UK, common genetic variants known to be associated with adult BMI, also were robustly associated with a greater weight gain in infancy, indicating that early growth is on the pathway to adult obesity risk[13]. Novel genetic determinants of early growth might provide insight in biological pathways and underlying mechanisms of these associations. Genome-wide association studies allow researchers to study many common genetic variants for association with a specific outcome in a hypothesis-free manner. In one analysis, the associations of 2.5 million common genetic variants can be explored. Common genetic variants are likely to have small effects, compared to rare mutations with large effects[14]. As a consequence, large sample sizes are needed to detect the small differences caused by common genetic variants. International consortia have been set up to provide the infrastructure for collaboration, which is needed to amass the sample size needed for successful genome-wide association study meta-analysis. The Early Growth Genetics (EGG) Consortium aims to identify common genetic determinants associated with various aspects of early growth.

Genome-wide association studies in adults have identified many common genetic variants associated with body mass index[15], waist circumference[16], body fat distribution[17] and height[18]. However, genome-wide association studies in children have been scarce. Only one genome-wide association meta-analysis was performed in children before the work presented in this thesis. Freathy et al. showed that common genetic variants in *ADCY5* (rs9883204) and near *CCNL1/LEKR1* (rs900400) were associated with birth weight. The *ADCY5* (rs9883204) locus was also implicated in the regulation of glucose levels and susceptibility of type 2 diabetes[19], providing evidence that genetic variation can at least partly explain the association between low birth weight and type 2 diabetes in later life. This is in line with the 'fetal insulin hypothesis', which states that that common genetic variants related to type 2 diabetes might partly explain the associations of low birth weight with metabolic diseases in adulthood[20]. In this thesis, we present the second genome-wide association study on birth weight performed in the EGG-consortium, in up to 69,308 individuals. We identified seven loci, including the two

previously published loci, associated with birth weight. Five of the loci are known to be associated with other outcomes: *ADCY5* (rs9883204) and *CDKAL1* (rs6931514) with type 2 diabetes; *ADRB1* (rs1801253) with adult blood pressure; and *HMGA2* (rs1042725)and *LCORL* (rs724577) with adult height. The results of this meta-analysis highlight biological pathways of relevance to the fetal origins of type 2 diabetes and blood pressure and demonstrate overlap between the genetics of fetal growth and adult height.

Common genetic variants are also likely to be associated with other aspects of early growth. Head circumference in infancy is used as a measure for brain size and development[21-22] and has a substantial heritability of 0.7-0.9[23]. Normal variation in head circumference seems to be associated with cognitive and behavioral development[24-26]. Larger head circumference in infancy is associated with higher IQ scores in childhood[26-28]. Several rare mutations with large effects on head circumference have been identified[29-32], including those resulting in microcephaly and intellectual disability[30-32]. In this thesis we present a genome-wide association study meta-analysis on infant head circumference. We identified two loci robustly associated with infant head circumference, one in *HMGA2* (rs1042725) and one near *SBNO1* (rs7980687)[33]. These two loci were previously associated with adult height[18], however conditional analyses showed that the effect on infant head circumference was largely independent of the effect on height. Another locus, in a region previously associated with Parkinson's disease and other neurodegenerative diseases[34-36], which includes the *MAPT* en *CRHR1* genes, also showed evidence for association with infant head circumference (rs11655470). Investigators from the CHARGE consortium showed that genetic variants in the same region were associated with intra cranial volume, which is a measure of maximum brain size[37]. This could indicate that common genetic variants in this region might link early brain growth with neurological disease in later life[33, 37].

It is likely that the gene-environment interactions also can affect birth weight, with common genetic variants only having an effect in a specific environmental setting. A previous study has suggested an interaction of maternal smoking with genetic variants in the nicotine receptor cluster, affecting birth weight[38]. In mothers who smoked, the risk allele of this variant was associated with a lower birth weight, while in non-smoking mothers the genotype did not have an effect on birth weight. Since birth weight is a crude end-point of fetal growth and can be the result of different growth patterns, we investigated whether this variant was associated with a fetal growth. In mothers who smoked, each maternal risk allele was associated with a reduced fetal growth, although the effect on birth weight itself was not significant[39]. These results suggest that the negative effect of maternal smoking during pregnancy is modified by this genetic variant in the nicotine receptor cluster. Since the findings on birth weight are non-significant, it also highlights the need of a well-powered sample of mothers and offspring to study this specific, and other possible gene-environment interactions.

Childhood obesity itself is one of the most important predictors of adult obesity and an increasingly prevalent problem in developed countries[40-41]. Since environmental exposures, such as nutrient rich diet, are present for a relatively short period, children provide an excellent opportunity to investigate the genetic contribution to the development of obesity. We performed a genome-wide association study meta-analysis on childhood obesity status. Many known common genetic variants associated with adult BMI were also associated with an increased risk of childhood obesity. More importantly, we identified two novel loci associated with childhood obesity, located near *OLFM4* and *HOXB5*. These two genes seem to be involved in different aspects of gut function[42]. Further functional characterization of these signals is needed to investigate the exact underlying mechanisms and function.

Cardiovascular development

Studies in children showed that left ventricular mass tracks from childhood to adulthood[43-44], implying that left cardiac structures also track during childhood and adolescence. However, a relatively smaller left ventricle and aortic root in early life may lead to insufficient cardiac functioning in later life. The heart may respond to this insufficiency by growth and adverse remodeling. Since the number of cardiomyocytes is established largely in fetal life, cardiac adaptations might lead to left ventricular hypertrophy and dysfunction[45-48]. Left ventricular hypertrophy is an important risk factor for cardiovascular morbidity and mortality in adulthood[49-50]. Therefore, fetal exposures who affect cardiac development may have consequences in later life. It has also been hypothesized that fetal growth restriction leads to impaired elastin synthesis in the blood vessel walls, leading to changes in the mechanical properties of these blood vessels[51]. Specific adverse fetal exposures might also lead to developmental changes in function and structure of blood vessels, which may predispose an individual to hypertension and cardiovascular disease in adulthood.

Growth in fetal life and early childhood seems to influence the susceptibility to cardiovascular disease in later life. Detailed studies in animals and humans have shown that fetal and childhood growth variation is associated with cardiovascular structural and hemodynamic adaptations in early life[5, 52-67]. In this thesis, we investigated the associations of different fetal, infant and child growth characteristics with cardiovascular structures and function at the age of 6 years and whether postnatal growth patterns modified the association of size at birth on childhood cardiovascular development. We showed that birth weight, and to a lesser extent gestational age, was associated with childhood blood pressure and fractional shortening, and positively associated with left cardiac structures. We also showed that postnatal catch-up and catch-down growth modified the associations between size at birth and childhood cardiovascular development. This

suggests that specific early growth patterns are associated with altered cardiovascular development.

One of the possible adverse fetal exposures is maternal smoking during pregnancy. Maternal smoking during pregnancy is one of the most important, potentially modifiable, adverse fetal exposures[68-69], and strongly related with increased risks of low birth weight and preterm birth[69-70]. Maternal smoking may lead to developmental adaptations with subsequent effects in postnatal life, such as higher blood pressure, obesity and type 2 diabetes[71]. However, it is not clear from previous studies whether these associations are due to direct intra-uterine effect of fetal smoke exposure or due to other, unmeasured confounders. We showed that maternal smoking during pregnancy is associated with childhood diastolic blood pressure and fractional shortening, but not with systolic blood pressure, carotid-femoral pulse wave velocity and left cardiac structures. To assess the role of confounding we also assessed whether paternal smoking during pregnancy was associated with the same outcome measurements[72]. Similar effect sizes for maternal and paternal smoking would suggest that these associations are explained by unmeasured environmental determinants[72]. Among mothers who did not smoke during pregnancy, paternal smoking was associated with aortic root diameter, but not with other cardiovascular outcomes. The stronger effect estimates for maternal smoking affecting diastolic blood pressure and fractional shortening, compared to paternal smoking might suggest that direct intra-uterine mechanisms are involved, but since paternal smoking was associated with childhood aortic root diameter, we must be careful to conclude direct intra-uterine effects.

Maternal psychological distress during pregnancy might also be one of the adverse fetal exposures leading to fetal developmental adaptations[73], possibly by dysregulation of the maternal hypothalamic-pituitary-adrenal (HPA) axis[74], and subsequent higher fetal cortisol exposure levels[74]. Previous animal and human studies have shown that excessive exposure to exogenous glucocorticoids is associated with higher blood pressure in the offspring[75-76]. Mild endogenous elevations in cortisol levels, e.g. due to maternal psychological distress during pregnancy, might also lead to developmental adaptations and affect cardiovascular function in the offspring[77]. We did not find evidence for association of maternal psychological distress with childhood blood pressure and carotid-femoral pulse wave velocity. High maternal overall psychological symptoms were associated with a lower childhood left ventricular mass, however paternal psychological showed a similar effect size. The similar effect sizes for maternal and paternal psychological distress with left ventricular mass suggest that these associations could be due to unmeasured shared social and environmental factors, rather than direct intra-uterine effects.

Exposure to extreme famine during pregnancy has been associated with a higher risk of adult diseases, such as hypertension[78-80]. The association of less extreme variations in maternal nutrition during pregnancy with blood pressure in the offspring has been

investigated in several studies, but results are inconclusive[81-91]. Possible underlying mechanisms are unclear, but might involve an effect on endothelial function, with subsequent changes in blood pressure development[92]. In this thesis, we found marginal evidence that low maternal iron intake and high vitamin B12 concentrations were associated with a higher childhood blood pressure, but the effect sizes were small and after taking into account multiple testing these associations were not statistically significant. Intake of other macronutrients and micronutrients was not associated with childhood blood pressure.

Apart from environmental exposures, genetic factors are also involved in blood pressure regulation from childhood to adulthood. The heritability of blood pressure is estimated to be 30-60%[93-94]. Previous genome-wide association studies identified several common genetic variants associated with adult blood pressure, only explaining ~1% of the variance of blood pressure in the investigated populations[95-96]. It has been shown that the presence of multiple variants affecting polygenic traits can be demonstrated by constructing genome-wide prediction models (genetic risk scores) of common variants[97-98]. We demonstrated that genetic risk scores incorporating many common variants explained more of the variance of blood pressure than genetic risk scores only based solely on common genetic variants reaching genome-wide significance in both adults and children. These results suggest that there are many more common genetic variants affecting blood pressure and that there are multiple genes involved in blood pressure regulation, which partly overlap in adults and children. Recently, the largest genome-wide association study meta-analysis on blood pressure in adults so far, identified 30 loci associated with blood pressure, confirming that blood pressure has a polygenic origin[99]. This indicates the need for larger genome-wide association studies, to identify more genetic variants involved in blood pressure regulation and improve the variance explained by genetic factors.

Renal development

The formation of nephrons (nephrogenesis) starts from 30 days of gestation onwards and continues until 36 weeks of gestational age. The number of nephrons is determined in fetal life as nephrogenesis ceases in postnatal life. On average about 750,000 nephrons per kidney are formed, but the nephron number can vary between 250,000 and 2,000,000 per kidney[100-101]. Adverse fetal environment might have negative effects on nephrogenesis, resulting in a lower number of nephrons. Animal studies have shown that various adverse intra-uterine environmental exposures, such as low protein intake and vitamin A deficiency, lead to fetal growth retardation and smaller kidneys with a lower nephron number[102-104]. Post mortem studies in humans showed that a lower nephron number is associated with both low birth weight and hypertension[101, 105-108]. It has been hypothesized that smaller kidneys with a reduced number of nephrons in low

birth weight children might lead to hyperfiltration of the remaining nephrons, eventually resulting in glomerular sclerosis ('the hyperfiltration hypothesis')[109-110]. This may predispose the individual to renal damage and development of higher blood pressure, impaired kidney function and end stage kidney disease in adulthood. Indeed, epidemiological studies clearly showed that low birth weight was associated with hypertension, impaired renal function and end stage renal disease in adulthood[3, 56, 111-113].The best surrogate marker for assessing nephron number in epidemiological studies seems to be kidney weight or kidney size measured by ultrasound[101, 105-106, 108, 114-119]. In this thesis we described normal kidney growth from fetal life until the age of two years, to enable identification of abnormal kidney size and growth[120]. Abnormal size or growth may have subsequent short and long-term consequences.

According to the hyperfiltration hypothesis, adverse fetal exposures, might lead to smaller kidneys with lower nephron numbers, with possible effects in later life. We have demonstrated that maternal smoking during pregnancy, as specific fetal exposure, is associated with an altered kidney volume in third trimester of pregnancy, but not in infancy. The direction and size of the effect depended on the timing and number of cigarettes smoked during pregnancy[121]. Children of mothers who smoked intensively (>10 sigarettes per day) during pregnancy, had smaller kidneys compared to children of mothers who did not smoke during pregnancy. This effect was independent of the effect of maternal smoking on fetal growth. These findings suggest that early kidney development may be an underlying mechanism for the associations between maternal smoking during pregnancy and increased blood pressure in later life[121-122].

Identification of maternal, fetal and infant determinants of childhood kidney volume and function may be important for early prevention of renal disease in adulthood. Impaired renal function in children, possibly as a consequence of smaller kidney with a lower number of nephrons, could be an early sign of developing renal disease. At the age of 5 to 6 years we examined which maternal, fetal and infant characteristics were important determinants of kidney volume and function. We showed that maternal anthropometrics, smoking during pregnancy, birth weight and gestational age were the main determinants of total kidney volume and function. This was in line with previous studies in a subsample of the Generation R Study which showed that maternal weight before pregnancy and maternal height were associated with kidney volume in fetal life and at the age of 2 years[119, 123]. Some of these factors are potentially modifiable and might provide targets for intervention to improve renal health on a population level. Furthermore, we also showed that smaller kidneys with reduced total kidney volume were associated with a decreased kidney function. This is in line with the hyperfiltration hypothesis and might indicate that kidney volume, which is easily measured, could be a tool to identify children at risk for developing impaired renal function and hypertension in later life.

The associations of smaller kidneys holding lower number of nephrons with high blood pressure and impaired kidney function might partly be explained by genetic factors. It seems likely that common genetic variants account for part of the normal variation in nephron endowment. It has been shown that common genetic variants in *PAX2*, *RET* and *ALDH1A2*, genes involved in kidney branching morphogenesis, are associated with newborn kidney volume[114, 124-125]. We did not find associations of common genetic variants in these genes with fetal kidney volume. We also showed that common genetic variants, previously associated with blood pressure or kidney function in adults[99, 126], were not associated with fetal kidney volume[127]. These findings suggest that these variants do not underlie the association of lower nephron number with higher blood pressure and impaired kidney function. However, it might be that our study was not adequately powered, or that other genetic factors might explain this association.

Methodological considerations

Specific methodological considerations of the studies have been discussed in the separate chapters of this thesis. Here, general methodological issues regarding selection bias, information bias and confounding are discussed. We also address methodological issues regarding genetic association studies.

Selection bias

Selection bias can occur if the association between the determinant and outcome of interest is different in subjects who participate in the study and those who did not participate in the study, but were eligible for the study. Selection bias may also occur when there is a selective loss to follow-up[128]. Of all children eligible at birth, the overall response to participate in the Generation R Study was 61%. The mothers of non-participating children more frequently belonged to an ethnic minority and had a lower socio-economic status, compared to mothers who were participating in the study. Participating mothers and children had less medical complications, such as preterm birth and low birth weight, suggesting a selection toward a relative more healthy study population[129]. This selection at baseline may reduce statistical power, due to lower prevalence rates, and possibly limit generalizability of our findings to other, less healthy, populations.

It has been described that biased estimates in large cohort studies mainly arise from loss to follow-up rather than non-response at baseline[130]. Selection bias due to selective loss to follow-up may occur if the associations studied differ between those lost to follow-up and those still included in the population for analysis. In the studies presented in this thesis, the response in at follow-up generally was around 70%. Approximately 10% of the children who were not included were loss to follow-up, the remainder either

withdrew consent for the study or was not able to visit the research center. Overall, mothers from children who did not visit the research center more frequently engaged in unhealthy life style habits and were less well educated than the total sample. This selective loss to follow-up towards a more healthy population may have biased our effect estimates, but is difficult to quantify.

Information bias

Information on the determinants and outcomes in the non-genetic studies described in this thesis are obtained by physical examinations and questionnaires. Random misclassification of the determinant or outcome can lead to bias towards the null. Differential misclassification, which occurs for instance when misclassification of the determinant is related to the outcome, could lead to biased effect estimates. Exposure data used in our studies were collected before assessment of the outcome, which makes differential misclassification of the exposure unlikely. However, underreporting of specific adverse exposures, such as maternal smoking during pregnancy and psychological distress, may still occur, although the mothers were not aware of specific research questions. Depending on the research question, this might lead to an overestimation (e.g. selective underreporting of heavy smokers when investigating a dose-response relationship) or underestimation (e.g. underreporting of smoking when investigating effect of smoking yes/no) of the effect. To overcome this limitation, studies have tried to use biomarkers of exposures superior to the use of questionnaires, but this has proven to be difficult[131-132]. In most of our studies, the outcome was assessed using standardized hands-on assessments of body composition and cardiovascular and renal development. Furthermore, the observers were blinded to the exposure status, which makes differential misclassification of the outcome less likely.

Confounding

A confounding factor is a factor associated with both the determinant and the outcome and if not taken into account, this may lead to a biased effect estimate of the association between the determinant and the outcome. In the non-genetic studies presented in this thesis we used multiple approaches to explore the role of confounding in the investigated association. First, we adjusted for multiple potential confounders in our analyses. In some of the studies, adjustment for potential confounders attenuated significant associations to non-significance indicating that the unadjusted association is confounded. In other studies, the association did not attenuate or only slightly, indicating the association is possibly a true association between the determinant and the outcome. Although there are many potential confounders available in the Generation R Study, it still may be that we did not measure all potential confounders. Also, measurement error of the confounding variables can occur, thus residual confounding might

still be an issue. Second, where possible, we assessed the associations of both maternal and paternal exposures during pregnancy. Similar effect sizes for maternal and paternal factors would suggest that an association of the maternal factor is likely to explained by unmeasured environmental factors, rather than direct intra-uterine mechanisms[72].

Genetic association studies

Different strategies have been used to identify genetic determinants of disease or other outcomes. In the past decades, many candidate gene studies investigated the association of one or several common genetic variants with a specific outcome. These studies used an 'a priori' hypothesis to select the genes and variants to investigate. In more recent years, genome-wide association studies have been the main approach to identify new common genetic variants associated with an outcome. Using genome-wide association studies one can test the association of a large number (~2.5 million) genetic variants in a hypothesis-free manner. Because of this large number of associations tested, false-positive findings are likely to occur when using a conventional statistical significance threshold. It has been shown that a genome-wide association analysis reflects approximately 1 million independent hypothesis tests[133]. Therefore the statistical significance threshold of genome-wide association studies has been set to $5x10^{-8}$, reflecting a Bonferroni correction of testing one million variants (0.05/1,000,000)[133]. Since common genetic variants are likely to have small effects, false-negative findings are likely to occur due to insufficient statistical power in a single study. It is therefore necessary to combine many subjects from different studies with genotypic data available, to generate sufficient power for identification of new common genetic variants. For this goal, consortia of collaborating studies, such as the Early Growth Genetics Consortium, have been set up to increase the sample size of the genome-wide association meta-analyses.

Although the selection of children with or without genetic data usually is not related to the outcome under investigation, population stratification may arise when genotype and outcome distribution is different between subgroups of the population in your study, and can give rise to false positive findings[134]. Several strategies have been proposed to correct for possible population stratification. Genomic control was applied in all of the genome-wide association studies presented in this thesis, to correct for population stratification. Misclassification or information bias can occur in the determination of the genetic determinants and the outcome, in each of the participating studies. Stringent quality control is applied when genotyping and imputing genotypic data of the participants in each study, which have been described in the genome-wide association studies in this thesis. General guidelines for sharing genome-wide association data and correct meta-analysis of this data have been described previously[135]. Outcome assessment can differ between the participating studies in a genome-wide association meta-analysis. This might lead to random misclassification, as it is not likely to be related to the geno-

type of the individuals, and therefore reduce statistical power to detect associations of genetic variants with the outcome. Outcome definition is important and should be assessed as similar as possible in the different studies participating.

Clinical implications and future perspectives

Early growth

Many studies have shown that catch-up growth in children born small for gestational age is associated with adverse metabolic effects in later life. However, children born appropriate size for gestational age and large size for gestational age, also exhibit postnatal growth patterns, which might affect their risk of being overweight. In comparison with catch-up growth in small for gestational age children, catch-down growth in large for gestational age children, has not been investigated often. We have shown that catch-up and catch-down growth in the first two years of life after being born small, appropriate or large size for gestational age modifies the risk for overweight and obesity at the age of four years. This study showed that children born large size for gestational age who do not show catch-down growth are at a high risk for developing childhood overweight. Also children born appropriate size for gestational age exhibiting postnatal catch-up growth, have an increased risk for overweight. These groups might be potential targets for early prevention of overweight in children, although evidence for interventions to prevent overweight in pre-school children is scarce. The effectiveness of such interventions should be determined first.

Genetics of early growth

In this thesis we describe several common genetic variants associated with different aspects of early growth; birth weight, infant head circumference and childhood obesity. The last few years, many common genetic variants have been identified with adult cardiovascular and metabolic disease by means of large scale genome-wide association studies. Early growth is an important determinant of the risk of adult disease. We identified several variants that are related both to early growth and adult disease (blood pressure, obesity and type 2 diabetes). This indicates that common genetic variants partly underlie the association between low birth weight and diseases in later life, and that the genetic variants act throughout the life course. Furthermore, it highlights that the fetal and early postnatal period provides a possible window of opportunity to improve adult health. Although the clinical implications of these findings are limited for know, future research might provide further insight and understanding of underlying pathogenic mechanisms. In turn, these findings might give rise to useful clinical applications or new drug targets.

Genetic risk prediction

At this stage, individual risk prediction is not feasible and personal genetic profiles that can be purchased on the internet are of little use so far[136]. The common genetic variants that have been identified usually have small effects and are not comparable to the risk of traditional risk factors, e.g. smoking or overweight for the risk of cardiovascular disease. The main reason for this is that these genetic variants explain a very small part of the variance in the investigated phenotypes, even when considering multiple genetic variants. Using information of multiple common genetic variants might improve genetic risk prediction, but several studies have shown that the additive value of these genetic risk profiles is limited so far, e.g. in type 2 diabets[137].

The genetic prediction of complex diseases will probably improve in the future by identifying more variants associated with the disease and the identification of rarer variants with larger effects. Genetic profiling might be a way of identifying subgroups at genetically high risk for increased blood pressure at a population level[138], but whether it will be enough for personalised medicine and early treatment of people at risk for adult diseases, remains to be determined. Work in this thesis indicates that this may require the identification of many more common variants with small effects on blood pressure.

Next steps after genetic findings

It is important to emphasize that the genetic variants associated with a specific outcome, are not necessarily the functional variants that cause the change in disease risk or phenotype. It is more likely that the variant, for which the association is observed, is in linkage disequilibrium with the causal variant. In order to find the causal variant, dense genotyping of all the genetic variants in this region is needed. Furthermore, studies in other ethnic groups might provide refinements of the associated signal. Also, sequencing in large sample sizes can help to investigate if rare variants are causing the association that was identified.

Identifying the causal gene or variant is also an important step to make findings of genome-wide association studies clinically relevant. The genome-wide association study approach does not identify the causal gene directly. To investigate which gene is causal for the association found, the effect on gene expression can be assessed. If the associated genetic variant also affects gene expression, this could point to the causal gene. It is important where expression of the gene is investigated since gene expression is tissue specific. Furthermore, animal knockout models could be used if available, and if these animals show the disease of interest, this could point to a causal effect of the gene. If there are genes known to be involved in Mendelian forms of the studied outcome this might provide clues for a possible causal gene and could be used to prioritize further research.

Future of genome-wide association studies

Increasing sample size is a key factor for the identification of novel genetic variants of complex polygenic phenotypes such as body mass index and blood pressure. In adults, large meta-analyses in up to 200,000 individuals have identified many genetic variants associated with several phenotypes. Some of these phenotypes are particularly interesting to study in children since environmental influences have been present for a relatively limited period of their life-time. In adults, the environmental influence on e.g. body mass index may be more important than genetic factors, which makes identification of variants associated with body mass index more difficult. However, although the number of subjects with genome-wide association studies and childhood data is likely to increase, other options to further increase the power to identify common genetic variants contributing to a complex disease or trait, can be considered.

It could be that within one locus, multiple common genetic variants are independently associated with the phenotype. These secondary signals can be found by conditioning on the identified variants as has been proposed recently[139]. Also, gene-environment interactions might add to explain the variance in complex phenotypes. Genome-wide approaches testing gene-environment interactions might be hampered by the different assessments and definitions of the environmental factor under study and the different periods of enrolment in the studies which can be decades apart. This heterogeneity reduces the power to find statistically significant gene-environment interactions. Ideally, future studies should aim to adopt similar assessment strategies in have the most fruitful meta-analysis. Another option might be to combine related traits in a multivariate genome-wide association study. This way, common genetic variants affecting both traits, i.e. having a pleiotropic effect, can be identified, beyond those already identified in 'regular' genome-wide association studies. These approaches can be used independently of each other, in an attempt to increase the number of identified genetic variants, which increases the possibility of findings that might be translated to relevant clinical applications.

Environmental exposures

In this thesis we describe associations of environmental exposures during pregnancy and postnatal development of the cardiovascular and renal system. Due to the observational design of our study we cannot establish whether this association is causal or not, due to possible residual confounding. A randomized controlled trial is the preferred study design to establish causation. However, with adverse environmental exposures, such as maternal smoking and maternal psychological distress, this is not possible. For other exposures, such as maternal nutrition, this might be feasible and randomized controlled trials could overcome this issue, possibly identifying mechanisms that underlie the associations.

Role of confounding

An approach that we have used for adverse fetal exposures in thesis is to asses the associations of paternal lifestyle habits. Comparing maternal and paternal effects might provide a way to separate intra-uterine effects from associations explained by social, familial and environmental factors[72]. Future research should aim to also involve and include fathers in the study with similar exposure assessment, to compare the associations of both maternal and paternal exposures during pregnancy with offspring health where possible.

Also, it might be hard to disentangle pre- and postnatal environmental exposures as these are likely to be highly correlated. For instance, mothers who smoke throughout pregnancy are likely to continue smoking in postnatal life. Exposure assessment should be similar in prenatal and postnatal life. Ideally, biomarkers of exposure could be determined in pre and postnatal life.

Cardiovascular development

Early markers of cardiovascular adaptations might provide insight in the underlying mechanism of the association between low birth weight with cardiovascular disease in later life. Subtle changes in cardiovascular development can be identified already in early life and follow-up studies should determine how these changes relate to cardiovascular disease in later life. New imaging techniques such as magnetic resonance imaging of the heart and three dimensional ultrasounds, can provide more detail in cardiovascular development at young ages and possibly identify other mechanisms important in the development of cardiovascular disease in adult life.

Also, endothelial dysfunction and structural vascular changes might underlie part of the association between low birth weight and cardiovascular disease in later life. In this thesis, we have measured pulse wave velocity as marker of arterial stiffness. We did not find associations of environmental exposures and pulse wave velocity. At this age, differences in arterial stiffness might be too small to detect. Future studies should focus on other markers of vascular adaptations such as flow-mediated arterial dilatation, intima media thickness and retinal arteriolar narrowing.

Renal development

Early renal development is important in determining the number of nephrons available for the remainder of the life time of the individual. Kidney volume, especially in children, seems to be a reliable surrogate for nephron endowment[140]. Future research should focus on more reliable ways of determing nephron number in population based studies. There is some promising evidence that magnetic resonance imaging techniques can provide individual counts of glomerular number in rats, which is equivalent to the nephron number[141]. If these methods could be extrapolated to humans it could provide

the means for large scale epidemiological studies, to directly assess determinants of nephron number and how this affects the development of hypertension and chronic kidney disease. Other earlier markers of kidney disease, such as mildly impaired kidney function, might also be subject to fetal programming. Identifying possible modifiable exposures associated with an impaired kidney function early in life, might give new biological insights in the development of hypertension and kidney disease.

Not only nephron endowment can be affected by adverse fetal exposures. Other important pathways, such as endocrine pathways, can be 'programmed' as well. The renin-angiotensin-aldosteron (RAS) system is important for blood pressure and organ development, and its activity can be altered by adverse fetal environment[142-143]. Other possible systems include the glucocorticoid metabolism and the renal tubular function (sodium reabsorption)[144]. Future studies should focus on identifying the underlying pathogenic mechanisms, including these pathways, as they may help to prevent the development of hypertension and kidney disease in an individual at risk. Subsequently, there is need for randomized clinical trials in humans if applicable, timed in pregnancy and aimed to prevent cardiovascular disease in later life, investigating if findings from epidemiological studies truly can add to the prevention or treatment of adult diseases.

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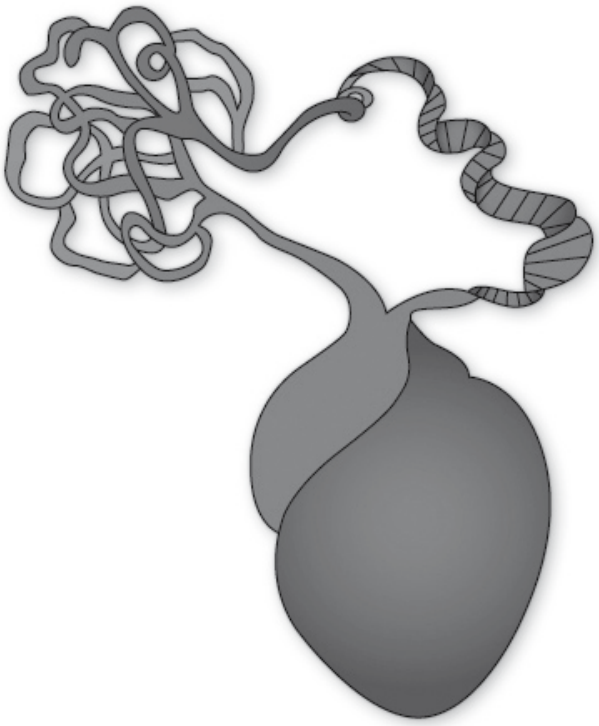
Chapter 6

Summary
Samenvatting



Summary

In **chapter 1** the background and rationale of the studies presented in this thesis are given. Many epidemiological studies suggested that early life events have an important role for the susceptibility to develop diseases in adult life, such as cardiovascular and metabolic diseases. These observations resulted in the "Developmental Origins of Health and Disease", recently adapted to a more general "developmental plasticity hypothesis", which proposes that an organism may develop in different ways, depending on the environment it is exposed to in fetal and early postnatal life. Numerous observational studies have shown that low birth weight and growth acceleration in the first months of life is associated with adverse outcomes in adolescence and adulthood, such as obesity, cardiovascular, renal and metabolic disease. We reasoned that investigating specific adverse fetal exposures and early growth may provide new insights in mechanisms underlying the associations of low birth weight with adult disease. Genetic factors associated with early growth might also partly explain the association of early growth with adult diseases. In this thesis, we performed studies to identify early environmental and genetic determinants of childhood growth, and cardiovascular and renal development. We focused on parental exposures during pregnancy and genetic determinants of early growth, cardiovascular and renal development.

In **chapter 2** we studied environmental and genetic determinants of different aspects of early growth. In **chapter 2.1**, we examined the postnatal growth of children born small (SGA), appropriate (AGA) and large size for gestational age (LGA). We showed that children born SGA and LGA display catch-up and catch-down growth respectively in the first months of life. Despite the compensatory growth these children still have persistent differences in head circumference, height and weight until the age of 4 years. In the same study, we also assessed whether postnatal growth patterns could modify the risk of childhood overweight and adverse body composition. We demonstrated that catch-up and catch-down growth in children born SGA and LGA modifies the association of size at birth with subcutaneous fat mass, body mass index (BMI) and the risk of being overweight or obese at the age of 4 years. Furthermore, effect modification of the association of birth weight with childhood overweight by postnatal catch-up and catch-down growth was also noted in children born AGA. Children born AGA with catch-up growth and children born LGA without catch-down growth have a higher subcutaneous fat mass and BMI in childhood, and are at increased risk for childhood overweight. Results from our study suggest that these groups could be potential targets for early prevention of childhood obesity.

In **chapter 2.2** we investigated which common genetic variants are associated with birth weight, using a genome wide association study approach. For this, we meta-

analyzed 18 genome-wide association studies and followed up 21 lead signals in 24 independent replication samples, including up to 69,308 individuals of European descent. We identified seven common genetic variants robustly associated with birth weight. Five of the loci were previously associated with other outcomes. Variants in or near *ADCY5* and *CDKAL1* were associated with type 2 diabetes. The variant in *ADRB1* was previously associated with blood pressure and *HMGA2* and *LCORL* with adult height. These results demonstrate that the well described association between low birth weight and type 2 diabetes and blood pressure has a genetic component, next to possible programming effects by the fetal environment.

Chapter 2.3 describes a genome-wide association meta-analysis on infant head circumference. We meta-analyzed GWAS from 7 different studies, and selected 3 variants for replication in an additional 6 studies. In total, we included more than 19 thousand individuals in this study. We identified two variants in or near *HMGA2* and *SBNO1* robustly associated with infant head circumference. Although these loci have previously been associated with adult height, their effects on infant head circumference were largely independent of their effect on infant height. We also found suggestive evidence that another variant, in a region previously associated with neurodegenerative diseases (17q21 including *MAPT* and *CRHR1*), was associated with infant head circumference. Other investigators found the same region to be associated with intra cranial volume. This could indicate that genetic variation in this region might link early brain growth with neurological disease in later life.

In **chapter 2.4** we studied whether genes and environment interact with each other to affect a specific outcome. We investigated whether maternal smoking during pregnancy modified the effect of a genetic variant in the nicotine receptor cluster on birth weight. We showed that in mothers who smoked during pregnancy, each maternal risk allele was associated with a reduced fetal growth. In mothers who did not smoke during pregnancy, we did not find an association of the genotype with birth weight. This indicates that the environment (smoking) and the genotype (nicotine receptor cluster) interact with each other to affect fetal growth.

Chapter 2.5 again uses a genome-wide association approach to identify common genetic variants associated with childhood obesity. We performed a collaborative meta-analysis of 14 studies consisting of 5,530 cases (≥95th percentile of BMI) and 8,318 controls (<50th percentile of BMI) of European ancestry. We followed up 8 novel signals for replication in 9 independent replication samples of European ancestry. We observed two loci robustly associated with childhood obesity, namely near *OLFM4* and within *HOXB5* located on chromosome 13 and 17 respectively. Both genes have been implicated in different aspects of gut function, but further functional characterization is needed to elucidate the precise underlying mechanisms.

In **chapter 3** we focused on fetal and infant growth patterns, parental exposures during pregnancy and genetic determinants and their association with childhood cardiovascular development.

In **chapter 3.1** we aimed to identify patterns of fetal and infant growth associated with childhood blood pressure, carotid-femoral pulse wave velocity and cardiac structures and function. We found that birth weight was inversely associated with cardiovascular function and positively associated with cardiac structures. These associations were modified by postnatal growth patterns. Children born SGA with catch-up growth had a higher systolic blood pressure, and LGA without catch-down growth had the largest left ventricular mass, compared to children born AGA without postnatal catch-up or catch-down growth. This suggests that specific fetal and infant growth patterns are associated with cardiovascular development.

In **chapter 3.2** we assessed the associations of both maternal and paternal smoking during pregnancy with childhood cardiovascular structures and function. We observed a dose-dependent association of the number of cigarettes smoked by the mother during pregnancy with diastolic blood pressure, but not with systolic blood pressure. Maternal smoking during pregnancy was not associated with childhood carotid-femoral pulse wave velocity or left cardiac structures. Maternal smoking of ten or more cigarettes per day was associated with a higher fractional shortening in childhood. Among mothers who did not smoke during pregnancy, paternal smoking was associated with aortic root diameter, but not with the other cardiovascular outcomes. The stronger effect estimates for maternal smoking compared to paternal smoking might suggest that direct intra-uterine adaptive responses are involved as underlying mechanisms.

In **chapter 3.3** we examined whether maternal and paternal psychological distress were associated with the cardiovascular outcome measurements in school age children. We assessed maternal and paternal psychological distress during pregnancy by questionnaire, using the Brief Symptom Inventory. At the child age of six years, we performed blood pressure and carotid- femoral pulse wave velocity measurements, and M-mode measurements of left cardiac structures and fractional shortening. We did not observe associations of high maternal and paternal psychological symptom scores with childhood blood pressure and carotid-femoral pulse wave velocity after adjustment for potential confounders. Maternal overall psychological symptoms were associated with a lower childhood left ventricular mass. The association of paternal overall psychological symptoms with childhood left ventricular mass showed a similar effect size. This may suggest that these associations could be due to unmeasured social and environmental factors, rather than direct intra-uterine effects.

In **chapter 3.4** we investigated the associations of maternal first trimester dietary intake with blood pressure in children at the age of six years. We assessed first trimester maternal daily dietary intake by a food frequency questionnaire and measured folate,

homocysteine and vitamin B12 concentrations in blood. Childhood systolic and diastolic blood pressure was measured using a validated automatic sphygmomanometer. Maternal intake of macronutrients was not associated with childhood blood pressure. We found associations of higher maternal iron intake with a lower childhood systolic blood pressure and higher maternal vitamin B12 concentrations with a higher diastolic blood pressure, although the effect sizes were small and borderline significant. Concentrations of folate and homocysteine in first trimester pregnancy were not associated with childhood blood pressure. The results of this study do not provide clear evidence for nutritional programming of childhood blood pressure at this age.

In **chapter 3.5** we aimed to assess to what extent multiple common genetic variants contribute to blood pressure regulation in both adults and children, and to assess overlap in variants between different age groups, using a technique called genome-wide profiling. We created sets of common genetic variants based on genome-wide association study meta-analyses on systolic and diastolic blood pressure performed by the Cohort for Heart and Aging Research in Genome Epidemiology consortium. Subsequently, genetic risk scores for systolic and diastolic blood pressure were calculated in an independent adult population and a child population. Genetic risk scores, including also many non-genome-wide significant genetic variants explained more of the variance than scores based only on very significant genetic variants in adults and children. These findings suggest the presence of many genetic loci with small effects on blood pressure regulation both in adults and children, indicating also a (partly) common polygenic regulation of blood pressure throughout different periods of life.

Studies focused on early environmental and genetic determinants of renal growth, function and development are presented in **chapter 4**. We constructed gender specific reference growth curves for kidney growth (left and right kidney length, width, depth and volume) from third trimester of pregnancy until the age of two years (**chapter 4.1**). Kidney size was significantly influenced by age and gender. These reference curves can be used for assessing kidney structures by ultrasound in fetal life and early childhood.

In **chapter 4.2**, we examined whether maternal smoking during pregnancy, as important adverse fetal exposure is associated with fetal (third trimester of pregnancy) and infant kidney volume (2 years of age) measured by ultrasound. Among continued smoking mothers, we observed dose-dependent associations between the number of cigarettes smoked during pregnancy with kidney volume in fetal life. Smoking less than 5 cigarettes per day was associated with larger fetal combined kidney volume, while smoking more than 10 cigarettes per day tended to be associated with smaller fetal combined kidney volume. These associations were not seen at the age of 2 years. Our results suggest that smoking during pregnancy affects kidney development in fetal life with a time-specific and dose-dependent relationship.

In **chapter 4.3** we aimed to identify early life environmental factors associated with kidney volume, kidney function and blood pressure at age of six years. We identified that maternal anthropometrics, smoking during pregnancy and birth weight for gestational age were the main determinants of total kidney volume and function (creatinine and cystatin C levels in serum). Furthermore, we showed that kidney volume at the age of six years is positively associated with kidney function in childhood. These results are in line with the 'hyperfiltration hypothesis' and indicate that the developing kidney is important for future adult health. Modifiable exposures that affect kidney development can be potential targets for future intervention studies.

In **chapter 4.4** we examined whether common genetic variants previously associated with adult blood pressure and kidney function might affect kidney volume in fetal life. After taking into account multiple testing, one variant near *ZNF652* was significantly associated with fetal kidney volume, but the effect showed the opposite direction as expected. The remaining common genetic variants were not associated with fetal kidney volume. We also did not find associations of genetic variants previously shown to affect newborn kidney volume, with third trimester fetal kidney volume. Our results suggest that common genetic variants, previously associated with adult blood pressure and kidney function, do not underlie the associations of smaller kidneys with a reduced nephron endowment in early life with higher blood pressure or impaired kidney function in later life.

In **chapter 5** we discussed the results of the aforementioned chapters in a broader perspective and discussed methodological considerations, important for this line of epidemiological research. Furthermore, we describe possible clinical implications of the results of these studies and make suggestions for future research possibilities.

Samenvatting

Epidemiologische studies hebben laten zien dat de eerste levensfase na de geboorte een belangrijke rol kan hebben in het ontwikkelen van ziekten op volwassen leeftijd, zoals hart- en vaatziekten en type 2 diabetes. Deze studies hebben geleid tot het formuleren van de "Developmental Origins of Health and Disease Hypothese", die stelt dat een organisme zich op verschillende manieren kan ontwikkelen, afhankelijke van de omgeving waarin het zich bevind tijdens het foetale en vroege postnatale leven. Adaptaties die gemaakt worden in deze fase, zijn gunstig op korte termijn, maar kunnen nadelige effecten hebben op lange termijn. Vele observationele studies hebben laten zien dat laag geboorte geweest en snelle postnatale groei in de eerste maanden van het leven zijn geassocieerd met veelvoorkomende ziekten bij volwassenen zoals obesitas, hart-, vaat- en nierziekten. Wij redeneerden dat het onderzoeken van specifieke nadelige omgevingsfactoren en vroege groei, en hun associatie met hart-, vaat- en nierontwikkeling op de kinderleeftijd tot nieuwe inzichten zou kunnen leiden, in de onderliggende mechanismes van de associatie van laag geboorte gewicht met ziekten bij volwassenen. Genetische factoren die geassocieerd zijn met vroege groei zouden ook een deel van deze associatie kunnen verklaren. In dit proefschrift hebben we studies uitgevoerd om vroege omgevings- en genetische factoren van vroege groei en hart-, vaat- en nierontwikkeling te identificeren. In **hoofdstuk 1** geven we de achtergrond en redenen voor de verschillende studies in dit proefschrift gegeven.

In **hoofdstuk 2** bestuderen we omgevingsfactoren en genetische determinanten die verschillende aspecten van vroege groei beïnvloeden. In **hoofdstuk 2.1** hebben we de posnatale groei beschreven van kinderen die met een te laag, normaal of te hoog gewicht voor de zwangerschapsduur zijn geboren. Een groot deel van de kinderen met een te laag of te hoog gewicht voor de zwangerschapsduur lieten 'catch-up' en 'catch-down' groei zien in de eerste maanden na de geboorte. Ondanks deze compensatoire groei hadden deze kinderen persisterende verschillen in hoofdomtrek, lengte en gewicht op de leeftijd van 4 jaar. In deze studie hebben we ook onderzocht welke postnatale groei patronen in deze groepen kinderen, het risico op nadelige lichaamssamenstelling en overgewicht bij kinderen verhogen of verlagen. 'Catch-up' of 'catch-down' groei modificeerde de associatie van het gewicht voor de zwangerschapsduur en subcutane vet massa, body mass index (BMI), en het risico op overgewicht of obesitas op de leeftijd van 4 jaar. Dit effect was ook zichtbaar in kinderen met een normaal gewicht voor de zwangerschapsduur. Kinderen met een normaal gewicht voor de zwangerschapsduur die 'catch-up' groei laten zien, en kinderen met een te hoog gewicht voor de zwangerschapsduur zonder 'catch-down' groei hadden een hoger subcutane vetmassa en BMI op de kinderleeftijd, en een hoger risico op overgewicht. Deze resultaten laten zien dat

deze twee groepen kinderen mogelijke risico groepen zijn waarin vroege preventie van overgewicht bij kinderen toegepast zou kunnen worden.

In **hoofdstuk 2.2** hebben we onderzocht welke genetische varianten geassocieerd zijn met geboorte gewicht. Dit hebben we gedaan door gebruik te maken van genoomwijde associatie studies. Met deze aanpak kunnen onderzoekers de relatie testen tussen 2,5 miljoen genetische varianten en een uitkomst van interesse, in een analyse. Deze vorm van onderzoek maakt geen gebruik van een voorafgestelde hypothese die onderzocht word maar is een hypothesevrije onderzoeksvorm. In deze studie hebben we een meta-analyse uitgevoerd van 18 genoomwijde associatie studies en hebben de 21 sterkst geassocieerde, onafhankelijke en nieuwe signalen (P-waarde < $1x10^{-5}$) opgevolgd in 24 onafhankelijke studie populaties. In totaal hebben we 69,308 individuen geïncludeerd in deze studie. We toonden zeven genetische varianten aan die geassocieerd waren met geboorte gewicht. Vijf van deze varianten zijn eerder geassocieerd met andere uitkomsten op volwassen leeftijd. Genetische varianten in of nabij de genen *ADCY5* en *CDKAL1* zijn eerder geassocieerd met type 2 diabetes. Een genetische variant in *ADRB1* is eerder geassocieerd met bloeddruk en varianten in *HMGA2* en *LCORL* zijn eerder in relatie gebracht met volwassen eindlengte. Deze resultaten laten zien dat de veel beschreven associatie tussen laag geboorte gewicht en ziekten op latere leeftijd een waarschijnlijk ook een genetische component heeft.

Hoofdstuk 2.3 beschrijft een genoomwijde associatie studie van hoofdomtrek op de peuterleeftijd; rond de leeftijd van 1,5 jaar. We hebben hiervoor een meta-analyse uitgevoerd van 7 verschillende studies en de 3 sterkste signalen van deze analyse geselecteerd voor replicatie in 6 onafhankelijke studie populaties (totaal aantal geïncludeerde kinderen = 19,089). We hebben aangetoond dat twee genetische varianten in of nabij *HMGA2* en *SBNO1* zijn geassocieerd met hoofdomtrek op de peuterleeftijd. We vonden ook sterke aanwijzingen dat een genetische variant in een regio op chromosoom 17 ook geassocieerd is met hoofdomtrek op de peuterleeftijd. Deze regio bevat onder andere het *MAPT* en *CRHR1* gen en is voorheen geassocieerd met neurondegeneratieve ziekten zoals de ziekte van Alzheimer en Parkinson. Genetische varianten in dezelfde regio zijn geassocieerd met intra cranieel volume, een maat voor maximaal bereikt breinvolume. Dit betekent mogelijk dat genetische variatie in deze regio vroege breingroei in verband zou kunnen brengen met neurologische ziekten op later leeftijd.

In **hoofdstuk 2.4** hebben we bestudeerd of genen en omgeving met elkaar kunnen interacteren en een effect kunnen hebben op foetale groei. We hebben onderzocht of het wel of niet roken van de moeder tijdens de zwangerschap, het effect van een genetische variant in de nicotine receptor op geboorte gewicht kan modificeren. In moeders die rookten tijdens de gehele zwangerschap, was elk risico allel van de genetische variant in de nicotine receptor geassocieerd met een verminderde foetale groei. In moeders die niet rookten tijdens de zwangerschap, vonden we geen associatie tussen het genotype

en foetale groei. Dit geeft aan dat de omgeving (roken van de moeder) en het genotype (nicotine receptor) samen interacteren en foetale groei vertragen.

Hoofdstuk 2.5 maakt opnieuw gebruik van een genoomwijde associatie studie aanpak, met het doel genetische varianten te identificeren die geassocieerd zijn met overgewicht bij kinderen. We hebben een meta-analyse uitgevoerd van 14 genoomwijde associatie studies, bestaande uit 5,530 gevallen van obesitas (>95ste percentiel van BMI) en 8,138 controles (<50ste percentiel voor BMI) van Europese afkomst. Vervolgens hebben we de 8 sterkst geassocieerde, niet eerder beschreven, genetische varianten geselecteerd voor replicatie in 9 onafhankelijke studies. In deze studie toonden we aan dat twee genetische varianten, nabij *OLFM4* en in *HOXB5* (liggend op respectievelijk chromosoom 13 en 17), geassocieerd zijn met overgewicht bij kinderen. Beide genen zijn eerder in verband gebracht met verschillende aspecten van de functie van de darmen, echter vervolgonderzoek is nodig om precies te achterhalen hoe deze genetische varianten leiden overgewicht op de kinderleeftijd.

In **hoofdstuk 3** hebben we de focus gelegd op patronen van foetale en vroege postnatale groei, blootstellingen tijdens de zwangerschap, genetische varianten en hun associatie met cardiovasculaire ontwikkeling van kinderen. In **hoofdstuk 3.1** hadden we als doel groeipatronen van foetale en vroeg postnatale groei te identificeren die geassocieerd waren met bloeddruk, carotis-femoralis pulse wave velocity en structuren en functie van het hart op zesjarige leeftijd. We vonden dat geboorte gewicht negatief geassocieerd was met cardiovasculaire functie en positief geassocieerd met cardiale structuren. Deze associaties werden gemodificeerd door postnatale groeipatronen. Kinderen met een klein gewicht voor de zwangerschapsduur met 'catch-up' groei hadden een hogere bloeddruk op de leeftijd van zes jaar, kinderen met een te hoog gewicht voor de zwangerschapsduur zonder 'catch-down' groei, hadden de grootste cardiale structuren, vergeleken met kinderen geboren met een normaal gewicht voor de zwangerschapsduur zonder 'catch-up' of 'catch-down' groei. Dit suggereert dat specifieke groeipatronen in het vroege leven geassocieerd zijn met cardiovasculaire ontwikkeling.

In **hoofdstuk 3.2** hebben we de associatie tussen roken van zowel moeder als vader tijdens de zwangerschap en cardiovasculaire structuren en functie onderzocht. Op de leeftijd van zes jaar hebben we bloeddruk metingen verricht, carotis-femoralis pulse wave velocity bepaald en middels M-mode echografie hebben we metingen verricht van de cardiale structuren en fractionele verkorting van de linker kamer. We vonden een dosisafhankelijke associatie tussen het aantal sigaretten gerookt door de moeder en een hogere diastolische bloeddruk bij de kinderen, maar niet met de systolische bloeddruk. Het roken tijdens de zwangerschap van de moeder was niet geassocieerd met carotis-femoralis pulse wave velocity of hartstructuren. Kinderen van moeders die meer dan tien sigaretten per dag rookten, hadden een hogere fractionele verkorting dan kinderen

van moeders die niet rookte tijdens de zwangerschap. In kinderen van moeders die niet rookte tijdens de zwangerschap, was roken van de vader geassocieerd met de diameter van de wortel van de aorta, maar niet met andere structuren van het hart. Het grotere effect grootte van roken van de moeder tijdens de zwangerschap op diastolische bloeddruk en fractionele verkorting, in vergelijking met de het roken van vader, suggereert dat er intra-uteriene adaptaties betrokken zijn als onderliggend mechanisme.

In **hoofdstuk 3.3** hebben we onderzocht of psychologische distress bij moeder en vader is geassocieerd met de cardiovasculaire uitkomstmaten bij kinderen rond de schoolleeftijd. We hebben psychologische distress gemeten met de 'Brief Symptom Inventory'-vragenlijst. We hebben geen associaties aangetoond van hoge psychologische symptoom scores van moeder of vader met bloeddruk en carotis-femoralis pulse wave velocity in de kinderen, na correctie voor potentieel verstorende factoren. Hoge scores van moeder op overall psychologische symptomen was geassocieerd met lagere linker ventrikel massa. De associatie van dezelfde scores van vader met linker ventrikel massa liet een zelfde effect grootte zien, wat suggereert dat de associaties verklaard kunnen worden door ongemeten sociale en omgevingsfactoren, in plaats van direct intra-uteriene mechanismes.

Hoofdstuk 3.4 beschrijft de associaties van het dieet van de moeder tijdens het eerste trimester van de zwangerschap en bloeddruk van 6 jarige kinderen. We hebben het dieet gemeten met behulp van een voedingsvragenlijst en hebben folaat, homocysteine en vitamine B12 concentraties gemeten in het bloed van de moeder. Systolische en diastolische bloeddruk bij de kinderen werd gemeten met een gevalideerde automatische sphygmanometer. De maternale intake van macronutriënten was niet geassocieerd met bloeddruk bij kinderen. Een hoge intake van ijzer in het eerste trimester van de zwangerschap was geassocieerd met een lagere systolische bloeddruk van de kinderen. Hogere concentraties van vitamine B12 waren geassocieerd met een hogere diastolische bloeddruk. Deze associaties hadden echter een klein effect grootte en waren marginaal significant. Concentraties van folaat en homocysteine in het eerste trimester van de zwangerschap waren niet geassocieerd met bloeddruk bij kinderen van zes jaar. De resultaten van deze studie geven geen duidelijk bewijs voor het programmeren van bloeddruk door bij kinderen van deze leeftijd door maternale voeding tijdens de zwangerschap.

In **hoofdstuk 3.5** hebben we bepaald of meerdere genetische varianten bijdragen tot de regulatie van bloeddruk in volwassenen en kinderen, en of er overlap in genetische varianten belangrijk voor bloeddruk, tussen deze leeftijdsgroepen. We hebben gebruik gemaakt van een techniek genaamd 'genome-wide profiling'. We hebben sets van genetische varianten gecreëerd op basis van een genoomwijde associatie studie op systolische en diastolische bloeddruk bij volwassenen, uitgevoerd door het CHARGE consortium. Vervolgens hebben we voor elk van deze sets, genetische risico scores

berekend voor individuen in twee onafhankelijke studies in volwassenen en kinderen. Genetische risico scores die informatie van veel genetische varianten bevatten, waren beter in het verklaren van de variatie in bloeddruk dan scores berekend op basis van alleen de sterkst geassocieerde genetische varianten, zowel in volwassenen als in kinderen. Deze bevindingen suggereren dat er veel genen/genetische varianten die een rol spelen bij de regulatie van bloeddruk in volwassenen en kinderen, en dat er een gedeeld gemeenschappelijke genetische regulatie van bloeddruk aanwezig is gedurende de beide levensfasen.

De studies die zich focussen op omgevingsinvloeden en genetische determinanten van niergroei, nierfunctie en nierontwikkeling worden gepresenteerd in **hoofdstuk 4**. We hebben geslachtsspecifieke referentie curven gecreëerd voor niergroei (linker en rechter nier lengte, wijdte, transverse diameter en volume) van het derde trimester van de zwangerschap to de leeftijd van 2 jaar (**hoofdstuk 4.1**). Leeftijd en geslacht hadden een significante invloed op de grootte van de nieren. Deze referentie curven kunnen gebruikt om de normale en abnormale groei van nieren in het foetale en vroeg postnatale leven te bestuderen.

In **hoofdstuk 4.2** hebben we bestudeerd of maternaal roken tijdens de zwangerschap, als een belangrijke potentieel veranderbare nadelige omgevingsfactor voor de foetus, is geassocieerd met niervolume in het derde trimester van de zwangerschap en op de leeftijd van twee jaar. In moeders die rookte tijdens de gehele zwangerschap, observeerden we een dosisafhankelijke associatie tussen het aantal sigaretten dat de moeder rookte en foetaal niervolume. Moeders die minder dan 5 sigaretten per dag rookten, hadden foetussen met een groter niervolume, terwijl bij moeders die meer dan 10 sigaretten per dag rookten de foetussen juist kleinere nieren leken te hebben. Deze associaties konden we niet aantonen op de leeftijd van 2 jaar. Deze resultaten suggereren dat roken tijdens de zwangerschap de foetale nierontwikkeling zou kunnen verstoren, maar dat dit afhangt van het moment en de hoeveelheid van het roken.

In **hoofdstuk 4.3** hadden we als doel vroege determinanten van nier volume, nierfunctie en bloeddruk op de leeftijd van zes jaar te onderzoeken. We vonden dat anthropometrie van de moeder, roken van de moeder tijdens de zwangerschap en geboortegewicht voor de zwangerschapsduur, de belangrijkste determinanten waren van niervolume en nierfunctie (serum creatinine en cystatine C). We vonden eveneens dat een groter niervolume sterk geassocieerd was met een betere nierfunctie op de leeftijd van zes jaar. Deze resultaten zijn in overeenstemming met de hyperfiltratie hypothese. Dit suggereert dat een goede nierontwikkeling belangrijk is voor de gezondheid op latere leeftijd. Beïnvloedbare omgevingsfactoren die de nierontwikkeling beïnvloeden, kunnen mogelijke 'targets' voor toekomstige interventie studies.

In **hoofdstuk 4.4** hebben we onderzocht of genetische varianten die eerder gerelateerd waren aan bloeddruk of nierfunctie in volwassenen, ook geassocieerd waren aan foetaal niervolume gemeten in het derde trimester van de zwangerschap. Nadat we hebben gecorrigeerd voor de meerdere geteste hypotheses, bleef een genetische variant nabij *ZNF652* significant geassocieerd met foetaal niervolume. Het effect liet echter de omgekeerde richting zien dan we verwachtten. De andere genetische varianten waren niet geassocieerd met foetaal niervolume. We vonden ook geen associatie met foetaal niervolume van genetische varianten die eerder geassocieerd waren met niervolume bij pasgeborenen. Deze resultaten laten zien dat genetische varianten, eerder geassocieerd met bloeddruk of nierfunctie bij volwassenen, de associatie tussen kleinere nieren met verminderd aantal nefronen en hoge bloeddruk en verminderde nierfunctie op latere leeftijd niet kunnen verklaren.

In **hoofdstuk 5** worden de resultaten van de studies in dit proefschrift in een breder perspectief besproken en bespreken we methodologische aspecten, belangrijk voor deze lijn van epidemiologisch onderzoek. Vervolgens beschrijven we mogelijke klinische implicaties van de resultaten uit dit proefschrift en doen suggesties voor toekomstige gebieden van onderzoek.

Chapter 7

About the author
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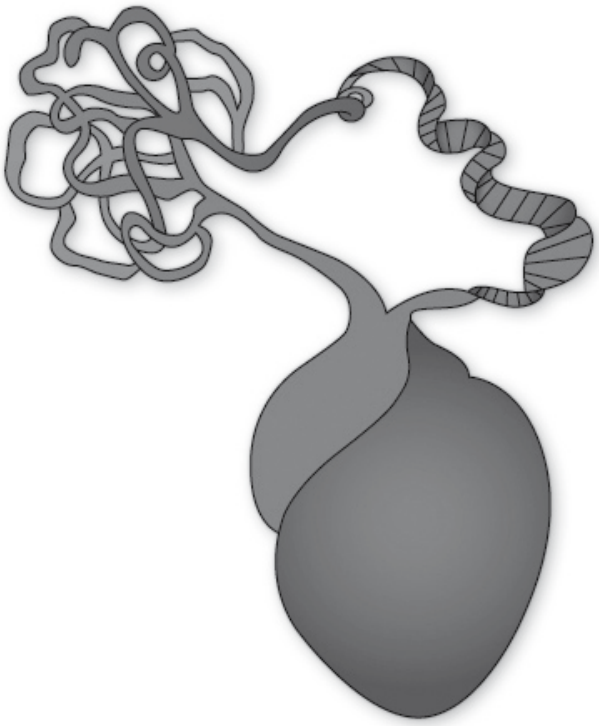

About the author

Hendrik Robert Taal was born on the 5th of July 1982, in The Hague, the Netherlands. He is the third son of Theo and Joke Taal. In 2000, he graduated from the Christelijk Gymnasium Sorghvliet in The Hague. Subsequently he started his medical education at the Erasmus University, Rotterdam, the Netherlands. As part of his medical training he performed research in the department of Internal Medicine at the Erasmus Medical Center with dr. W. de Herder which resulted in his first scientific paper in 2005. In January 2007 he graduated from Medical School and started working as a resident in pediatrics (ANIOS) at the Reinier de Graaf Gasthuis in Delft (dr. N. van der Lely). Nine months later he continued his residency (ANIOS) at the Sophia Childrens Hospital in Rotterdam (prof. A.J. van der Heijden, dr. M. de Hoog). During this period he was involved in clinical research with dr. Govaert, which resulted in two scientific papers on neonatal stroke. In 2009, he started working as a PhD student at the Generation R Study in the Growth and Development group (co-promotor Dr. V.W.V. Jaddoe and prof. E.A.P. Steegers) in collaboration with the departments of Pediatrics (promotor prof. A.J. van der Heijden) and Epidemiology (promotor prof. A. Hofman) at the Erasmus Medical Center in Rotterdam. His research focused on genetic and environmental factors that influence early growth, cardiovascular and renal development. In August 2012 he resumed his work as resident at the department of Pediatrics in the Sophia Childrens Hospital in Rotterdam and from April 2013 onwards he will start his training in Pediatrics (AIOS) in the Amphia Hospital in Breda (dr. A.A.P.H. Vaessen-Verberne).

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Submitted

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PhD Portfolio

Summary of PhD training and teaching activities

Name PhD student:	Hendrik Robert Taal
Erasmus MC Department:	Epidemiology and Pediatrics
Research School:	Netherlands Institute for Health Sciences
PhD period:	Jan 2009 – July 2012
Promotors:	Prof dr. A.J. van der Heijden and Prof. Dr. A. Hofman
Supervisor:	Dr. V.W.V. Jaddoe

1. Phd Training

	Year	Workload (ECTS)
General research skills		
- Master's Degree Clinical Epidemiology, NIHES	2009-2011	
- Principles in Research in Medicine		0.7
- Clinical Decision Analysis		0.7
- Methods of Clinical Research		0.7
- Clinical Trials		0.7
- Topics in Meta-analysis		0.7
- Health Economics		0.7
- Case-control Studies		0.7
- Principles of Genetic Epidemiology		1.4
- Introduction to Decision-making in Medicine		0.7
- Topics in Health and Diseases in the Elderly		0.7
- Demography of Ageing		0.7
- Markers in Prognostic Research		0.7
- Study Design		4.3
- Classical Methods for Data-Analysis		5.7
- Clinical Epidemiology		5.7
- Methodologic Topics in Epidemiologic Research		1.4
- Modern Statistical Methods		4.3
In-depth courses		
- Advances in genome-wide association studies	2008	1.4
- Advanced Topics in Clinical Trials	2009	0.7
- Introduction to Clinical and Public Health Genomics	2009	0.7
- Mendelian Randomisation	2010	0.7
- Mendelian Randomization and Bayesian Modelling in Genetic Epidemiology	2011	0.7
- Winterschool Dutch Kidney Foundation	2012	1.4
- Stralings cursus	2010	0.4
- Echo cursus Fontys	2009	1.0
Symposia		
- Metabolomics of the Obese	2009	0.7
- Generation R Symposium 2009	2009	0.7
- Generation R Symposium 2010	2010	0.7
- Surveying Children in Longitudinal Studies	2011	0.7

(Inter)national conferences and presentations:

- European Society of Pediatric Research, Hamburg	2009	0.7
- Developmental Origins of Health and Disease, Santiago, Chili	2009	1.4
- Generation R research meeting, Rotterdam	2010	0.2
- Gelre Ziekenhuizen, Apeldoorn	2010	0.2
- Generation R symposium: Genetics in Child Cohort Studies	2010	0.2
- Early Genetics & Lifecourse Epidemiolgy meeting, Oslo	2010	0.7
- Methodology working group, Department of Public Health, Rotterdam	2011	0.2
- Early Growth Genetics meeting, Rotterdam	2011	0.7
- Conference of Epidemiological Longitudinal Studies Europe, Cyprus	2011	1.4
- European Society of Human Genetics Conference, Amsterdam	2011	0.7
- Research Day Pediatrics, Sophia Children's Hospital, Rotterdam	2011	0.2
- Jonge onderzoekersdag NVK, Velthoven	2011	0.2
- Early Growth Genetics meeting, London	2012	0.7
- Developmental Origins of Health and Disease, Rotterdam, the Netherlands	2012	0.7

Reviewing papers

- Review papers for various international journals (the Lancet, European Journal of Epidemiology, Human Molecular Genetics, Plos One, Pediatric Research, Archives of Diseases in Childhood, International Journal of Epidemiology, Early Human Development)

2. Teaching

	Year	Workload (ECTS)
Supervising Master's and Bachelor Theses		
- Supervisor Denise Heppe, Clinical Research, NIHES. Thesis topic: "Bone age assessment by dual-energy X-ray absorptiometry in children"	2009	2.0
- Supervisor Lisan Leermakers, Clinical Research, NIHES. Thesis topic: "A Common Genetic Variant at 15q25 Modifies the Associations of Maternal Smoking during Pregnancy with Fetal Growth"	2011	2.0
- Supervisor Liset Grootten, Student Human Health Sciences, Bachelor Thesis Topic: "Protein intake during pregnancy and fetal and infant kidney volume"	2011	2.0
- Supervisor Leontine van den Hil, Medical student, Bachelor Thesis topic: "Maternal diet in first trimester of pregnancy and childhood blood pressure"	2011-2012	3.0

Dankwoord

Voordat ik begin aan het deel van dit proefschrift met de grootste (sociale) impact factor, wil ik allereerst graag alle deelnemers aan het Generation R onderzoek bedanken. Zonder jullie geen data, geen succes en geen promotie – kortom: onmisbaar. Ik hoop dat alle deelnemers nog lang betrokken blijven bij het onderzoek om de gezondheid voor de komende generaties te bevorderen.

Ik wil op deze plaats graag mijn beide promotoren bedanken. Professor van der Heijden, beste Bert, als promotor hebt u mij in discussies over manuscripten zeer scherp gehouden en hield altijd oog voor de eventuele klinische implicaties van de resultaten. U hebt me laten zien hoe je de wetenschap met de kindergeneeskunde kan combineren en ik kijk ernaar uit ook op klinisch vlak veel van u te leren. Professor Hofman, beste Bert, bedankt voor de begeleiding. Als geen ander kunt u promovendi en studenten motiveren tot het doen van onderzoek. Dank voor uw enthousiasme en begeleiding tijdens dit project.

Dr. Jaddoe, beste Vincent. Nadat ik vier en een half jaar geleden teleurgesteld in de arts-assistenten kamer binnen kwam en vertelde over mijn gefrustreerde pogingen een promotieplek te bemachtigen, heb je mij als promovendus binnen gehaald bij Generation R. Dat wij elkaar kenden maakte de start makkelijker en onze samenwerking is altijd recht door zee geweest. Ik wil je heel erg bedanken voor alle begeleiding op wetenschappelijk gebied, interesse op persoonlijk vlak en je hulp bij de voortzetting van mijn loopbaan en toekomst als kinderarts!

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Drie en een half jaar heb ik mijn werkplek gehad in het Ae-gebouw, kamer Ae-026, als een van de weinigen zonder kamerswitch. Ik wil graag al mijn kamergenoten bedanken voor de fijne en gezellige tijd met jullie. Hanan, jij hebt me opgevangen toen ik als wetenschappelijk groentje arriveerde uit de kliniek. Ik kon altijd met vragen bij je terecht, dank nogmaals voor alle hulp. Buiten je hulp, was het bovenal natuurlijk erg gezellig om af en toe even te kletsen over andere zaken in het leven (en ik hoop dat er

nog vele kamerlunches zullen volgen!). Layla, ruim 3 jaar zijn wij elkaars kamergenoten geweest. Een superleuke tijd, die ik voor geen goud had willen missen. We gingen al snel met elkaar op congres naar Hamburg en Chili en later volgden ook London en Cyprus. Je bent altijd vrolijk, vooral 's ochtends vroeg als ik nog maar net wakker ben. Dank voor je interesse in alles wat ik wel en niet deed. Ik ben ervan overtuigd dat je een uitstekende radioloog zult worden! Nina en Ank, jullie verblijf in Ae-026 was relatief kort, maar ook jullie bedankt voor alle gezelligheid! Ik ben ervan overtuigd dat jullie goede onderzoekers zijn en een uitdagend project als dat van jullie tot een uitstekend einde zullen brengen. Selma, de laatste anderhalf jaar ben jij Ae-026 komen versterken. Na Hanan en Ank, beide rustig, was het een kleine cultuurshock en moest ik wel even wennen aan de snelheid van onze gesprekken. Samen met Layla hebben we het Ae-026 diner ingevoerd, ik hoop dat er nog vele volgen!

Natuurlijk zat ik niet gekluisterd in een kamer en wil ik graag ook alle andere collega promovendi en studenten bedanken met wie ik samen heb gewerkt. Met het risico een paar namen te missen (bij voorbaat: excuses) wil ik hier graag noemen; Agnes (buurvrouw!), Ankie (goed voorbeeld doet volgen), Claudia (lunch buddy), Denise (mijn mede kindergeneeskunde geïnteresseerde), Dennis (genetisch expert), Edith (1 van de 5 ;)), Fleur (London altijd gezellig!), Hanneke (stress, stress, maar onze paper is er uiteindelijk toch bij! Dank!!), Jolien de G-S (superuitje), Leontine (keigezellig), Lisan (ook keigezellig), Marieke en Nienke (gillend door de muren heen te horen), Miranda (organisatietalent), Monica (si!), Ralf (ja dit is goed genoeg voor NG!) en Romy (GWAS is echt leuk hoor).

Uiteraard wil ik hier ook mijn paranimfen eren. Arjan en Layla, ik kan mij geen beter duo voorstellen om me bij te staan tijdens de verdediging. Arjan, dank voor al je belangstelling de afgelopen jaren. Onze discussies over de politiek, economie, financiën en andere gewichtige zaken (uiteraard onder genot van), en onze stapavonden waren zeer welkome intermezzo's in het onderzoekersleven. Ik hoop dat er nog vele van deze avonden volgen in het leven als arts in opleiding! Lieve Layla, we hebben al zoveel gedeeld de afgelopen jaren dat je uiteraard ook mijn paranimf moet zijn. Dank voor alle steun de afgelopen tijd!

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