Early Influences on Cardiovascular and Renal Development

The Generation R Study

Jacomina Jessica Miranda Geelhoed

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The Generation R Study

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List of abbreviations

AC	Abdominal circumference
A-wave	Active atrial contraction filling peak velocity
BMI	Body mass index
BP	Blood pressure
CI	Confidence interval
CO	Cardiac output
CV	Coefficient of variation
DBP	Diastolic blood pressure
EFW	Estimated fetal weight
E-wave	Passive early ventricular filling peak velocity
FL	Femur length
GA	Gestational age
HC	Head circumference
ICC	Intraclass correlation coefficient
OR	Odds ratio
PI	Pulsatility index
PSV	Peak systolic velocity
SD	Standard deviation
SDS	Standard deviation score
SBP	Systolic blood pressure
Yrs	Years

Introduction

1

Introduction

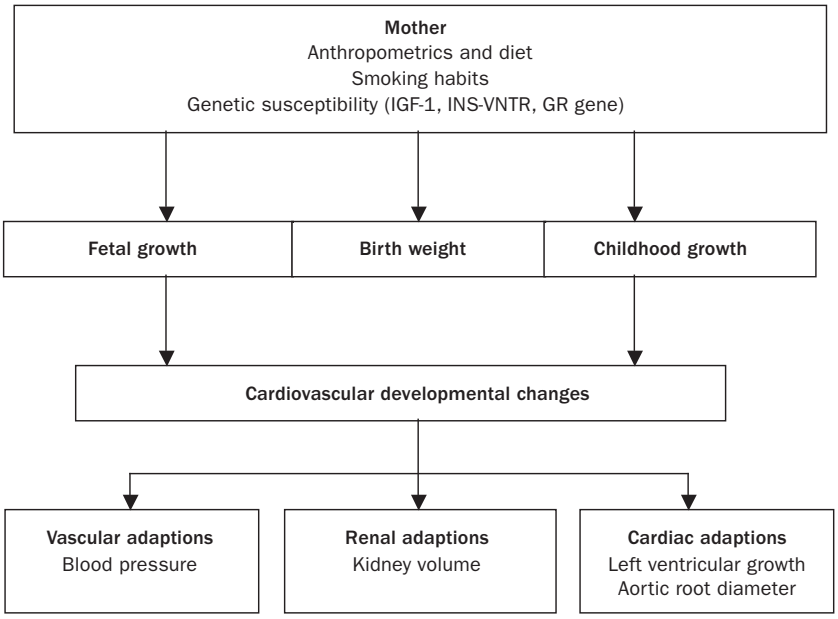
Rationale

Cardiovascular disease is common in the general population, affecting 40-60% of adults older than 60 years of age. Many lines of evidence indicate an important role of early life events in influencing later susceptibility to cardiovascular disease. Barker and Osmond demonstrated that areas of Britain with the highest neonatal mortality rates early in the 20th century also tended to have the highest rates of coronary heart disease many decades later.[1] After this, observational studies showed that low birth weight and weight at one year were associated with an increased risk of later cardiovascular disease[2], especially in subjects who show a postnatal catch-up growth and become obese as adults.[3,4] The most commonly studied risk factors for cardiovascular disease include blood pressure and total cholesterol levels.[5,6] Several studies showed small but consistent effects.[7-9] These observations resulted in the "fetal origins of adult disease" hypothesis. More recently, this hypothesis has been transformed to a more general "developmental plasticity hypothesis"[10], which suggests that an organism may develop in various ways, depending on the particular environment or setting.[11] In this process, adverse environmental exposures in fetal and early postnatal life lead to adaptations that permanently program the fetus' structure, physiology and metabolism. These adaptations may be beneficial in the short term but have adverse consequences at birth and in postnatal life, leading to both low birth weight and cardiovascular disease in adulthood. Thus, cardiovascular disease may at least partly originate in early fetal life.

Birth weight has been the most widely studied measure of fetal growth in studies examining the associations of birth-related measures with risk factors of disease in later life.[12-14] The effect size of low birth weight on cardiovascular disease and blood pressure in adulthood seems to be small and the major limitation of current studies is that the specific adverse exposures and underlying mechanisms are not known.[5] Also, residual confounding may still be an issue. To further explore the underlying mechanisms and to assess whether these associations are causal, research has to move onwards from birth weight association studies to adverse fetal exposure, and detailed early cardiovascular development studies.

We designed a prospective cohort study from early fetal life until the age of two years to identify mechanisms underlying the associations between low birth weight and cardiovascular disease (Figure 1). We used both maternal and fetal characteristics as specific exposures of both fetal growth retardation and cardiovascular developmental changes in fetal life and early childhood. The main focus was on cardiovascular and renal development, since adaptations in these organs may be associated with cardiovascular and renal disease in adulthood.

Figure 1. Associations studied in this thesis



Aims

The specific aims of this thesis were to study:

1. Genetic determinants of growth characteristics in fetal and early postnatal life. The main interest was in genetic variants related to insuline and insuline-like growth factors, and to glucocorticoid sensitivity.

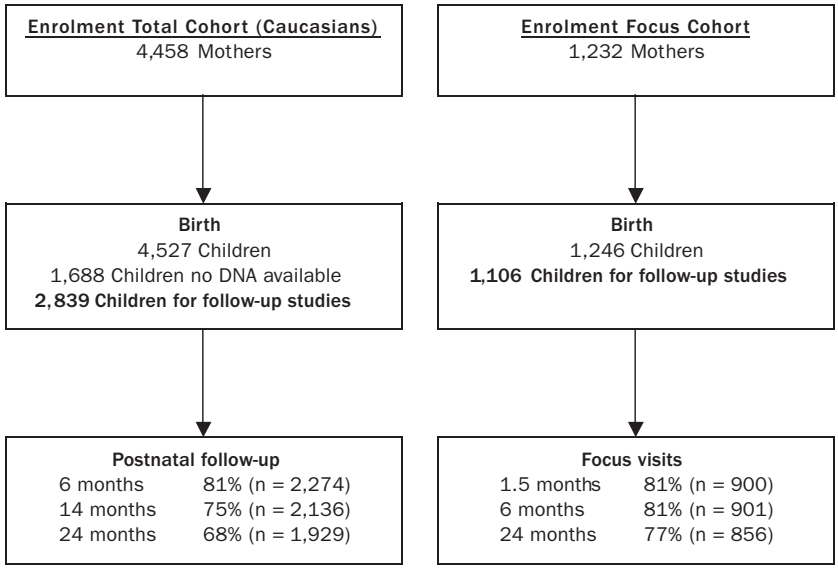
2. Early determinants of kidney growth and development from fetal life until childhood. Determinants of interest were maternal smoking and anthropometrics, as a measure of fetal nutrition, and fetal growth and blood flow characteristics measured in second and third trimester of pregnancy.

3. Genetic and environmental determinants of growth and development of the heart and blood vessels in fetal life and early childhood. Determinants of interest were maternal smoking and anthropometrics, and fetal growth and blood flow characteristics measured in second and third trimester of pregnancy

General design

Most studies 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.[15,16] The Generation R Study is designed to identify early environmental and biological determinants of growth, development and health in fetal life and childhood. All pregnant women living in the study area with a delivery date between April 2002 and January 2006 were eligible for enrolment in the Generation R Study. While the aim was to have participants enrole during pregnancy, it was possible until the birth of the child. Measurements were planned in early pregnancy (<18 weeks of gestation), mid-pregnancy (18-25 weeks of gestation), and late pregnancy (≥25 weeks of gestation). In total, 9,778 women were included, of whom 8,880 were enrolled in the prenatal part of the study (Figure 2). Detailed assessments of fetal growth and development were conducted in the Generation R Focus cohort.[15,16] In this subgroup of 1,232 Dutch pregnant women and their children, we performed detailed assessments, such as Doppler ultrasounds of the placental, and fetal cerebral and cardiac arteries, and renal ultrasounds in prenatal life and cardiac and renal ultrasounds at the postnatal ages of 1.5, 6 and 24 months. This subgroup is ethnically homogeneous to exclude confounding or effect modification by ethnicity.

Figure 2. Flow chart Generation R Cohort



One study was performed in the Avon Longitudinal Study of Parents and Children (ALSPAC). ALSPAC is a similar population-based prospective cohort study as the Generation R Study and investigates the health and development in children (www.alspac.bris.ac.uk).[17] Detailed information about the children has been collected from questionnaires administered through childhood, and clinical measurements have been performed on the entire cohort annually since the age of seven years. In total, 6,668 mother-offspring pairs, approximately 65% of those invited, attended the nine year follow-up clinic.

Outline of thesis

In **chapter 2**, the associations between genetic determinants with fetal and postnatal growth are presented. These genetic determinants include variation in the insulin-like growth factor-1 (*IGF1*) gene (**chapter 2.1**), insulin gene variable number of tandem repeats (*INS*-VNTR) (**chapter 2.2**) and genetic variants in the glucocorticoid receptor gene (**chapter 2.3**). **Chapter 3** demonstrates early determinants of kidney growth and development from third trimester of pregnancy until the age of two years. Reliability of kidney ultrasound measurements and renal reference growth curves for kidney growth from third trimester of pregnancy until the age of two years are presented in **chapters 3.1 and 3.2**. Studies focused on kidney growth determinants, such as maternal smoking during pregnancy, fetal and placental blood flow characteristics and fetal and postnatal growth characteristics are presented in **chapters 3.3, 3.4 and 3.5**. In **chapter 4** we studied early determinants of cardiac growth and development until the age of two years. Reliability of cardiac ultrasound measurements is demonstrated in **chapter 4.1**. Studied genetic and environmental determinants included glucocorticoid receptor-9β polymorphism, maternal smoking and anthropometrics during pregnancy, and fetal and postnatal growth. These results are presented in **chapters 4.2, 4.3, 4.4 and 4.5**. In **chapter 4.6**, the associations of hypertensive disorders of pregnancy with blood pressure in the offspring are presented. Finally, **chapter 5** provides a general discussion focused on our and other epidemiological studies designed to identify mechanisms underlying the associations of low birth weight with development of cardiovascular disease in adulthood. This discussion concludes with implications for future research and clinical practice.

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Genetic influences on fetal and early childhood growth

2

Chapter 2.1

Variation in the insulin-like growth factor-1 gene and growth in fetal life and infancy

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Abstract

Background: The objective of this study was to examine whether variants of the insulin-like growth factor-1 (*IGF1*) gene are associated with growth patterns from fetal life until infancy.

Methods: This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life. Fetal growth (head circumference, abdominal circumference, femur length, estimated fetal weight) was assessed by ultrasounds in early, mid- and late pregnancy. Growth in infancy was assessed at birth (weight) and at the ages of 6 weeks, 6 months and 14 months (head circumference, length, weight). *IGF1* promoter region genotype was determined in 738 children.

Results: Eight alleles of the *IGF1* promoter region were identified. In total, 43% of the subjects were homozygous for the most common 192-bp allele (wild-type), 45% were heterozygous, and 12% were non-carrier of the 192-bp allele. No differences were found in birth weight between the three groups. However, non-carriers had a lower estimated fetal weight in mid-pregnancy ($p = 0.040$), followed by an increased growth rate until 6 months ($p < 0.005$) in comparison to 192-bp homozygotes. A similar difference in growth rate was found for length ($p < 0.001$).

Conclusions: Variants of the *IGF1* promoter region are not associated with birth weight. However, non-carriers of the 192-bp allele tend to have a smaller fetal size, followed by an increased growth rate from mid-pregnancy to early infancy. Studies in larger cohorts are necessary to replicate our findings and to examine whether these effects persist throughout childhood.

Introduction

Birth weight is inversely associated with type 2 diabetes and cardiovascular disease in adulthood.[1] The fetal insulin hypothesis proposes that these associations are explained by common genetic variants.[1] Insulin and insulin like growth factors are important fetal growth factors.[2] Genetic factors related to insulin or insulin-like growth factors production and sensitivity may lead to both impaired fetal growth and to type 2 diabetes and cardiovascular disease in later life.[1]

Insulin-like growth factor-1 (IGF-1) is an essential regulator of both fetal and postnatal growth.[3-5] IGF-1 is also important for growth of insulin-producing β cells in the pancreas.[6] It has been suggested that IGF-1 is involved in postnatal changes in body composition, insulin sensitivity and development of type 2 diabetes and cardiovascular disease.[7, 8] A polymorphism in the promoter region of the *IGF1* gene has been identified, which has been associated with circulating IGF-1 levels.[9, 10] Previous studies have shown that the absence of the common 192-bp allele of the *IGF1* gene is associated with lower IGF-1 levels, lower birth weight and adult height, and an increased risk of type 2 diabetes and myocardial infarction.[7, 11-14] However, other studies were not able to replicate these findings.[15, 16] The inconsistent findings of studies examining the associations of this *IGF1* gene variant with birth weight may be explained by the fact that birth weight is an inappropriate measure of fetal growth. It has been shown that not children with low birth weight per se but those who are small at birth and have a subsequent postnatal accelerated weight gain leading to a normal or increased weight from childhood onwards are at increased risk of development of type 2 diabetes and cardiovascular disease.[17-19] Recently, the absence of the 192-bp allele was shown to increase the risk for accelerated weight gain in the first year of life.[20]

We hypothesized that variants of the *IGF1* gene may be stronger related to longitudinally measured growth in pre- and postnatal life than to one specific growth characteristic such as birth weight. Therefore, we examined in the Generation R study, a prospective prenatally recruited birth-cohort study, the associations of the *IGF1* promoter polymorphism with growth measured in different periods from fetal life to infancy.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. This study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail.[21, 22] In total, the cohort includes 9,778 mothers and their children living in Rotterdam, the Netherlands. A vast majority (69%) of all mothers was enrolled in the first trimester of pregnancy.[22] Assessments in pregnancy included physical examinations,

fetal ultrasounds, biological samples and questionnaires. These were planned in early (gestational age <18 weeks), mid- (gestational age 18-25 weeks) and late pregnancy (gestational age ≥25 weeks) to collect information about fetal growth and its main determinants. Their partners were assessed once during this period. The children were born between April 2002 and January 2006 and form a prenatally recruited birth-cohort that is currently being followed until young adulthood. Of all eligible children, 61% participated in the study at birth. Additional, more detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of 1,232 parents and their children, referred to as the Generation R Focus cohort. This subgroup is ethnic homogeneous to exclude possible confounding or effect modification by ethnicity. Of all approached women, 80% was enrolled in this subgroup study in late pregnancy (gestational age of 30 weeks). In this subgroup, postnatal examinations were performed at the ages of 6 weeks, 6 months, and 14 months. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants.

Population for analysis

In total, 1,232 women were enrolled in the Generation R Focus Study. Twin pregnancies (n = 15) and pregnancies leading to perinatal death (n = 2) were excluded from the present analysis. Of the remaining singleton 1,215 live births, *IGF1* genotyping was achieved in 61% (n = 738) of the subjects. Growth characteristics in mid- and late pregnancy, and birth weight were available in all these subjects. In total, 554 (75%), 551 (75%) and 538 (73%) of these subjects participated in the postnatal assessments at the ages of 6 weeks, 6 months and 14 months, respectively.

Genotyping assay *IGF1* gene

DNA was collected from cord blood samples of the children at birth. Cord blood for DNA isolation was available in 85% of all children participating in the Focus cohort. Missing of cord blood samples was mainly due to logistic constraints. Polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify the polymorphic cytosine-adenine (CA) repeat 1 kb upstream of the human *IGF1* gene.[23] The reaction was carried out in a final volume of 10 ml containing 50 ng of genomic DNA obtained from peripheral blood cells, 0.5 nmol/l forward primer (‘5-ACCACTCTGGGAGAAGGGTA-3’), 0.5 nmol/l reverse primer (‘5-GCTAGCCAGCTGGTGTTATT-3’), 0.25 mmol/l 2’-dNTP, 2.2 mmol/l MgCl2, 0.01% W1 (Gibco BRL), and 0.4 Taq DNA polymerase (Gibco BRL). PCR was performed in 284 well plates (94˚C 10 min; 35 PCR cycles of 30 s at 94˚C, 30 s on 55˚C, and 30 s on 72˚C; 72˚C 10 min; 4˚C hold). Forward primers were labeled with FAM, HEX or NED to determine the size of PCR products by autosequencer (ABI 3100, POP 4, filter set D, collecting time array 36 cm 7 s, peak-height between 100 and 2000, each lane containing three samples). The size of the PCR products was determined in comparison with internal ROX-size

standard (Perkin Elmer). Eight different alleles of the *IGF1* promoter region were identified (Table 1). The genotype frequency was similar to those found in previous studies and the frequency distribution did not deviate from the Hardy-Weinberg equilibrium (Chi-square = 0.005, p >0.9).[7, 20] As in previous studies, *IGF1* genotypes were categorized in the following categories based on their 192-bp allele: homozygous (wild-type), heterozygous and non-carrier.[11, 15, 16, 20] In our study, IGF-1 levels were not available due to logistical and financial constraints.

Table 1. *IGF1* promoter polymorphism- and genotype frequency distributions of the study population (n = 738)

Polymorphism			
Allele (base pairs)	**$(CA)_n$**	**Boys** (n = 376)	**Girls** (n = 362)
186	16	2 (0.3)	0
188	17	20 (2.7)	17 (2.4)
190	18	37 (4.9)	39 (5.4)
192 (wild-type)	19	494 (65.7)	474 (65.5)
194	20	146 (19.4)	129 (17.8)
196	21	51 (6.7)	55 (7.6)
198	22	2 (0.3)	9 (1.2)
200	23	0	1 (0.1)
Genotypes			
Homozygous 192-bp		166 (44.1)	151 (41.7)
Heterozygous 192-bp		162 (43.1)	172 (47.5)
Non-carriers 192-bp		48 (12.8)	39 (10.8)

Values are number of children (%). The allele distribution is based on 2 alleles per infant.
(CA)n: number of cytosine-adenine repeats; wild type allele: most frequent allele in this population.

Fetal growth and birth characteristics

Fetal ultrasound examinations were carried out at the visits in one of the research centers in early, mid- and late pregnancy. These fetal ultrasounds were used for establishing gestational age and for assessing fetal growth characteristics.[24] Pregnancy dating curves were constructed on subjects in the study with complete data on gestational age measured by ultrasound and last menstrual period. Crown-rump length was used for pregnancy dating in early pregnancy (gesta-

tional age until 12 weeks and 5 days, crown-rump length smaller than 65 mm) and biparietal diameter was used for pregnancy dating thereafter (gestational age from 12 weeks and 5 days onwards, biparietal diameter larger than 20 mm).[25, 26] Fetal growth measurements used for the present study included head circumference (HC), abdominal circumference (AC) and femur length (FL) in mid- and late pregnancy, measured to the nearest mm using standardized ultrasound procedures.[27] Estimated fetal weight (EFW) was calculated using the formula by Hadlock using head circumference, abdominal circumference and femur length ($\log_{10}$ EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC) + 0.171 (FL) + 0.00034 $(HC)^2$ – 0.003685 (AC * FL)).[28] Early pregnancy was not included since these fetal ultrasound examinations were primarily performed to establish gestational age. Gestational age-adjusted standard deviation scores (SDS) were constructed for all fetal growth measurements. These were based on reference growth curves from the whole study population.

Postnatal growth characteristics

Birth weight, date of birth and gender were obtained from community midwife and hospital registries. At the age of 6 weeks, 6 months and 14 months, subjects visited the research center. Anthropometrics were measured without clothes. Weight was measured to the nearest gram using electronic scales. Length was measured to the nearest millimeter in supine position using a neonatometer at the ages of 6 weeks and 6 months, and in up-right position at the age of 14 months. Head circumference was measured to the nearest millimeter.

Covariates

Information on maternal age, parity and weight before pregnancy was obtained by the first questionnaire at the enrolment in the study. Maternal smoking habits were assessed in each questionnaire. Maternal height and weight (in mid- and late pregnancy) was measured without shoes at our research center, and body mass index (weight/$height^2$ (kg/m^2)) was calculated. The occurrence of gestational diabetes was obtained from midwife or obstetric records. Information about duration of breastfeeding was obtained by postnatal questionnaires at the ages of 2, 6 and 12 months.

Statistical analysis

First, differences of maternal, fetal and postnatal characteristics between the *IGF1* genotype groups were assessed by the independent sample t-test or Mann-Whitney U test for continuous variables and the chi-square test for categorical variables. Subsequently, we performed multiple linear regression models to assess the associations of the *IGF1* gene with weight and length at

each age cross-sectionally (prenatally: mid- and late pregnancy; at birth; postnatally: 6 weeks, 6 months and 14 months). For length, we used femur length as a proxy for body length in the prenatal data and to compare the effect estimates, we used standard deviation scores for the cross-sectional analyses.[29] These models were adjusted for (gestational) age and gender of the child, maternal weight and height before pregnancy, increase in maternal weight during pregnancy, smoking, and breastfeeding (only postnatal growth). Since no data about maternal diet was available for this study, maternal weight gain was used as a proxy for nutritional status.[30] Age of mother, parity and the occurrence of gestational diabetes did not change the effect estimates and were therefore not included from the analyses.

Second, to assess longitudinally measured weight and length patterns from fetal life to infancy, we performed repeated measures regression analysis with (estimated) weight and (femur) length in fetal life and infancy as outcome. In these models, *IGF1* genotype group was included as both intercept and interaction with age. To account for (gestational) age at each specific measurement, these analyses were conducted with age-adjusted standard deviation scores. The models can be written as:

Weight (SDS) or length (SDS) = $\beta_0 + \beta_1$*age + β_2**IGF1* genotype + β_3**IGF1* genotype*age.

In this model, the term including 'β_0' reflects the intercept and the term including 'β_1' reflects the slope of growth (in weight or length) per week for the reference group (192-bp homozygous individuals). The terms including 'β_2' and 'β_3' reflect the age independent difference and the growth differences in weight (and length) between the different categories of the *IGF1* genotype, respectively. These models were adjusted for the same covariates as in the multiple linear regression models.[31]

Thirdly, using binary logistic regression analyses and taking the covariates into account, the associations of *IGF1* genotype with weight realignment in different periods were examined. Main interest was in realignment towards an increase in weight in infancy ("positive growth realignment"), which was defined as a change of weight of more than +0.67 SDS (meaning, for example, an increase from the 3rd to the 10th percentile).[32-34]

All effect estimates are presented with their 95% confidence interval (95% CI). Statistical analyses were performed using the Statistical Analysis System version 8.2 (SAS, Stata corporation, College station, TX, USA), including the PROC MIXED module for unbalanced repeated measurements and the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics for the three genotype groups are shown in Tables 2, 3 and 4. In total, 317 (43%) subjects were 192-bp homozygous, 334 (45%) were 192-bp heterozygous and 87 (12%) subjects were non-carriers. No differences in maternal, fetal and postnatal growth characteristics were observed between these groups.

Table 2. Maternal characteristics according to the fetal *IGF1* genotype

	Homozygous 192-bp allele (n = 317)	**Heterozygous 192-bp allele** (n = 334)	**Non-carrier 192-bp allele** (n = 87)
Maternal characteristics			
Age (years)	31.6 (4.0)	31.5 (4.4)	31.8 (3.8)
Weight before pregnancy (kg)	69.2 (12.7)	68.4 (12.7)	70.6 (13.5)
Height (cm)	171.1 (6.5)	170.9 (6.4)	171.8 (7.0)
Body mass index before pregnancy (kg/m^2)	23.7 (4.2)	23.3 (3.9)	23.8 (4.5)
Weight gain during pregnancy (kg)	10.0 (4.6)	10.1(4.5)	10.3 (4.4)
Parity (% nulliparous)	59.0	59.3	56.3
Gestational diabetes (%)	0.3	1.5	0
Smoking (%)			
No	76.0	76.6	80.5
Yes, until pregnancy	8.8	9.6	5.7
Yes, throughout pregnancy	15.1	13.8	13.8

Values are means (standard deviation) or percentages. Differences were tested using independent sample t-test or Chi-square test (all p-values >0.05). Of the total group, data were missing on maternal weight and body mass index before pregnancy (n=175), maternal weight gain (n=189), parity (n=4), gestational diabetes (n=4), and smoking (n=1).

Table 3. Fetal characteristics according to the fetal *IGF1* genotype

	Homozygous 192-bp allele (n = 317)	**Heterozygous 192-bp allele** (n = 334)	**Non-carrier 192-bp allele** (n = 87)
Fetal characteristics mid-pregnancy			
Gestational age (weeks)	20.6 (1.0)	20.5 (1.0)	20.5 (0.9)
Head circumference (mm)	178.6 (12.9)	178.1 (12.9)	177.5 (11.9)
Abdominal circumference (mm)	157.4 (13.5)	157.2 (13.8)	154.7 (11.6)
Femur length (mm)	33.2 (3.3)	32.9 (3.2)	32.6 (2.7)
Estimated fetal weight (grams)	379 (83)	374 (83)	360 (66)
Fetal characteristics late pregnancy			
Gestational age (weeks)	30.5 (1.1)	30.4 (1.0)	30.5 (0.9)
Head circumference (mm)	287.0 (11.7)	285.9 (11.6)	286.2 (11.5)
Abdominal circumference (mm)	266.6 (16.5)	266.8 (16.0)	265.2 (14.9)
Femur length (mm)	57.5 (3.0)	57.1 (2.8)	57.4 (2.9)
Estimated fetal weigh (grams)	1649 (265)	1639 (248)	1626 (228)

Values are means (standard deviation). Differences were tested using independent sample t-test or Chi-square test (all p-values >0.05). Of the total group, data were missing on mid-pregnancy gestational age (n=8), head circumference (n=13), abdominal circumference (n=13), femur length (n=13), estimated fetal weight (n=18), late pregnancy head circumference (n=3), abdominal circumference (n=3), femur length (n=3), and estimated fetal weight (n=6).

Table 4. Birth and postnatal characteristics according to the *IGF1* genotype

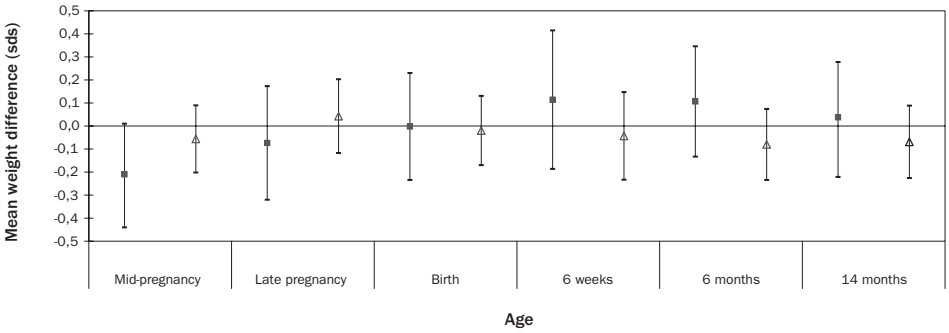
	Homozygous 192-bp allele (n = 317)	**Heterozygous 192-bp allele** (n = 334)	**Non-carrier 192-bp allele** (n = 87)
Birth			
Weight (grams)	3536 (494)	3546 (511)	3565 (452)
Gestational age (weeks)	40.1 (36.6 - 42.4)	40.4 (36.8 - 42.5)	40.6 (35.8 - 42.3)
Gender (% boy)	52.4	48.5	55.2
Postnatal characteristics 6 weeks			
Age at visit (weeks)	6.3 (4.5 - 12.0)	6.1 (4.6 - 13.1)	6.3 (4.2 - 10.9)
Head circumference (cm)	38.7 (1.3)	38.6 (1.6)	38.6 (1.5)
Weight (grams)	4946 (599)	4907 (762)	4970 (701)
Length (cm)	56.9 (2.5)	56.7 (2.8)	57.0 (2.3)
Postnatal characteristics 6 months			
Age at visit (weeks)	27.6 (23.7 - 34.8)	27.3 (23.6 - 35.6)	27.1 (23.9 - 34.9)
Head circumference (cm)	43.9 (1.4)	43.9 (1.4)	44.0 (1.1)
Weight (grams)	7980 (842)	7886 (900)	8100 (941)
Length (cm)	68.7 (2.6)	68.7 (2.7)	69.1 (2.8)
Postnatal characteristics 14 months			
Age at visit (months)	14.5 (13.4 - 16.4)	14.4 (13.4 - 17.6)	14.4 (13.1 - 18.9)
Head circumference (cm)	47.4 (1.4)	47.3 (1.4)	47.3 (1.7)
Weight (grams)	10556 (1042)	10481 (1074)	10675 (1263)
Length (cm)	79.0 (2.9)	79.1 (3.0)	79.4 (3.0)
Breastfeeding			
Never (%)	15.2	14.0	14.5
Yes, shorter than 6 months (%)	37.1	41.7	50.0
Yes, 6 months or longer (%)	25.8	22.7	19.7
Yes, duration unknown (%)	21.9	21.7	15.8

Values are means (standard deviation), medians (95% range) for variables with skewed distribution or percentages. Differences were tested using independent sample t-test, Mann-Whitney U test, or Chi-square test (all p-values >0.05). Of the total group, data were missing at the age 6 of weeks on head circumference 6 weeks (n=207), weight (n=198), length (n=201), at the of age 6 months on head circumference (n=194), on weight (n=191), on length (n=193), at the age of 14 months on head circumference (n=238), weight (n=208), length (n=214), and breastfeeding (n=79).

Differences in weight (SDS) and length (SDS) between the genotype groups at each age are given in Figure 1 and 2. These estimated differences were based on multiple linear regression models. In non-carriers, we found a lower weight in mid-pregnancy (difference: -0.24 (95% CI: -0.50, -0.01)

SDS) than in the 192-bp homozygous group. Similar non-significant differences were found for length (SDS), where femur length was shorter in mid-pregnancy in non-carriers (difference: -0.17 (95% CI: -0.42, 0.08) SDS). Moreover, length (SDS) at 6 months was significantly greater in non-carriers (difference: 0.32 (95% CI: 0.04, 0.61) SDS). Birth weight was similar in all three genotype groups and no differences in pre- and postnatal growth characteristics were observed between the 192-bp homozygous and 192-bp heterozygous groups.

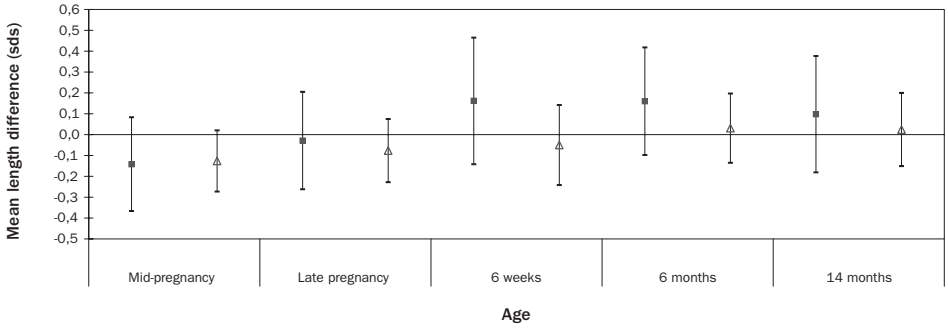
Figure 1. Mean weight difference between the three genotype groups using the homozygous 192-bp genotype as the reference group



Values represent estimated differences in SDS (95% confidence interval) based on multiple linear regression models, adjusted for (gestational) age and gender of the child, maternal weight and height before pregnancy, increase in maternal weight during pregnancy, smoking, and breastfeeding (only postnatal growth).

■ *: difference between non-carrier en homozygous genotypes. Δ : difference between heterozygous and homozygous genotypes.*

Figure 2. Mean length difference between the three genotype groups using the homozygous 192-bp genotype as the reference group



Values represent estimated differences in SDS (95% confidence interval) based on multiple linear regression models, adjusted for (gestational) age and gender of the child, maternal weight and height before pregnancy, increase in maternal weight during pregnancy, smoking, and breastfeeding (only postnatal growth). For the prenatal data, femur length was used as a proxy for body length.29

■ *: difference between non-carrier en homozygous genotypes. Δ : difference between heterozygous and homozygous genotypes.*

Table 5 shows the associations between *IGF1* genotype and longitudinally measured weight and length of all children. Compared to the 192-bp homozygous group, the non-carriers had a significantly higher growth rate from mid-pregnancy to 6 months in both weight and length. After the age of 6 months, no differences were found in growth rate between the genotype groups.

Table 5. Differences in weight and length gain between *IGF1* genotypes

Weight	**Difference in weight gain mid-pregnancy to 6 months ((SDS / 10 weeks) * 10^{-2})**	**Difference in weight gain From 6 to 14 months ((SDS / 10 weeks) * 10^{-2})**
Homozygous 192-bp	Reference	Reference
Heterozygous 192-bp	2.1 (-2.7, 7.0) p = 0.3981	0.4 (-6.1, 6.9) p = 0.8997
Non-carriers 192-bp	11.5 (3.5, 19.6) p = 0.0048	-2.2 (-12.8, 8.3) p = 0.6752
Length	**Difference in length gain mid-pregnancy to 6 months ((SDS / 10 weeks) * 10^{-2})**	**Difference in length gain From 6 to 14 months ((SDS / 10 weeks) * 10^{-2})**
Homozygous 192-bp	Reference	Reference
Heterozygous 192-bp	6.9 (2.0, 11.8) p = 0.0059	-1.6 (-8.5, 5.4) p = 0.6562
Non-carriers 192-bp	14.4 (6.4, 22.4) p = 0.0004	-2.5 (-13.9, 8.8) p = 0.6610

Estimates are based on repeated measures regression analysis.
*Values are regression coefficients (95% confidence interval) and reflect the difference in weight and length gain ((SDS / 10 weeks) * 10^{-2}) for each IGF1 genotype adjusted for (gestational) age and gender of the child, maternal weight and height before pregnancy, increase in maternal weight during pregnancy, and smoking.*

The odds ratio's for the associations between *IGF1* genotype group and positive growth realignment are shown in Table 6. Non-carriers showed positive growth realignment between mid-pregnancy and the age of 14 months more frequently compared to 192-bp homozygous subjects. An increased

frequency of positive growth realignment was also found between mid-pregnancy and birth amongst non-carriers, though this association was not significant.

Table 6. Association of *IGF1* promoter polymorphism with "positive growth realignment"

Genotype	Odds ratio for positive growth realignment		
	From mid-pregnancy to birth (SDS)	From birth to 14 months (SDS)	From mid-pregnancy to 14 months (SDS)
Homozygous 192-bp	Reference	Reference	Reference
Heterozygous 192-bp	1.38 (0.92, 2.05)	1.43 (0.95, 2.17)	1.79 (1.10, 2.90)*
Non-carriers 192-bp	1.77 (0.96, 3.23)	1.23 (0.63, 2.40)	2.28 (1.12, 4.67)*

**p-value <0.05 vs. homozygous 192-bp using binary logistic regression analyses, adjusted for (gestational) age and gender of the child, maternal weight and height before pregnancy, increase in maternal weight during pregnancy, smoking, and breastfeeding (only postnatal growth). Positive growth realignment was defined as growth realignment >+0.67 SDS.*

Discussion

We showed that non-carriers of the 192-bp allele tend to be smaller in mid-pregnancy and have a higher growth rate onwards compared to homozygous 192-bp individuals. This accelerated weight and length gain continued until the age of 6 months, after which growth rate was equivalent in all three groups. No differences were found between homozygous and heterozygous 192-bp individuals.

To our knowledge, this study is the first prospective cohort that examined the association between *IGF1* gene promoter polymorphism with fetal growth and growth in infancy. DNA for genotyping was available in 738 subjects (61%). Of all genotyped subjects at baseline, 73-75% participated in the follow-up measurements in infancy, which may lead to a loss of power and underestimation of the effect of the *IGF1* gene promoter polymorphism. Children who were not genotyped had a shorter gestational age (difference: -0.34 (95% CI: -0.55, -0.12) weeks, p = 0.002) and were lighter at birth (difference: -70.0 (95% CI: -146.6, 6.7) grams, p = 0.074) than subjects who were genotyped. Our effect estimates would be biased if the associations between genotypes and growth characteristics differ between those with and without DNA available for genotyping studies. This seems unlikely but cannot be excluded.

Several *IGF1* polymorphisms have been shown to influence IGF-1 levels and postnatal growth in small for gestational age children, though few have focused on the affect of the *IGF1* gene promoter region.[35] Vaessen *et al.* found that the absence of the 192-bp allele was associated with a decreased adult height.[7] In the same cohort, non-carriers of the 192-bp allele had a lower

birth weight.[11] In their study, information on birth weight was retrospectively collected and not adjusted for gestational age.[11] In two other large cohorts studies, no associations were found between this polymorphism and birth weight.[15,16] Recently, in a retrospective study in children, it was found that the absence of the 192-bp allele had no effect on birth weight, but was an independent risk factor for accelerated weight gain in the first year of life.[20]

In our study, we found that non-carriers of the 192-bp allele were smaller in mid-pregnancy than homozygous 192-bp carriers, which may be an indication of growth restriction in early pregnancy. Subsequently, non-carriers had an increased growth rate until the age of 6 months. We also found an increased risk of positive growth realignment between mid-pregnancy and 14 months amongst non-carriers compared to the homozygous group. We did not find differences in birth weight or postnatal growth characteristics between the genotype groups.

We could not replicate the previously demonstrated associations of *IGF1* gene promoter polymorphism with birth weight or postnatal catch-up growth.[11,20] These differences could be explained by differences in study design. One of the major strengths of our study is than we examined the association of *IGF1* gene promoter polymorphism with various growth characteristics and patterns rather than, as previously performed, with only birth or postnatal weight. We believe that if *IGF1* gene promoter polymorphism is truly associated with growth in early life, associations with longitudinally measured growth patterns are expected to be stronger than with only one or two growth measurements. However, the number of subjects in our and previous studies may be too small to show the longitudinal effects. Landmann *et al.* found accelerated weight gain from birth during the first year amongst non-carriers, while we found an increased risk of positive growth realignment between mid-pregnancy and 14 months.[20] We only found a non-significant trend towards an increased growth rate from birth to 14 months. Thus our results are not similar as those of Landmann *et al.*, but is interesting that both studies found an increased growth rate in early life among non-carriers. We cannot explain the differences in results. Since Landmann's study does not provide fetal growth data, it is not possible to replicate our major findings in their study. Thus far, our and previous studies show rather inconsistent associations with growth characteristics in early life.[11,16,20] Chance finding can therefore not be excluded. Further studies in larger cohorts are necessary to study the effect of this *IGF1* gene promoter polymorphism with growth from fetal to early postnatal life.

Although associations of this *IGF1* genotype with growth characteristics and type 2 diabetes have been suggested, conflicting results have been published on the biological effect of this polymorphism and the functionality of this gene is not yet known.[7,9,10,15] It has been suggested that, due to its close location to the transcription site, the *IGF1* promoter region may have a regulatory function.[7,9] The absence of the wild-type 192-bp allele might therefore alter the transcription rate of *IGF1*. Alternatively, it has been suggested that the *IGF1* promoter region may be in linkage disequilibrium with other regulatory elements, which in turn influence IGF-1 levels.[7,9] Furthermore, it has been shown that placental *IGF1* gene expression is altered in intra-uterine growth restric-

tion.[36] The *IGF1* gene promoter polymorphism may play an important role in placental development and function in early pregnancy, thus influencing early fetal growth. We are not aware of any previous studies relating this *IGF1* genotype with IGF-1 levels measured in cord blood in fetal life or at birth. Further studies are necessary to establish the functionality of this *IGF1* gene polymorphism and its association with circulating IGF-1 levels at birth.

In summary, our results suggest for the first time that the *IGF1* promoter polymorphism may influence growth from early fetal life to infancy. Because of the inconsistent findings in studies examining the associations of this genotype with growth characteristics in early life, studies in larger cohorts seem to be needed and the functionality of this gene needs to be further explored. Probably, more importantly, systematic searches for common genetic variants by means of genome-wide association studies may enable us to obtain a more complete understanding of the functionality of the entire *IGF1* region and its relation to growth and morbidity in childhood and later life.

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Chapter 2.2

Insulin gene variable number of tandem repeats and weight in fetal life and infancy

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Abstract

Background: The aim of this study was to examine whether the insulin gene variable number of tandem repeats (*INS* VNTR) is associated with growth patterns in fetal life and infancy.

Methods: This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. Fetal growth was assessed by ultrasounds in early, mid-, and late pregnancy. Anthropometry in infancy was assessed at birth and at the ages of 6 weeks, 6 months and 14 months. DNA for genotyping of the *INS* VNTR promoter region was available in 859 children.

Results: The genotype distribution was I/I 50.8%; I/III 40.0%; and III/III 9.2%. III/III individuals had a shorter gestational age ($p < 0.005$ vs. I/I) and a lower birth weight ($p < 0.05$ vs. I/I). There were no differences in birth weight after adjusting for gestational age. Class III homozygotes had a smaller abdominal circumference / head circumference ratio ($p < 0.005$ vs. I/I) in mid-pregnancy but not in late pregnancy. Also, III/III subjects had a relative decrease in head circumference (SDS) from mid-pregnancy to the age of 14 months ($p < 0.05$ vs. I/I). No other differences in pre- and postnatal growth characteristics and patterns were found.

Conclusions: Class III homozygotes were born at an earlier gestational age. No association was found between *INS* VNTR and birth weight adjusted for gestational age. Our data suggest that the III/III genotype may be associated with asymmetrical growth in mid-pregnancy, but not in late pregnancy.

Introduction

Insulin is the most important fetal growth factor.[1] Previous experimental and observational studies have shown that reduced secretion of fetal insulin and insulin-like growth factors is associated with low birth weight.[1,2] Several rare monogenic defects that affect insulin secretion have been shown to be related to altered fetal growth.[1,3] It has been suggested that also more common genetic polymorphisms related to insulin secretion and metabolism may explain part of the differences in birth weight in the normal population.[3]

Variation at the insulin gene variable number of tandem repeats (*INS* VNTR) minisatellite has been shown to influence pancreatic insulin gene transcription, both in the fetus and in adulthood.[4,5] The VNTR lies upstream of the imprinted insulin and insulin growth factor II genes on chromosome 11p15.5 and has been suggested to influence transcription rate of these genes.[5-7] *INS* VNTR has been suggested as a candidate genetic variant which influences fetal and early postnatal growth in a normal population.[8,9] There are two main classes of the VNTR, namely class I (30-44 repeat units) and class III (around 150 repeat units); class II is very rare in the Caucasian population.[5] Studies in the human pancreas have suggested that *INS* expression is lower in the VNTR class II homozygous than in the VNTR class I homozygous individuals.[5,10] At birth, *INS* VNTR class III homozygous individuals have been shown to have a larger mean head circumference.[8] Among those subjects without postnatal growth realignment, birth weight and length were also increased.[8] However, other cohort studies found a lower birth weight in class III homozygotes or no difference in birth weight at all.[9,11,12] It has also been suggested that this genotype is involved in childhood obesity and the development of metabolic syndrome[13,14] ,polycystic ovary syndrome[15] and type 2 diabetes in adulthood.[16,17] Nevertheless, two large cohort studies were unable to demonstrate effects of *INS* VNTR on body composition or the risk of metabolic syndrome in adulthood.[18,19]

The inconsistent findings from studies examining the association between *INS* VNTR genotype with birth size may be explained by the fact that size at birth alone is an inappropriate measure for fetal growth. We hypothesized that variants of the *INS* VNTR may be stronger related to longitudinally measured growth in pre- and postnatal life than to one specific growth characteristic such as birth weight. Therefore, we examined in the Generation R Study, a prospective prenatally recruited birth-cohort study, the associations of the *INS* VNTR genotype with growth parameters measured in different periods from fetal life until infancy.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. This study is designed to identify early environmental and

genetic determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail.[20, 21] In total, the cohort includes 9,778 mothers and their children living in Rotterdam, the Netherlands. A vast majority (69%) of all mothers were enrolled in the first trimester of pregnancy.[20] Assessments in pregnancy included physical examinations, fetal ultrasounds, biological samples and questionnaires. These were planned in early (gestational age <18 weeks), mid- (gestational age 18-25 weeks) and late pregnancy (gestational age ≥25 weeks) to collect information about fetal growth and its main determinants. Their partners were assessed once during this period. The children were born between April 2002 and January 2006 and form a prenatally recruited birth-cohort that is currently being followed until young adulthood. Of all eligible children, 61% participated in the study at birth. Additionally, more detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of 1,232 parents and their children, referred to as the Generation R Focus cohort. This subgroup is completely Caucasian to exclude possible confounding or effect modification by ethnicity. Of all approached women, 80% was enrolled in this subgroup study in late pregnancy (gestational age of 30 weeks). In this subgroup, postnatal examinations were performed at the ages of 6 weeks, 6 months, and 14 months. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants or their parents.

Population for analysis

In total, 1,232 women were enrolled in the Generation R Focus Study. Twin pregnancies (n = 15) and pregnancies leading to perinatal death (n = 2) were excluded from the present analysis. Of the remaining singleton 1,215 live births, *INS* VNTR genotyping was achieved in 71% (n = 859) of the subjects. Growth characteristics in mid- and late pregnancy, and birth weight were available in all these children. In total, 653 (76%), 653 (76%) and 605 (70%) children participated in the postnatal assessments at the age of 6 weeks, 6 months and 14 months, respectively.

Genotyping *INS* VNTR gene

DNA was collected from cord blood samples at birth. Cord blood for DNA isolation was available in 85% of all children participating in the Focus cohort. Missing cord blood samples were mainly due to logistic constraints at the delivery. Polymerase chain reaction (PCR) was performed to amplify the –23/*Hph*I single nucleotide polymorphism (A/T), which is known to be in almost complete linkage disequilibrium with *INS* VNTR class.[22]

The genotype distribution (AA 50.8%; AT 40.0%; and TT 9.2%) was similar to those found in previous studies and the frequency distribution did not deviate from the Hardy-Weinberg equilibrium (Chi-square = 1.28, p >0.1).[8, 11, 19]

Fetal growth and birth characteristics

Fetal ultrasound examinations were carried out in one of the research centers in early, mid- and late pregnancy. These fetal ultrasounds were used for both establishing gestational age and assessing fetal growth characteristics.[23] Pregnancy dating curves were constructed on subjects in the study with complete data on gestational age measured by ultrasound and last menstrual period. Crown-rump length was used for pregnancy dating in early pregnancy (gestational age until 12 weeks and 5 days, crown-rump length smaller than 65 mm) and biparietal diameter was used for pregnancy dating thereafter (gestational age from 12 weeks and 5 days onwards, biparietal diameter larger than 20 mm).[24,25] Fetal growth measurements used for the present study included head circumference (HC), abdominal circumference (AC) and femur length (FL) in mid- and late pregnancy, measured to the nearest mm using standardized ultrasound procedures.[26] Abdominal circumference / head circumference ratio was calculated, which has been shown to be useful in distinguishing symmetrical from asymmetrical growth.[27] Estimated fetal weight was calculated using the formula by Hadlock using head circumference, abdominal circumference and femur length ($\log_{10}$ EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC) + 0.171 (FL) + 0.00034 $(HC)^2$ – 0.003685 (AC * FL)).[28] Early pregnancy was not included since these fetal ultrasound examinations were primarily performed to establish gestational age. Gestational age-adjusted standard deviation scores (SDS) were constructed for all fetal growth measurements. These were based on reference growth charts from the whole study population.

Postnatal growth characteristics

Date of birth, gender, and birth weight were obtained from community midwife and hospital registries. At the age of 6 weeks, 6 months and 14 months, anthropometrics were measured without clothes. Weight was measured to the nearest gram using electronic scales. Length was measured in supine position to the nearest millimeter at the ages of 6 weeks and 6 months using a neonatometer, and it up-right position at the age of 14 months. Head circumference was measured to the nearest millimeter.

Covariates

Information about maternal age, parity and weight before pregnancy was obtained by the first questionnaire at enrolment in the study. Maternal height was measured without shoes at our research center, and body mass index (weight/$height^2$ (kg/m^2)) was calculated. Information on the occurrence of hypertension, pre-eclampsia, gestational diabetes, and labour details (induced or primary caesarean section, spontaneous) was obtained from midwife and obstetrician records.

Statistical analysis

First, differences of maternal, fetal and postnatal characteristics between the *INS* VNTR genotypic groups were assessed by independent sample t-test or Mann-Whitney's U test for continuous variables and the Chi-square test for categorical variables. Since the *VNTR-INS-IGF2* region is imprinted, with its paternally-inherited allele being expressed, the I/III heterozygote group can be considered as an indeterminate group, consisting partly of individuals in whom the I allele is expressed, and partly of individuals in whom the III allele is expressed. Main interest in our analyses considering etiological associations was on the difference between I/I and III/III heterozygous subjects. For all analyses, the I/III and III/III genotype groups were both separately compared to the I/I group (reference group). Subsequently, we used multiple linear regression models to assess the associations of *INS* VNTR with gestational age at birth, adjusting for maternal age, parity, hypertension, pre-clampsia, gestational diabetes, induced labour and primary caesarean section. To assess the association without the extremes of gestational age or birth weight, we also performed this analysis, excluding those children born prematurely (gestational age <37 weeks) and small or large for gestational age (-2 SDS or +2 SDS, respectively). Multiple linear regression models were also performed to study the association between genotype and (estimated) weight, head circumference and abdominal circumference / head circumference ratio (AC/HC ratio) at each age cross-sectionally (prenatally: mid- and late pregnancy; at birth; postnatally: 6 weeks, 6 months and 14 months). These models were adjusted for gender and age and postnatal data was additionally adjusted for gestational age at birth. Next, to examine the associations of *INS* VNTR with prospectively measured growth patterns, rather than growth characteristics at one age, we studied the differences in (estimated) weight change (SDS) and head circumference (SDS) change from mid-pregnancy to 14 months between genotypes using multiple linear regression models. Since it has been suggested that in children from multiparous mothers and/or in children with no postnatal growth realignment, the genetic contribution to birth weight is greater than in first born and/or in children with growth realignment, we also examined the associations of *INS* VNTR genotype and birth weight in strata of birth order and growth realignment.[6] As previously described, growth realignment was defined as a change, either increase or decrease, of weight between birth and 14 months of more than 0.67 SDS ("changers" means, for example, a 3rd to 10th percentile increase, and "non-changers" means a growth realignment less than 0.67 SDS).[8, 17, 29] Finally, using Pearson's Chi-square, we compared the prevalence of catch-up growth between genotypes, where catch-up growth was defined as a positive growth realignment of more than 0.67 SDS. All effect estimates are presented with their 95% confidence interval (CI). Statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics for the three genotypes are shown in Tables 1, 2 and 3. Genotype frequency distribution was I/I 50.8%; I/III 40.0%; and III/III 9.2%. No differences between genotypes were found in maternal characteristics. Gender distribution was similar in the three genotypes. In mid-pregnancy, class III homozygotes had a smaller abdominal circumference and a reduced abdominal circumference / head circumference compared to class I homozygotes. Subjects with the III/III genotype were born at a significantly shorter gestational age and had a lower birth weight than the I/I individuals. After adjusting for maternal age, parity, hypertension, pre-clampsia, gestational diabetes, induced labour and primary caesarean section, gestational age remained shorter in the homozygous III/III group (differences: -0.39 (95% CI: -0.71, -0.08) weeks versus I/I). Also, when we excluded large and small for gestational age births and preterm births from the analysis, the difference in gestational age was significant (differences: -0.34 (95% CI: -0.62, -0.05) weeks versus I/I). The odds ratio for preterm birth in the III/III group versus the I/I group was not significant (1.86 (95% CI: 0.58, 5.94)). No differences in birth weight SDS were found.

Table 1. Maternal characteristics according to fetal insulin gene VNTR class genotype

	I / I (n = 436)	**I / III** (n = 344)	**III / III** (n = 79)
Maternal characteristics			
Age (years)	31.7 (4.1)	31.5 (4.3)	31.5 (4.0)
Weight (kg)	68.6 (11.9)	69.1 (13.1)	69.0 (12.3)
Height (cm)	171.0 (6.6)	171.1 (6.4)	170.6 (6.2)
Body mass index (kg/m^2)	23.4 (3.9)	23.6 (4.1)	23.7 (3.9)
Parity (% nulliparous)	59.1	59.9	59.0
Hypertension (%)	5.1	4.4	7.7
Pre-eclampsie (%)	1.2	1.2	1.3
Gestational diabetes (%)	0.2	1.2	1.3
Induced labour (%)	26.2	24.1	34.2
Primary caesarean section (%)	3.1	3.1	5.1

Values are means (SDS) or percentages. Differences were tested using independent sample t-test or Chi-square test. Of the total group, data were missing on maternal height before pregnancy (n=8), weight and body mass index (n=146), parity (n=12), hypertension (n=4), pre-eclampsia (n=4), gestational diabetes (n=5), induced labour (n=35) and primary caesarean section (n=42).

Table 2. Fetal characteristics according to fetal insulin gene VNTR class genotype

	I / I (n = 436)	I / III (n = 344)	III / III (n = 79)
Fetal characteristics mid-pregnancy			
Gestational age (weeks)	20.6 (1.0)	20.5 (1.0)	20.4 (0.9)
Head circumference (mm)	179 (12.6)	178 (12.8)	178 (11.0)
Abdominal circumference (mm)	157 (13.1)	156 (13.1)	154 (12.0)*
Femur length (mm)	33.1 (3.1)	32.9 (3.1)	32.6 (3.0)
Abdominal / head circumference ratio	0.880 (0.043)	0.880 (0.040)	0.865 (0.035)†
Estimated fetal weight (grams)	377 (80)	371 (80)	359 (69)
Fetal characteristics late pregnancy			
Gestational age (weeks)	30.4 (1.0)	30.4 (1.0)	30.5 (1.0)
Head circumference (mm)	286 (11.7)	286 (11.7)	287 (13.0)
Abdominal circumference (mm)	265 (15.7)	267 (16.7)	267 (18.0)
Femur length (mm)	57.1 (3.1)	57.4 (2.9)	57.3 (2.8)
Abdominal / head circumference ratio	0.928 (0.042)	0.933 (0.044)	0.931 (0.046)
Estimated fetal weight (grams)	1622 (255)	1648 (261)	1648 (267)

Values are means (SDS). Differences were tested using independent sample t-test. Of the total group, data were missing on mid-pregnancy gestational age (n=10), head circumference (n=14), abdominal circumference (n=12), femur length (n=14), abdominal circumference / head circumference ratio (n=36), estimated fetal weight (n=16), late pregnancy gestational age (n=6), head circumference (n=8), abdominal circumference (n=10), femur length (n=9), abdominal circumference / head circumference ratio (n=22) and estimated fetal weight (n=10).

**p-value <0.05 vs. I/I genotype, †p-value <0.005 vs. I/I genotype*

Table 3. Birth and postnatal characteristics according to insulin gene VNTR class genotype

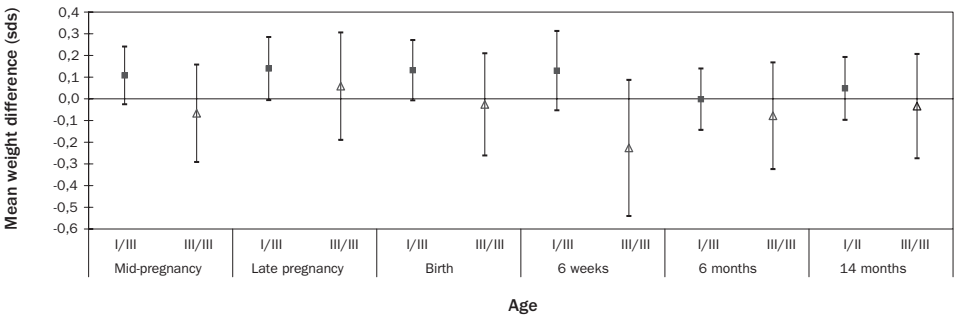
	I / I (n = 436)	I / III (n = 344)	III / III (n = 79)
Birth			
Gestational age (weeks)	40.2 (36.7 - 42.4)	40.2 (37.6 - 42.4)	39.6 (36.0 - 42.3)*
Gestational age <37 weeks (%)	3.0	1.7	5.1
Weight (grams)	3510 (512)	3591 (478)	3396 (504)†
Weight (SDS)	0.01 (0.99)	0.14 (0.98)	-0.01 (0.90)
Weight <-2 SDS (%)	3.2	2.3	2.5
Weight >+2 SDS (%)	2.1	3.2	1.3
Gender (% boy)	50.0	55.2	50.6
Postnatal characteristics 6 weeks			
Age at visit (weeks)	6.9 (4.6 - 12.7)	6.5 (4.4 - 10.3)	7.1 (4.2 - 14.9)
Head circumference (cm)	38.6 (1.4)	38.6 (1.4)	38.5 (1.8)
Weight (grams)	4919 (710)	4918 (615)	4817 (802)
Length (cm)	56.7 (2.6)	57.0 (2.4)	56.3 (2.8)
Body mass index (kg/m^2)	15.2 (1.5)	15.1 (1.4)	15.1 (1.4)
Postnatal characteristics 6 months			
Age at visit (weeks)	27.7 (23.3 - 34.3)	28.4 (23.7 - 36.4)	28.3 (24.1 - 35.6)
Head circumference (cm)	43.8 (1.3)	44.0 (1.3)	43.9 (1.7)
Weight (grams)	7889 (859)	7998 (897)	7878 (907)
Length (cm)	68.4 (2.6)	69.0 (2.7)	68.6 (2.7)
Body mass index (kg/m^2)	16.8 (1.3)	16.8 (1.3)	16.8 (1.5)
Postnatal characteristics 14 months			
Age at visit (months)	14.7 (13.4 - 17.5)	14.5 (13.3 - 16.5)	14.6 (13.5 - 17.3)
Head circumference (cm)	47.3 (1.4)	47.4 (1.3)	47.0 (1.6)
Weight (grams)	10490 (1074)	10563 (1053)	10431 (1181)
Length (cm)	79.1 (2.9)	79.3 (3.0)	78.8 (3.2)
Body mass index (kg/m^2)	16.8 (1.3)	16.8 (1.1)	16.8 (1.2)

Values are means (SDS), medians (95% range) for variables with skewed distribution, or percentages. Differences were tested using independent sample t-test, Mann-Whitney's U test, or Chi-square test. Of the total group, data were missing at 6 weeks head circumference (n=242), weight (n=232), length (n=235); at 6 months head circumference (n=234), weight (n=230), length (n=232); at 14 months head circumference (n=293), weight (n=262), and length (n=270).

**p-value <0.05 vs. I/I genotype, †p-value <0.005 vs. I/I genotype*

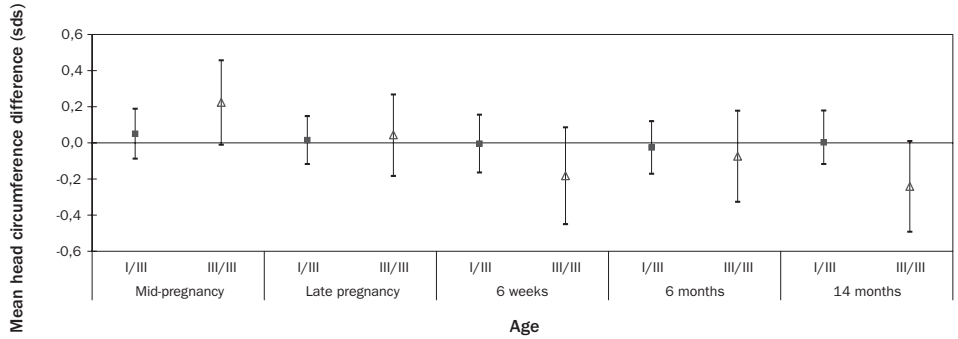
Results from the cross-sectional multiple regression analyses of weight, head circumference and abdominal circumference / head circumference ratio (prenatal) at each age are shown in Figures 1, 2, and 3. No differences in weight (SDS) were found at any age. In class III homozygous subjects, head circumference (SDS) tended to be larger in mid-pregnancy and smaller at the age of 14 months compared to class I homozygotes (differences: 0.23 (95% CI: 0.00, 0.46) SDS and -0.25 (95% CI: -0.49, 0.00) SDS, respectively) though these differences were not significant. Abdominal circumference / head circumference ratio was significantly lower in III/III subjects than in I/I individuals in mid-pregnancy (difference: -0.28 (95% CI: -0.47, -0.09) SDS), but not in late pregnancy.

Figure 1. Mean weight difference between the three genotypes using the I/I genotype as reference group



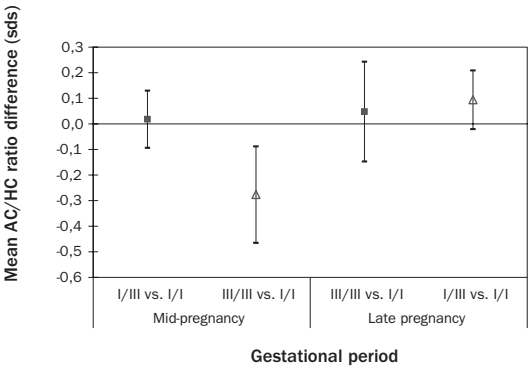
Values represent differences between genotypes in SDS (95% confidence interval) based on multiple linear regression models, adjusted for (gestational) age and gender. Postnatal estimates are additionally adjusted for gestational age at birth.

Figure 2. Mean head circumference difference between the three genotypes using the I/I genotype as reference group



Values represent differences between genotypes in SDS (95% confidence interval) based on multiple linear regression models, adjusted for (gestational) age and gender. Postnatal estimates are additionally adjusted for gestational age at birth.

Figure 3. Mean abdominal circumference / head circumference (AC/HC) ratio difference (in SDS) between the three genotypes using the I/I genotype as reference group



Values represent differences between genotypes in SDS (95% confidence interval) based on multiple linear regression models, adjusted for gestational age and gender.

From mid-pregnancy to 14 months, the III/III individuals had a significant mean decrease in head circumference SDS compared to I/I subjects (Table 4). No difference between the genotype groups in weight change were found during any period between mid-pregnancy and the age of 14 months.

Table 4. Differences in weight change and head circumference change from mid-pregnancy to 14 months using the I/I genotype as a reference group

Genotype	Weight change (SDS)	Head circumference change (SDS)
I/I	Reference	Reference
I/III	-0.083 (-0.292, 0.127)	-0.027 (-0.236, 0.181)
III/III	-0.029 (-0.378, 0.320)	-0.443 (-0.799, - 0.086)

Values represent differences in change in SDS (95% confidence interval). Differences were tested using multiple linear regression models.

The associations between *INS* VNTR genotypes and birth weight, stratified by birth order and weight realignment, are shown in Table 5. Amongst the firstborn children, subjects with the I/III genotype were significantly heavier than I/I individuals. Birth weight was similar in the three in genotype groups amongst children born from multiparous mothers. No difference between the three genotypes was found in birth weight amongst the "non-changers". Amongst the "changers", however, the I/III subjects had a significantly higher birth weight after adjusting for age and gender than I/I subjects. And finally, no differences were found between the three genotypes in the prevalence of catch-up growth between birth and the age of 14 months (p >0.5, using Pearson's Chi-square).

Table 5. Birth weight per genotype, stratified by birth order and growth realignment

Genotype	1st pregnancy	2nd+ pregnancy	Non-changers	Changers
I/I	3421 (516)	3633 (476)	3498 (413)	3555 (541)
I/III	3512 (490)*	3668 (449)	3510 (421)	3695 (493)*
III/III	3398 (502)	3616 (467)	3462 (524)	3463 (526)

Values represent mean in grams (SDS). Differences were tested using multiple linear regression models, adjusting for gestational age and gender. Change is defined as postnatal growth realignment between birth and 14 months, positive or negative, of more than 0.67 SDS.

**p-value <0.05 vs. I/I genotype*

Discussion

We showed that III/III individuals of *INS* VNTR had a shorter gestational duration compared to the I/I subjects. No differences were found in birth weight adjusted for gestational age. Class III homozygous subjects had a smaller abdominal circumference / head circumference ratio in mid-pregnancy but not in late pregnancy compared to I/I individuals. In III/III, we also found a decreased growth rate in head circumference from mid-pregnancy to 14 months of age. No differences were found for any other growth characteristics or patterns.

To our knowledge, this study is the first prospective cohort that examined the associations between *INS* VNTR and growth in fetal life and infancy. DNA for genotyping was available in 859 Caucasian subjects (71%) and of all genotyped subjects at baseline about 70-75% participated in the follow-up measurements in infancy. Children who were not genotyped had a shorter gestational age at birth (difference: -0.47 (95% CI: -0.72, -0.21) weeks, p <0.001) and a lower birth weight (difference: -52.2 (95% CI: -143.7, 39.6) grams, p = 0.18). Our effect estimates would be biased if the associations between *INS* VNTR genotype and growth characteristics differ between those with and without complete data. This seems unlikely but cannot be excluded.

The underlying mechanism explaining how *INS* VNTR influences growth remains unclear. In the pancreas, *INS* expression has been found to be lower in the VNTR class III homozygous than in the VNTR class I homozygous subjects.[5,10] It has also been hypothesized that the VNTR influences *IGF2*, a neighbouring gene on chromosome 11p15.5[8,10], though studies have been conflicting. The VNTR class I allele is associated with higher *IGF2* expression in the human placenta[7], while VNTR class III homozygotes have been shown to have higher IGF-II cord blood levels.[6] *INS* and *IGF2* are both imprinted genes.[30] In the human yolk sac, *INS* has been shown to be exclusively paternally expressed[30], and the imprinting of the *IGF2/H19* region is known to affect growth in syndromes with early growth disorders.[31] Therefore, several studies have focused on paternal-specific allele transmission of the *VNTR-INS-IGF2* region in relation to growth and diseases. Paternally derived VNTR class III allele has been shown to be associated with type 2 diabetes[16] and polycystic ovary syndrome[32], while paternally derived VNTR class I allele may be a risk factor for childhood obesity.[33] These findings suggest that the greatest phenotypic difference would be between class I and class III homozygous individuals, since, by definition, they have inherited an active paternal allele. Other studies, however, show no parent-of-origin effect on birth weight or type 2 diabetes.[6,9] Finally, *IGF2* in mice plays an important role in placental development and regulation.[34] The VNTR may affect the imprinting of *IGF2* and subsequently impair placental circulation.[34]

Several studies have examined the association between *INS* VNTR and growth. In the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort, Dunger *et al*. was the first to show an association between *INS* VNTR and birth size.[8] In those individuals who had no postnatal weight realignment and in children from multiparous mothers, the class III genotype was associated with a larger birth size.[6,8] It has been proposed that in these children the maternal uterine factors are less important and the genetic contribution to growth is amplified.[8] On the other hand, in a Pima Indian population the class III genotype was associated with lower birth weight and an increased prevalence of type 2 diabetes.[9] Two other studies, one of which was performed in a large Finnish cohort, could not replicate any of the results regarding birth weight, also after stratifying for postnatal realignment.[11,12]

In our study, we found no differences in weight in fetal life, at birth or in infancy. We also did not find associations between patterns in weight growth from fetal life until infancy. Unexpectedly, class III homozygous subjects had a shorter gestational age at birth than class I homozygous subjects. No such association has previously been described. This difference remained significant after adjusting for factors that may explain a shorter gestational period, such as birth weight and pregnancy and delivery complications. Furthermore, this association was still present after excluding all large and small for gestational age births and preterm births, and therefore cannot be explained by outliers or skewed distributions. We have no explanation for this finding. It could be hypothesized that altered intra-uterine growth patterns resulting in earlier maturation or changes in placental function may cause an earlier delivery. However, this association remained significant after adjustment for birth weight. Also additional adjustment for weight change between 20 weeks

and birth did not materially affect our effect estimate (results not shown). On the other hand, this association turned up without a previous hypothesis. Therefore, this finding could be due to chance and further studies in other population-based cohorts are needed for replication.

Class III genotype subjects also had a considerably smaller abdominal circumference / head circumference ratio in mid-pregnancy. We found that class III homozygote subjects tended to have a larger head circumference and a smaller abdominal circumference in mid-pregnancy. However, by late pregnancy head circumference SDS was decreased and abdominal circumference SDS was increased in these fetuses, resulting in a similar abdominal circumference / head circumference ratio as class I/I subjects. Abdominal circumference / head circumference ratio has been shown to be useful in distinguishing symmetrical from asymmetrical growth.[27] Asymmetrical growth with a relatively large head circumference is also known as brain sparing. Our findings may suggest that brain sparing occurs in early pregnancy in these individuals. Based on current literature, we did not hypothesize beforehand to find such an association specifically. Therefore, further studies are necessary to replicate these findings.

Epidemiological studies have demonstrated an inverse relationship between birth weight and the risk of developing type 2 diabetes and cardiovascular disease in adulthood.[35, 36] The fetal insulin hypothesis proposes that genetic variants that regulate fetal insulin or sensitivity may lead to both impaired fetal growth and to increased morbidity in later life.[3] Several studies, however, have suggested that not low birth weight per se but rather postnatal accelerated weight gain in subjects with a small size at birth, leading to a normal or increased weight from childhood onwards, increases the risk for adult disease.[37-39] Animal models have shown that an altered fetal growth trajectory may also lead to an increased risk of adult morbidity, even when birth weight is normal.[40] We only found differences in early fetal growth patterns between *INS* VNTR genotype variants. In our study postnatal growth characteristics were available until the age of 14 months. Studies with a longer follow-up period are needed to assess whether this genotype is associated with growth patterns in childhood that are related to development of type 2 diabetes and cardiovascular disease in adulthood. It has also been suggested that prematurity, regardless of birth weight, can lead to reduced insulin sensitivity and possibly type 2 diabetes in later life.[41] Our finding that shows an association between *INS* VNTR and gestational age is in line with the hypothesis that common genetic variations may underlie the association between preterm birth and increased risk of development of type 2 diabetes and cardiovascular disease.

In conclusion, this study demonstrates that *INS* VNTR is not associated with weight from fetal life and until infancy. Our data suggest that *INS* VNTR is associated with asymmetrical growth in early and mid-pregnancy, but not in late pregnancy. We found for the first time an association between *INS* VNTR and gestational age at birth. Studies in larger cohorts are necessary to replicate our findings. Also, systematic searches by genome-wide association studies may enable us to obtain a more complete understanding of the functionality of the entire *VNTR-INS-IGF2* region and its relation to growth and morbidity in childhood and later life.

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Chapter 2.3

Genetic variants in the glucocorticoid receptor gene and growth in fetal life and early childhood

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Abstract

Background: Glucocorticoids have an important role in early growth and development. Glucocorticoid receptor gene polymorphisms have been identified that contribute to the variability in glucocorticoid sensitivity. We examined whether these glucocorticoid receptor gene polymorphisms are associated with growth in fetal and early postnatal life.

Methods: This study was embedded in a population-based prospective cohort study from fetal life onwards. The studied glucocorticoid receptor gene polymorphisms included *Bcl*I (rs41423247), *TthIII*I (rs10052957), GR-9β (rs6198), N363S (rs6195) and R23K (rs6189 and 6190). Fetal growth was assessed by ultrasounds in second and third trimester of pregnancy. Anthropometric measurements in early childhood were performed at birth and at the ages of 6, 14 and 24 months postnatally. Analyses were based on 2,414 healthy, Caucasian children and focused on weight, length and head circumference.

Results: Glucocorticoid receptor gene polymorphisms were not associated with fetal weight, birth weight and early postnatal weight. Also, no associations were found with length and head circumference. Neither were these polymorphisms associated with the risks of low birth weight or catch-up growth from birth to 24 months of age.

Conclusions: We found in a large population-based cohort no evidence for an effect of known glucocorticoid receptor gene polymorphisms on fetal and early postnatal growth characteristics. Further systematic searches for common genetic variants by means of genome-wide association studies will enable us to obtain a more complete understanding of what genes and polymorphisms are involved in growth in fetal life and infancy.

Introduction

Glucocorticoids are important regulators of growth, development and metabolism. The effects of these hormones, including cortisol, are mediated by glucocorticoid receptors. The sensitivity to glucocorticoids is known to show a large interindividual variation.[1] Polymorphisms in the glucocorticoid receptor gene have been suggested to contribute to this difference in sensitivity and thereby to differences in growth, development and metabolism. These glucocorticoid receptor genes may also explain part of the previously demonstrated associations between growth characteristics in early life and metabolic disease, including type 2 diabetes, in adult life.[2,3]

Five different variants in the glucocorticoid receptor gene have been described to be associated with cortisol sensitivity.[4-6] Few studies analyzed the associations of these glucocorticoid receptor gene variants with body composition and obesity in adults. The R23K variant (two Single Nucleotide Polymorphisms in complete linkage disequilibrium) was found to be associated with higher serum cortisol concentrations as well as a smaller decrease in cortisol after dexamethasone suppression tests. Furthermore, carriers showed lower fasting insulin levels and lower LDL cholesterol levels. These data suggest that R23K carriers are relatively more cortisol resistant than non-carriers, which results in a better metabolic health profile in adults.[7,8] By contrast, the *Bcl*I and N363S polymorphisms were found to cause the opposite effects.[9-11] In healthy subjects over 55 years, these polymorphisms were associated with hypersensitivity to glucocorticoids, resulting in an increased body mass index (BMI).[10] However, results were not consistent.[12,13] The *TthIII*I polymorphism was associated with elevated diurnal cortisol levels, but not with any anthropometric or glucose related phenotype.[14] Recently, the GR-9β polymorphism was found to be related to an increased sensitivity to glucocorticoids, leading to an increased risk of cardiovascular disease.[15] These results suggest that common functional variants of the glucocorticoid receptor gene may affect body composition. However, the exact mechanisms have not been confirmed. Also, thus far no studies did assess the effects of these glucocorticoid receptor gene polymorphisms in young children. However, the effect of these glucocorticoid receptor gene polymorphisms might be stronger on anthropometric measures in early life than on body mass index in adult life, because of very limited life style influences.

We hypothesised that genetic variants leading to increased glucocorticoid sensitivity are associated with fetal growth retardation and postnatal growth acceleration. This would be in line with well-known associations of high cortisol exposures with low birth weight and higher postnatal weight. Therefore, we studied in a population-based prospective cohort study from fetal life until the age of 2 years the effects of the *Bcl*I, *TthIII*I, GR-9β, N363S and R23K polymorphisms on anthropometrics in second and third trimester of pregnancy, at birth and postnatally until the age of 24 months.

Methods

Design

This study was embedded in the Generation R Study, a prospective cohort study from early fetal life onwards. This study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail.[16,17] Fetal and postnatal growth and their main determinants were repeatedly measured by physical examinations, fetal ultrasounds and questionnaires. We have previously shown that of all eligible children born in the study area 61% participated in the study.[17] The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all parents.

Population for analysis

Analysis were restricted to Caucasian children (n = 4,527) and of whom DNA was available for genotyping (n = 2,839). Reasons for non-availability of DNA were mainly due to logistical constraints at birth. Fetal growth measurements were available in 2,746 and 2,791 children in second and third trimester of pregnancy, respectively. A total of 81% (n = 2,274), 75% (n = 2,136) and 68% (n = 1,929) participated in the postnatal assessments at the ages of 6, 14 and 24 months. Information about anthropometrics of at least one of the postnatal visits was available in 2,414 subjects of whom 72%, 91% and 100% had measurements at least three, two and one visit. In total, analyses were based on more than 6,000 measurements.

Genotyping

DNA was collected from cord blood samples at birth. All participants were genotyped for five known glucocorticoid receptor gene polymorphisms which are known to be associated with changes in glucocorticoid sensitivity: *Bcl*I (rs41423247), *TthIII*I (rs10052957), GR-9β (rs6198), N363S (rs6195) and R23K (rs6189 and 6190).[4,5] Figure 1 schematically shows the specific nucleotide variations and allele frequencies of these polymorphisms. Genotyping of the five glucocorticoid receptor gene polymorphisms was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C (15 minutes), then 40 cycles of 94° C (15 seconds) and 60° C (1 minute)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems).

Figure 1. Schematic overview of the glucocorticoid receptor gene polymorphisms and haplotypes

Haplotype	Polymorphism						Allele frequency (%)
1		T	GG	A	C	A	42,4
2	*Bcl*1	T	GG	A	**G**	A	22,7
3	*TthIII*I + *Bcl*1	**C**	GG	A	**G**	A	14,3
4	GR-9β + *TthIII*I	**C**	GG	A	C	**G**	13,4
5	N363S	T	GG	**G**	C	A	4,1
6	ER22/23EK + GR-9β + *TthIII*I	**C**	**AA**	A	C	**G**	3,1

Haplotypes are numbered in order of decreasing frequency.
The nucleic acid changes are indicated; C = Cytidine, G = Guanine, A = Adenosine, T = Thymine.

Genotyping was successful in 97-99% of the samples for the five genotypes. To confirm the accuracy of the genotyping results 276 randomly selected samples were genotyped for a second time with the same method. The error rate was less than 1% for all genotypes. We used the genotype data for each of the 5 polymorphisms to infer the haplotypes present in the population using the program PHASE, which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data.[18] Instead of individual polymorphisms, we studied the haplotype structure of the glucocorticoid receptor gene to encompass a major proportion of variation in the gene. We excluded very rare polymorphisms because they have potential to explain only a very small fraction of variation in response to glucocorticoids seen between individuals. For each haplotype, 3 genotype combinations were distinguished as carrying 0, 1, or 2 copies of the haplotype allele. Haplotype 1 carries the major alleles of the polymorphisms; therefore, the reference allele is defined as carrying 2 copies of haplotype 1. Genotype and allele frequencies were in Hardy Weinberg equilibrium ($p > 0.01$).

Growth measurements

Fetal growth characteristics

Fetal ultrasound examinations were carried out during visits at the research centers. The respective median (95% range) gestational ages for these visits were 12.6 weeks (9.6 – 16.9), 20.4 weeks (18.6 – 22.5) and 30.2 weeks (28.5 – 32.5). The second and third visits were considered as second and third trimester measurements. These fetal ultrasounds were used both to establish gestational age (first ultrasound) and to assess fetal growth characteristics.[19] Fetal growth

measurements used for the present study comprised head circumference (HC), abdominal circumference (AC) and femur length (FL) in the second and third trimester measured to the nearest millimeter using standardized ultrasound procedures.[20-22] Estimated fetal weight was calculated using the formula by Hadlock using head circumference, abdominal circumference and femur length: EFW (grams) = 10**(1.326 – 0.00326*AC*FL + 0.0107*HC + 0.0438*AC + 0.158*FL).[23] Growth measurements in early pregnancy (gestational age <18 weeks) were not included, since these fetal ultrasound examinations were performed primarily to establish gestational age. Gestational age-adjusted standard deviation scores were constructed for these fetal growth measurements.

Postnatal growth characteristics

Birth weight, date of birth and gender were obtained from community midwife and hospital registries. Well-trained staff in community health centers obtained postnatal growth characteristics using standardized procedures. Based on the routine health care program, visits for these growth characteristics were grouped into three age periods: 6 (range 5 - 8.99) months; 14 (range 12 - 18.38) months and 24 (range 23 - 28.93) months. Anthropometrics were measured without clothes. Weight was measured to the nearest gram using electronic scales. Length was measured to the nearest millimeter in the supine position using a neonatometer at the age of 6 months, and in the upright position at the ages of 14 and 24 months. Head circumference was postnatally measured at the age of 6 and 14 months.

Statistical analysis

Differences in baseline characteristics between boys and girls were examined by independent samples t-tests (continuous variables) or Pearson's chi-square (categorical variables). Because of the low number of homozygous subjects for haplotype 5 (n = 8) and 6 (n = 1), these haplotypes were analyzed as carriers (1 or 2 copies) and non-carriers (0 copies).

The associations of the glucocorticoid receptor haplotypes with pre- and postnatal growth characteristics (weight, length and head circumference) were analyzed in three different time-intervals: from second trimester to birth, from birth to 24 months of age and from second trimester to 24 months of age. Since, no data were available for head circumference at 24 months of age, head circumference was analyzed until the age of 14 months. With these outcomes, the effects of glucocorticoid receptor polymorphisms on skeletal and non-skeletal growth and head circumference can be studied. We used femur length as measure of skeletal growth in fetal life (correlation femur length in third trimester of pregnancy and length at 1 month of age: r = 0.30, p-value <0.001). To assess longitudinally measured growth patterns from fetal life to infancy, we performed repeated measures regression analysis. This regression technique takes the correlation of multiple measurements within one subject into account, assesses both the time-independent

and time-dependent effect of the glucocorticoid receptor genotypes, and allows for incomplete outcome data.[24, 25] In these models, haplotype was included as both intercept and interaction with age. To account for (gestational) age at each specific measurement, these analyses were conducted with age-adjusted standard deviation scores. The models can be written as:

Weight (SDS) = β_0 + β_1*age + β_2* glucocorticoid receptor haplotype + β_3* glucocorticoid receptor haplotype*age.

Similar models were used for length and head circumference growth. The term including 'β_0' reflects the intercept and the term including 'β_1' reflects the growth per week for the reference group. The terms including 'β_2' and 'β_3' reflect the age independent and dependent growth differences between the different categories of the glucocorticoid receptor genotype, respectively.[25] To study the dominant effects of the glucocorticoid receptor gene polymorphisms, we merged the group heterozygous and homozygous variant subjects and performed the same analyses.

Furthermore, we performed multiple logistic regression models to analyze the associations of the different glucocorticoid receptor haplotypes with prenatal growth retardation (growth deceleration) and postnatal growth acceleration. We defined growth deceleration as a decrease in weight from second trimester of pregnancy until birth of <-0.67 standard deviation and growth acceleration as an increase in weight from birth to 24 months of age of more than 0.67 standard deviation.[26] Each anthropometric outcome was analyzed using gender and age adjusted standard deviation scores (SDS). These were based on reference growth curves from the whole study population. Variables were included in these models when they changed the effects estimates of interest on pre- and postnatal growth substantially (>10%).

With a sample size in the Generation R Study of 2,839 subjects and assuming a statistical power level (1 – β) of 0.80 and a level of significance (α) of 0.05, we were able to detect differences in growth characteristics of about 0.05 SDS. All effect estimates are presented with their 95% confidence interval (95% CI). Statistical analyses were performed using the Statistical Package of 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 distribution of the different glucocorticoid receptor haplotypes within our study population is presented in Table 1. Haplotype 1, 2, 3 and 4 were most frequent with allele frequencies of 42.4%, 22.7%, 14.3% and 13.4%, respectively. Haplotypes 5 and 6 had allele frequencies of 4.1% and 3.1%, respectively. Comparison of means of baseline characteristics between carriers of 0, 1 or 2 copies of haplotype 1 to 6 revealed no significant differences for the covariates age at visit and gender.

Table 1. Distribution of the different haplotype alleles of the glucocorticoid receptor gene within our study population (n = 2,414)

Glucocorticoid receptor haplotype (copies)	N (%)
Haplotype 1	
0	531 (22.0)
1	1,379 (57.1)
2	504 (20.9)
Haplotype 2	
0	1,265 (52.4)
1	1,017 (42.1)
2	132 (5.5)
Haplotype 3	
0	1,668 (69.1)
1	699 (29.0)
2	47 (1.9)
Haplotype 4	
0	1,688 (70.0)
1	677 (28.0)
2	49 (2.0)
Haplotype 5	
0	2,196 (91.0)
1 or 2	218 (9.0)
Haplotype 6	
0	2,242 (92.9)
1 or 2	172 (7.1)

Values are number of persons (%).

Table 2 presents the baseline characteristics of infants who participated in the postnatal visits. The percentage of boys and girls was both 50%. The overall median ages (95% range) in infants at their visits were 6.2 months (5.2 – 7.9), 14.3 months (13.5 – 16.1) and 24.8 months (23.4 – 28.1). Anthropometrics in second and third trimester of pregnancy, at birth and at the postnatal ages of 6, 14 and 24 months were larger in boys than in girls.

Table 2. Fetal and infant characteristics (n = 2,414)

	Boys (n = 1,233)	**Girls** (n = 1,181)	**P-value**
Second trimester			
Gestational age (weeks)	20.6 (18.7 – 23.3)	20.4 (18.5 – 23.1)	<0.01
Estimated fetal weight (grams)	386 (91)	374 (86)	<0.01
Femur length (mm)	33.3 (3.2)	33.2 (3.3)	0.55
Head circumference (mm)	181.0 (13.1)	177.2 (13.0)	<0.01
Third trimester			
Gestational age (weeks)	30.4 (28.6 – 33.0)	30.3 (28.3 – 32.8)	<0.01
Estimated fetal weight (grams)	1646 (258)	1620 (268)	<0.01
Femur length (mm)	57.4 (2.9)	57.4 (2.9)	0.64
Head circumference (mm)	288.8 (11.8)	283.7 (11.2)	<0.01
Birth			
Gestational age (weeks)	40.1 (35.0 – 42.4)	40.1 (35.2 – 42.1)	0.42
Weight (grams)	3518 (596)	3400 (574)	<0.01
Length (cm)*	55.1 (2.4)	54.0 (2.1)	<0.01
Head circumference (cm)*	38.1 (1.3)	37.3 (1.2)	<0.01
Weight <2500 grams (%)	119 (5.3)	123 (5.5)	0.77
Preterm birth (<36 weeks) (%)	133 (6.0)	129 (5.8)	0.81
Age 6 months			
Age at visit (months)	6.2 (5.2 – 7.9)	6.2 (5.2 – 7.9)	0.28
Weight (grams)	8,078 (845)	7,540 (812)	<0.01
Length (cm)	68.5 (2.4)	66.8 (2.3)	<0.01
Head circumference (cm)	44.2 (1.3)	43.1 (1.7)	<0.01
Age 14 months			
Age at visit (months)	14.3 (13.6 – 16.2)	14.3 (13.4 – 16.0)	0.05
Weight (grams)	10,824 (1,070)	10,191 (1,018)	<0.01
Length (cm)	78.9 (2.6)	77.5 (2.6)	<0.01
Head circumference (cm)	47.7 (1.3)	46.6 (1.1)	<0.01
Age 24 months			
Age at visit (months)	24.8 (23.4 – 28.1)	24.8 (23.4 – 28.1)	0.48
Weight (grams)	13,140 (1,376)	12,651 (1,412)	<0.01
Length (cm)	88.9 (3.4)	87.7 (3.4)	<0.01

*Values are means (SDS) or medians (95% range). Differences between boys and girls were compared using independent sample t-tests. *Measured at 1 months of age.*

Table 3 shows the associations of glucocorticoid receptor haplotypes with pre- and postnatal weight until the age of 24 months. No consistent associations were found between the different haplotypes and repeatedly measured weight in the three different time-intervals.

Table 3. Associations of glucocorticoid receptor haplotype with repeatedly measured weight from second trimester of pregnancy until 24 months of age

	Weight SDS change 2nd trimester – birth (95% CI)	**Weight SDS change Birth – 24 months (95% CI)**	**Weight SDS change 2nd trimester – 24 months (95% CI)**
Glucocorticoid receptor haplotype (copies)			
Haplotype 1			
0	0.03 (-0.06, 0.12)	-0.02 (-0.12, 0.08)	0.05 (-0.05, 0.15)
1	0.02 (-0.05, 0.09)	-0.03 (-0.11, 0.05)	0.03 (-0.04, 0.11)
2	Reference	Reference	Reference
Haplotype 2			
0	Reference	Reference	Reference
1	-0.06 (-0.12, 0.01)	-0.02 (-0.09, 0.06)	-0.10 (-0.17, -0.03)
2	-0.01 (-0.15, 0.13)	-0.01 (-0.17, 0.16)	-0.02 (-0.18, 0.15)
Haplotype 3			
0	Reference	Reference	Reference
1	0.06 (-0.01, 0.14)	0.06 (-0.02, 0.14)	0.09 (0.01, 0.17)
2	0.02 (-0.21, 0.25)	0.02 (-0.26, 0.29)	0.14(-0.14, 0.42)
Haplotype 4			
0	Reference	Reference	Reference
1	-0.02 (-0.09, 0.05)	0.02 (-0.07, 0.10)	0.12 (-0.15, 0.40)
2	-0.21 (-0.43, 0.02)	0.07 (-0.21, 0.36)	0.16 (-0.10, 0.44)
Haplotype 5			
0	Reference	Reference	Reference
1 or 2	-0.01 (-0.18, 0.16)	-0.36 (-1.02, 0.29)	-0.16 (-0.75, 0.43)
Haplotype 6			
0	Reference	Reference	Reference
1 or 2	-0.20 (-0.41, 0.01)	0.97 (-0.99, 2.93)	0.54 (0.13, 0.96)

Values are regression coefficients (95% confidence interval) and reflect the difference in standard deviation score of weight from 2nd trimester until 24 months of age for the different glucocorticoid haplotypes. SDS, gestational age-adjusted standard deviation score; CI, confidence interval. Models are adjusted for age at visit and gender.

Also, no consistent associations were found with length and head circumference (Table 4 and 5). In the dominant models, the associations between the glucocorticoid receptor gene polymorphisms were also not significant.

Table 4. Associations of glucocorticoid receptor haplotype with repeatedly measured length from second trimester of pregnancy until 24 months of age

	Length SDS change 2nd trimester – birth (95% CI)	**Length SDS change Birth – 24 months (95% CI)**	**Length SDS change 2nd trimester – 24 months (95% CI)**
Glucocorticoid receptor haplotype (copies)			
Haplotype 1			
0	0.01 (-0.09, 0.10)	-0.01 (-0.13, 0.12)	0.01 (-0.09, 0.10)
1	0.02 (-0.05, 0.09)	-0.01 (-0.10, 0.09)	0.01 (-0.07, 0.08)
2	Reference	Reference	Reference
Haplotype 2			
0	Reference	Reference	Reference
1	0.02 (-0.08, 0.12)	0.02 (-0.07, 0.11)	-0.07 (-0.14, 0.01)
2	0.08 (-0.08, 0.24)	0.03 (-0.19, 0.26)	0.10 (-0.06, 0.26)
Haplotype 3			
0	Reference	Reference	Reference
1	0.09 (0.02, 0.16)	0.05 (-0.05, 0.15)	0.07 (-0.01, 0.14)
2	0.16 (-0.08, 0.40)	0.13 (-0.24, 0.50)	0.12 (-0.15, 0.38)
Haplotype 4			
0	Reference	Reference	Reference
1	-0.03 (-0.10, 0.05)	-0.07 (-0.17, 0.04)	-0.02 (-0.10, 0.06)
2	-0.23 (-0.47, 0.01)	-0.02 (-0.40, 0.34)	-0.15 (-0.42, 0.11)
Haplotype 5			
0	Reference	Reference	Reference
1 or 2	0.14 (0.02, 0.25)	0.02 (-0.14, 0.19)	0.18 (0.05, 0.30)
Haplotype 6			
0	Reference	Reference	Reference
1 or 2	-0.10 (-0.23, 0.03)	-0.05 (-0.23, 0.14)	-0.11 (-0.25, 0.03)

Values are regression coefficients (95% confidence interval) and reflect the difference in standard deviation score of length from 2nd trimester until 24 months of age for the different glucocorticoid haplotypes. SDS, gestational age-adjusted standard deviation score; CI, confidence interval. Models are adjusted for age at visit and gender.

Table 5. Associations of glucocorticoid receptor haplotype with repeatedly measured head circumference from second trimester of pregnancy until 14 months of age

	Head circumference SDS change 2nd trimester – birth (95% CI)	Head circumference SDS change Birth – 24 months (95% CI)	Head circumference SDS change 2nd trimester – 24 months (95% CI)
Glucocorticoid receptor haplotype (copies)			
Haplotype 1			
0	-0.10 (-0.20, 0.01)	-0.16 (-0.33, 0.01)	-0.09 (-0.18, 0.01)
1	-0.05 (-0.13, 0.03)	-0.11 (-0.24, 0.02)	-0.06 (-0.13, 0.01)
2	Reference	Reference	Reference
Haplotype 2			
0	Reference	Reference	Reference
1	-0.07 (-0.16, 0.01)	0.01 (-0.11, 0.12)	-0.05 (-0.12, 0.02)
2	-0.12 (-0.30, 0.06)	0.08 (-0.18, 0.35)	-0.02 (-0.17, 0.13)
Haplotype 3			
0	Reference	Reference	Reference
1	0.11 (0.03, 0.20)	0.12 (-0.01, 0.25)	0.10 (0.02, 0.17)
2	0.11 (-0.17, 0.40)	-0.12 (-0.53, 0.29)	0.08 (-0.16, 0.33)
Haplotype 4			
0	Reference	Reference	Reference
1	0.01 (-0.07, 0.10)	0.08 (-0.05, 0.21)	0.12 (-0.15, 0.40)
2	0.18 (-0.12, 0.47)	0.19 (-0.22, 0.60)	0.17 (-0.10, 0.44)
Haplotype 5			
0	Reference	Reference	Reference
1 or 2	-0.10 (-0.33, 0.13)	-0.17 (-0.38, 0.03)	0.02 (-0.10, 0.14)
Haplotype 6			
0	Reference	Reference	Reference
1 or 2	0.07 (-0.09, 0.22)	0.07 (-0.15, 0.29)	0.06 (-0.07, 0.19)

Values are regression coefficients (95% confidence interval) and reflect the difference in standard deviation score of head circumference from 2nd trimester until 14 months of age for the different glucocorticoid haplotypes. SDS, gestational age-adjusted standard deviation score; CI, confidence interval. Models are adjusted for age at visit and gender.

Associations of the different haplotypes with the risks of prenatal growth retardation (growth deceleration) and postnatal growth acceleration are presented in Table 6. No significant differences

were found in risks of prenatal growth deceleration and postnatal growth acceleration for the different haplotypes. However, children who showed prenatally an increased risk of growth retardation compared to the reference, tend to have an increased risk of growth acceleration in postnatal life as well.

Table 6. Associations of glucocorticoid receptor haplotype with the risks of prenatal catch-down growth and postnatal catch-up growth until 24 months of age

	Prenatal growth deceleration (95% CI)		**Postnatal growth acceleration (95% CI)**	
Glucocorticoid receptor haplotype (copies)	Reference (-0.67 to 0.67 SDS)	Growth deceleration (<-0.67 SDS)	Reference (-0.67 to 0.67 SDS)	Growth acceleration (>0.67 SDS)
Haplotype 1				
0	Reference	1.09 (0.84, 1.42)	Reference	1.07 (0.78, 1.48)
1	Reference	1.16 (0.95, 1.41)	Reference	1.11 (0.87, 1.42)
2	Reference	Reference	Reference	Reference
Haplotype 2				
0	Reference	Reference	Reference	Reference
1	Reference	0.88 (0.73, 1.07)	Reference	0.99 (0.79, 1.26)
2	Reference	1.03 (0.68, 1.55)	Reference	1.20 (0.73, 1.97)
Haplotype 3				
0	Reference	Reference	Reference	Reference
1	Reference	1.17 (0.95, 1.43)	Reference	1.05 (0.82, 1.35)
2	Reference	0.90 (0.45, 1.84)	Reference	0.42 (0.13, 1.39)
Haplotype 4				
0	Reference	Reference	Reference	Reference
1	Reference	0.88 (0.71, 1.09)	Reference	0.97 (0.74, 1.25)
2	Reference	0.93 (0.47, 1.84)	Reference	0.80 (0.27, 2.39)
Haplotype 5				
0	Reference	Reference	Reference	Reference
1 or 2	Reference	0.96 (0.69, 1.34)	Reference	0.96 (0.65, 1.42)
Haplotype 6				
0	Reference	Reference	Reference	Reference
1 or 2	Reference	1.26 (0.86, 1.87)	Reference	1.28 (0.80, 2.07)

Values are odds ratios (95% confidence interval) and reflect the difference in risk of prenatal growth deceleration and postnatal growth acceleration until 24 months of age for the different glucocorticoid haplotypes. SDS, gestational age-adjusted standard deviation score; CI, confidence interval. Models are adjusted for age at visit and gender.

Discussion

In our population-based prospective cohort study we showed that glucocorticoid receptor gene polymorphisms are not consistently associated with growth in fetal and early postnatal life. Furthermore, we demonstrated that these polymorphisms were not related to size at birth or growth acceleration during the first 2 years of life.

The major strengths of our study are its prospective design from early fetal life and the size of the population-based cohort. Our analyses were based on over 6,000 growth measurements. Furthermore, the relative effect of variants of the glucocorticoid receptor gene on growth measurements might be larger in childhood, when the effect of various environmental factors, such as life style habits, is limited. A possible limitation is that the current study was performed in a healthy, population-based cohort study. DNA for genotyping was available in 59% of all subjects and was isolated from cord-blood. Missing cord-blood was mainly caused by logistical restraints at delivery. Of all genotyped eligible subjects at baseline, 22% did not participate in follow-up measurements. Our study was designed to assess pre- and postnatal growth in a relatively healthy group of children. As a consequence, the group of children born with small size for gestational age (n = 55) was too small for specific analyses focused on this group. Thus generalizability is limited with respect to children born preterm or with low birth weight.

Glucocorticoid receptor gene polymorphisms have been identified that contribute to the variability in glucocorticoid sensitivity. This sensitivity to glucocorticoids is known to show a large interindividual variation.[1] Persons vary considerably in their response to both endogenous and exogenous glucocorticoids. So it is likely that these polymorphisms are to some extent responsible for the variability in the sensitivity to glucocorticoids. Glucocorticoids are important regulators of the immune system, inflammatory processes and many other processes involved in fat and glucose metabolism. Previous studies examined the potential role of glucocorticoids in the development of adult disease. Studies in rats showed that activity of placental 11β-hydroxysteroid dehydrogenase type 2, which converts physiological glucocorticoids to inactive products, correlates positively with birth weight and negatively with placental weight.[27] Thus, fetuses with the greatest exposure to growth-retarding maternal glucocorticoids have low birth weight and high placental weight. In human studies, it is demonstrated that these fetuses might be at a higher risk of subsequent hypertension.[28] In addition, administration of low-dose dexamethasone to pregnant rats not only reduces birth weight but also leads to high blood pressure in young adult offspring.[27] Increased exposure to cortisol in adults leads again to increased risks of cardiovascular disease, type 2 diabetes and obesity.[2, 3] Therefore, these polymorphisms in the glucocorticoid receptor gene could, by increasing glucocorticoid sensitivity in the fetus for maternal glucocorticoids, lead to intrauterine growth retardation and metabolic and cardiovascular diseases in adulthood. Genetically established differences between individuals in glucocortcoid sensitivity may also be associated with these diseases.

The effect of glucocorticoids is mediated by the glucocorticoid receptor, which is thought to be the connection between HPA axis function and early life conditions. Rautanen *et al.* reported a common glucocorticoid receptor haplotype to be associated with short length and low weight at birth and higher indices of HPAA function later in life.[29] In humans, the possible importance of glucocorticoid sensitivity on fetal growth and HPA programming has not been previously investigated. However, previous studies have examined the associations of different polymorphisms in the glucocorticoid receptor gene and sensitivity to glucocorticoids. The results of these studies are conflicting. A few studies report positive associations between the N363S and *Bcl*I polymorphisms and hypersensitivity to glucocorticoids[9-11], while other studies found the opposite effect.[12, 13] The R23K polymorphism was associated with relative resistance to glucocorticoids.[7, 8] No associations were found yet with the *TthIII*I polymorphism.[4, 14] These studies suggest that genetically established differences in glucocortcoid sensitivity are important for various growth, development and health related outcomes. In addition, it is known that environmental, dietary, and socioeconomic factors also play an important role in determinants of body composition and metabolic factors. Associations with polymorphisms depend on many additional factors, for example differences in characteristics between populations, prevalence of the polymorphism, and interactions with other genetic polymorphism. All these factors may play a role in the discrepancies found between studies so far.

Our results suggest a recessive effect of the glucocorticoid receptor gene polymorphisms, which is in line with the heritability modes seen in earlier papers. We hypothezised that genetic variants leading to increased glucocorticoid sensitivity are associated with fetal growth retardation and postnatal growth acceleration. This hypothesis is based on previous observations showing associations of cortisol levels and low birth weight. Low birth weight and postnatal growth acceleration are again associated with obesity and other metabolic diseases. However, we did not find any consistent effect on pre- and postnatal weight, length and head circumference between the different glucocorticoid receptor haplotypes in our population-based study. Neither did we find associations with prenatal growth deceleration or postnatal growth acceleration. Therefore, we may conclude that our results do not support our hypothesis. Other recent identified glucocorticoid related polymorphisms, such as the brain-derived neurotrophic factor (BDNF) and the mineral corticoid gene, may affect pre- and postnatal growth by influencing the glucocorticoid metabolism. Further systematic searches for common genetic variants by means of genome-wide association studies will enable us to obtain a more complete understanding of what genes and polymorphisms are involved in growth in fetal life and infancy.

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Kidney growth and development during early childhood

3

Chapter 3.1

Reliability of kidney ultrasound measurements in children

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Abstract

Background: To assess the intraobserver and interobserver variability of renal measurements in children.

Methods: The study comprised 56 paired measurements in 28 children with a median age of 7.5 years (range: 3.0 – 15.0) and without renal or ureterovesical anomalies. Intraobserver and interobserver reproducibility was assessed by repeated measurements of left and right renal length, width and thickness. Intraclass correlation coefficients (ICCs) with the corresponding 95% confidence interval (CI) were calculated. Bland and Altman plots were computed to analyze agreement for the measurements. Limits of agreement ± 2 standard deviation for the mean differences in renal measurements were derived.

Results: 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 renal 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).

Conclusions: Overall, this study demonstrated good reproducibility and agreement of most renal dimensions in children measured by ultrasound. Ultrasound is an appropriate measure to assess renal dimensions in both clinical and epidemiological studies.

Introduction

Renal size is an important part of the assessment of the renal tract in childhood. Many epidemiological studies use renal ultrasound measurements to examine the associations of various determinants with renal size in children.[1, 2] This information may be relevant for identifying critical periods in life for renal growth and development.[3]

Ultrasound is widely recognized as method of choice for visualizing organs in children as it is noninvasive, cost-effective and efficient. It is known from literature that ultrasound, compared to CT or MRI, is an accurate method for visualising kidneys and evaluating renal growth in children.[4, 5] Bakker *et al.* concluded that renal volume calculations obtained by using ultrasound with ellipsoid formula resulted is a substantial systematic underestimation compared with MR imaging with the voxel-count method. However, a systematic underestimation of the renal volume is also reported by use of the ellipsoid formula with MR imaging.

Although the clinical relevance and research importance of renal ultrasound measurements in children is well known, little has been published about the reliability of these measurements. To compare renal ultrasound measurements as an outcome of different studies, it is necessary to minimize intraobserver and interobserver variability. A few studies assessed reproducibility of renal ultrasound measurements in children. Sargent *et al.* demonstrated an interobserver variation in the sonographic estimation of renal volume of approximately 30-40%.[6, 7] Schlesinger *et al.* concluded that the observed variability in ultrasound measurements of renal length is similar to the expected annual increase in length of the kidneys during childhood.[8] However, less is known about the reliability of renal ultrasound measurements in young children in both clinical and epidemiological research projects.

The aim of the present study was to evaluate the intra- and interobserver variability of renal ultrasound measurements in children.

Methods

Design

Children were selected from the outpatient clinic of the Erasmus Medical Center – Sophia Children's Hospital, a university hospital in Rotterdam, the Netherlands. In total, of the 30 subjects we asked, 28 subjects (participation 93%) participated in this study to validate renal ultrasound measurements. Non-response was mainly due to lack of time of the parents. No renal or ureterovesical anomalies were present in the study population. Written informed consent was obtained.

Two experienced sonographers (VE Kleyburg, SPE Snijders) performed all examinations at the same visit. The first observer scanned the ultrasound measurements in no specific order. Subsequently, the other observer did the same, after which the first examiner repeated the process.

The sonographers left the ultrasound room during each other's assessment. The time interval for the first observer to rescan the patient depended on the time the second observer took for the measurements, being about 10 minutes. Measurement results were blinded to both operators; the caliper read out on the screen was hidden. Printouts of the measurements were not read by the observers and were saved on hard disc for later analyses.

Ultrasound measurements

The examination was carried out in a quiet room with the child quietly awake in a supine position. Renal size was measured during each ultrasound examination using a transabdominal probe. Two-dimensional ultrasounds of the kidneys were performed in all the children. In a sagittal plane, the maximum longitudinal renal length was measured placing the calipers on the outer edges of the caudal and cranial side. Additionally, the phase of respiration was used to acquire the sagittal image. We asked the children to breathe out and hold shortly their breath to get a clear picture of the kidneys. Antero-posterior and transverse renal diameter were measured perpendicular to each other, outer to outer, in an axial plane.[9] Measures of maximal bipolar renal length, width and thickness were obtained from both the left and right kidney. Renal width and thickness were measured at the level of the renal hilum.[9, 10] All dimensions were measured to the nearest millimeter. Ultrasound examinations were performed using an ATL-Philips Model HDI 5000 (Seattle, Washington, USA) equipped with a 2.0 - 5.0 MHz curved array transducer.

Statistical analysis

To compare observers in detail we used the methods described by Bland and Altman.[11, 12] The first step was to plot the data and draw the line of equality. This visualizes the degree of agreement.[11] The consensus between and among observers was analyzed using the intraclass correlation coefficient (ICC) for all renal measurements. The ICC is defined as the ratio of the variance between subjects to total variance. The ICC measures the strength of the agreement of the variables, independent of the dimension of the variable considered. Additionally, the 95% confidence interval (CI) was calculated for all renal measurements.

Next, agreement was tested to investigate intra- and interobserver reproducibility.[11] We created Bland and Altman plots by plotting the differences of all the measurements against their mean with the 2 SD of the mean to see the distribution and to find any possible differences from the mean within or between the observers.[11] The average differences between duplicate measures were tested using the paired sample t-test to see if there was systemic bias. If the differences are Gaussian distributed, 95% of the differences will lie between the mean ±2 SD limits. These are the limits of agreement, and the measures between and among observers can be assumed to be interchangeable within these limits. An advantage of the Bland and Altman plots over the

statistical testing is that one can visualize between which boundaries a measurement is interchangeable and how large the measurement differences between operators are in proportions. How small the limits of agreement should be is a clinical, not a statistical, decision that should be made in advance of the analysis. A priori, we considered renal measurements in children reproducible and valid in case of ICC over 0.80 and mean differences within 10% from the mean of two different measurements. Statistical analysis was performed using Statistical Package for the Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Median age of the children was 7.5 years (range: 3.0 – 15.0) and 17 (61%) were male. A total of 14 children (50%) was younger than 8 years of age. Table 1 presents the descriptive statistics of the ultrasound measurements of left and right renal dimensions assessed in this study. Figure 1 shows plots of measurements of left and right renal dimensions between observers against the line of equality. All points seem to lie randomly around this line, meaning that there seems to be no bias. The variables are close to the line of equality as well, indicating good agreement and suggesting small differences between observers.

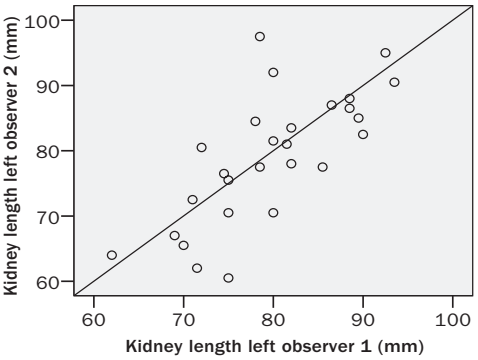
Table 1. Descriptive statistics for ultrasound measurements of left and right renal dimensions in children

	Sonographer 1				Sonographer 2			
	1st observation		2nd observation		1st observation		2nd observation	
Renal measurements (mm)	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Left kidney								
Length	79.4	8.0	79.9	7.9	78.5	10.2	79.2	9.8
Width	34.4	3.4	34.3	3.3	33.5	3.9	33.5	3.8
Thickness	35.3	3.3	35.5	3.7	38.8	4.2	39.0	4.1
Right kidney								
Length	78.1	8.3	78.4	8.8	76.7	11.1	77.9	10.8
Width	34.7	3.4	34.0	3.4	33.9	4.1	34.2	3.7
Thickness	36.1	3.4	35.6	3.3	40.1	4.7	40.5	4.2

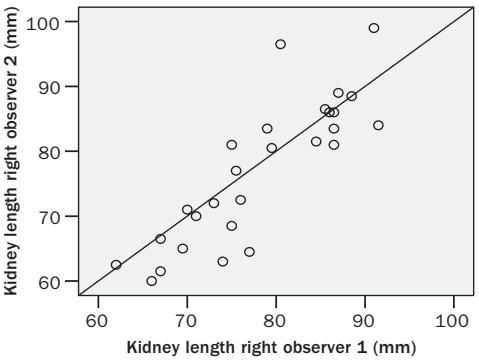
SD, standard deviation. Analyses were based on a group of 28 children.

Figure 1. Renal ultrasound measurements of observers with the line of equality

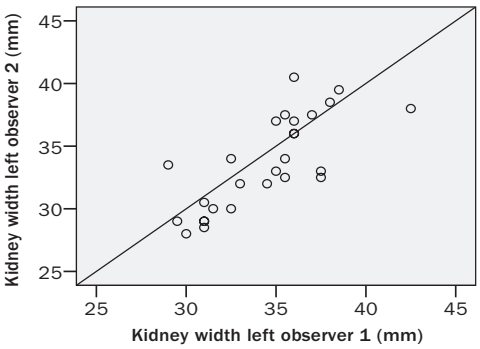
a. Left renal length measurements



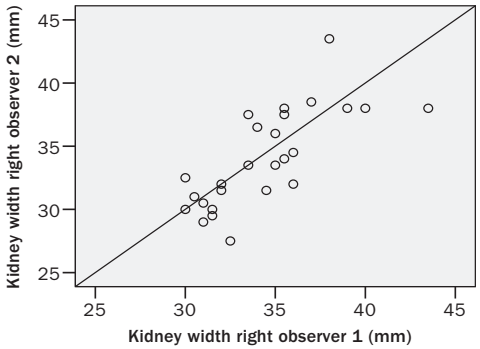
b. Right renal length measurements



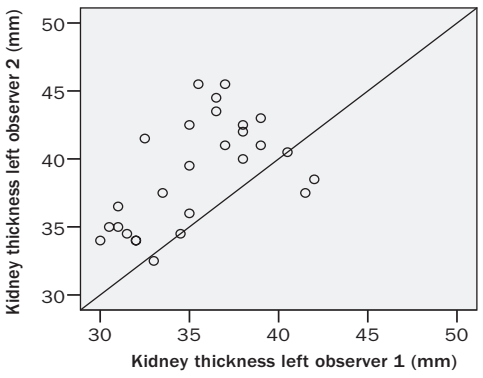
c. Left renal width measurements



d. Right renal width measurements



e. Left renal thickness measurements



f. Right renal thickness measurements

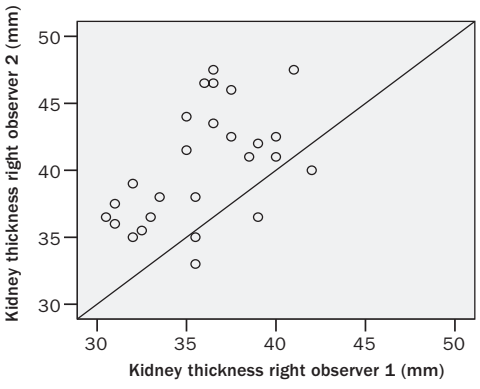


Table 2 presents the intra-class correlation coefficients (ICCs) with their 95% confidence interval. Intraobserver ICC ranged from 0.93 (left and right renal width and right renal thickness) to 0.99 (left renal length) and interobserver ICC ranged from 0.64 (right renal thickness) to 0.90 (right renal length).

Table 2. Intra- and interobserver intraclass correlation coefficient (ICC) and 95% confidence interval for left and right renal dimensions in childhood

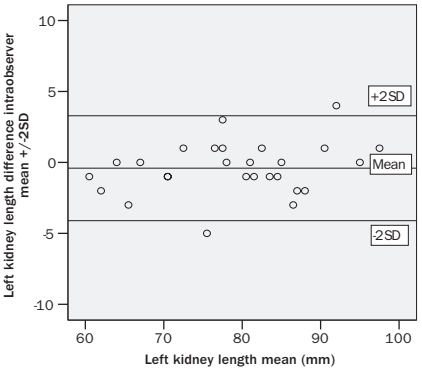
	Intraobserver ICC (95% confidence interval)	**Interobserver ICC (95% confidence interval)**
Left kidney		
Length	0.99 (0.97, 0.99)	0.81 (0.57, 0.91)
Width	0.93 (0.85, 0.97)	0.83, 0.63, 0.92)
Thickness	0.96 (0.91, 0.98)	0.66 (0.26, 0.85)
Right kidney		
Length	0.97 (0.93, 0.99)	0.90 (0.78, 0.95)
Width	0.93 (0.84, 0.97)	0.83 (0.63, 0.93)
Thickness	0.93 (0.84, 0.97)	0.64 (0.20, 0.84)

ICC, intraclass correlation coefficient

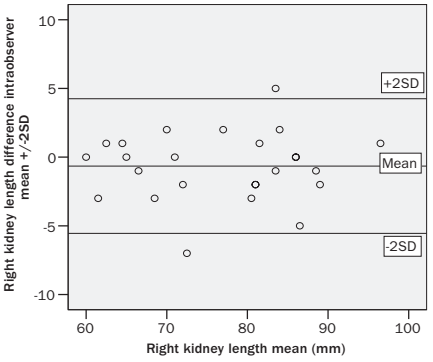
Figure 2 shows Bland and Altman plots for the differences in left and right renal measurements among or between observers against the mean. The limits of agreement (+/-2 SD) are plotted in the figures. Most differences lie between the limits of agreement.

Figure 2. Intra- and interobserver Bland and Altman plots of variation in renal ultrasound measurements among and between observers

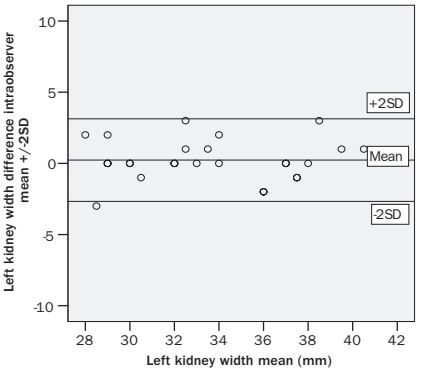
a. Intraobserver agreement left kidney length



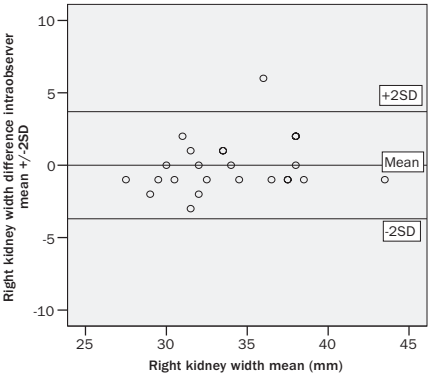
b. Intraobserver agreement right kidney length



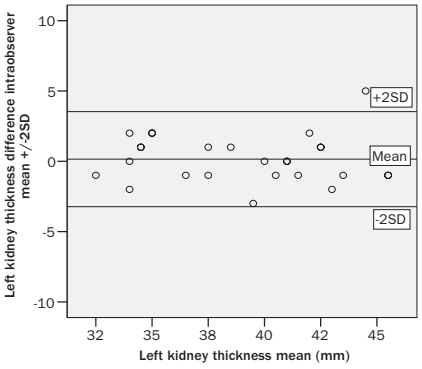
c. Intraobserver agreement left kidney width



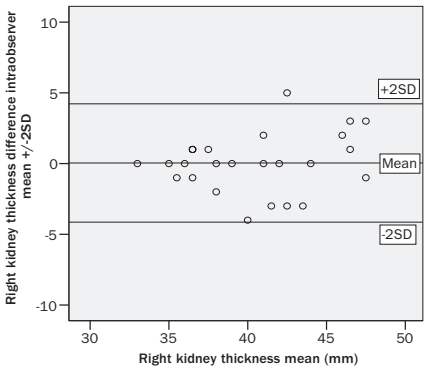
d. Intraobserver agreement right kidney width



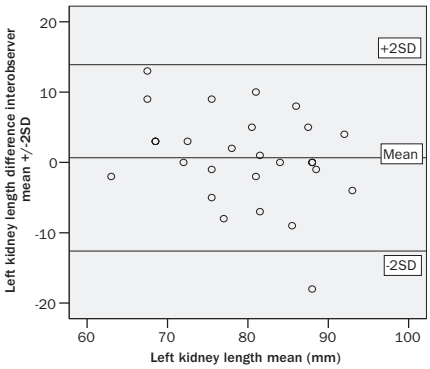
e. Intraobserver agreement left kidney thickness



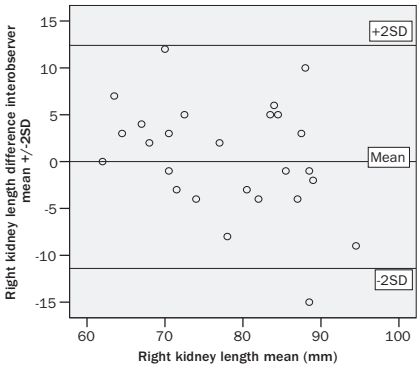
f. Intraobserver agreement right kidney thickness



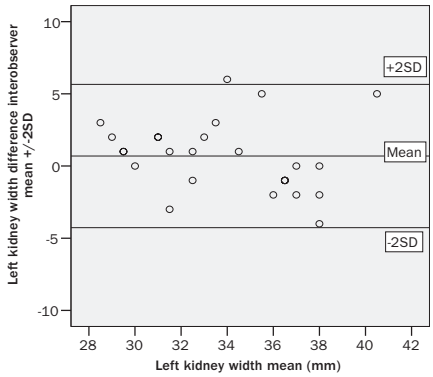
g. Interobserver agreement left kidney length



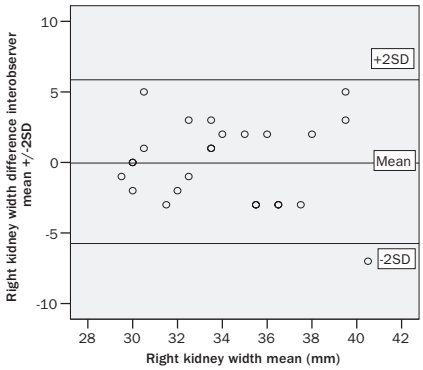
h. Interobserver agreement right kidney length



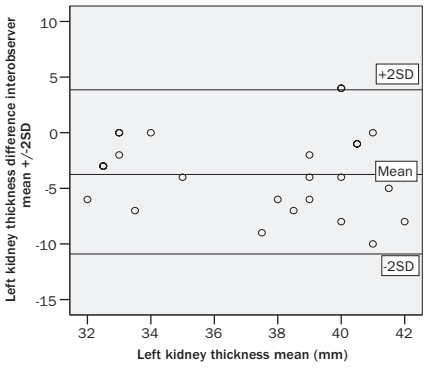
i. Interobserver agreement left kidney width



j. Interobserver agreement right kidney width



k. Interobserver agreement left kidney thickness



l. Interobserver agreement right kidney thickness

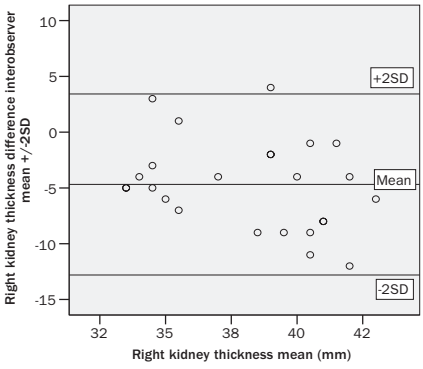


Table 3 shows the mean differences of the left and right renal dimensions among and between observers and the 95% limits of agreement (M_d +/-2 SD_d). The observed difference from the mean for left and right renal dimensions ranged from 0 mm (0%) (intraobserver right renal width) to -4.68 mm (12%) (interobserver right renal thickness). The mean difference between duplicate measures was not significantly different from zero. This suggest that no systematic difference between the pairs of results. The limits of agreement ranged from –2.67 (8.0%) to 3.13 mm (9.2%) (intraobserver left renal width) to the widest limit from –14.3 (18.0%) to 15.5 mm (19.2%) (interobserver left renal length).

Table 3. Intra- and interobserver measurement variation in absolute number and in proportions of the mean with 95% limits of agreement

Intraobserver agreement observer 1	**Mean difference**		**95% limits of agreement**	
	(mm)	**(%)**	**Lower limit**	**Upper limit**
Left kidney				
Length	-0.44	0.6	-4.00	3.12
Width	0.15	0.4	-3.55	3.55
Thickness	-0.22	0.6	-2.96	2.52
Right kidney				
Length	-0.12	0.2	-6.04	5.80
Width	0.73	2.1	-2.87	4.33
Thickness	0.46	1.3	-3.00	3.92
Intraobserver agreement observer 2	**Mean difference**		**95% limits of agreement**	
	(mm)	**(%)**	**Lower limit**	**Upper limit**
Left kidney				
Length	-0.41	0.5	-4.11	3.29
Width	0.23	0.7	-2.67	3.13
Thickness	0.15	0.4	-3.23	3.53
Right kidney				
Length	-0.65	0.8	-5.55	4.25
Width	0	0	-3.70	3.70
Thickness	0.04	0.1	-4.14	4.22

Table 3. continued

Interobserver agreement observation 1	Mean difference (mm)	(%)	95% limits of agreement Lower limit	Upper limit
Left kidney				
Length	0.63	0.8	-14.3	15.5
Width	0.70	2.0	-4.76	6.16
Thickness	-3.78	10.2	-11.2	3.60
Right kidney				
Length	1.37	1.8	-10.6	13.4
Width	0.73	2.1	-5.03	6.49
Thickness	-4.27	11.2	-12.7	4.13
Interobserver agreement observation 2	**Mean difference (mm)**	**(%)**	**Limits of agreement Lower limit**	**Upper limit**
Left kidney				
Length	0.67	0.8	-12.6	13.9
Width	0.69	2.0	-4.27	5.65
Thickness	-3.50	9.4	-10.9	3.86
Right kidney				
Length	0.46	0.6	-11.4	12.4
Width	-0.04	0.1	-5.94	5.86
Thickness	-4.68	12.3	-12.8	3.42

T-tests were performed to compare the difference from the mean; none of the measurements was significantly different from zero.

Discussion

Ultrasound is widely used to visualize organs in children. Left and right renal dimensions can be measured in children with 2-dimensional (2D), sonography. The aim of this study was to assess the reproducibility of left and right renal dimensions, length, width and thickness, in children without renal or ureterovesical anomalies.

Both reproducibility, whether two observers using the same measurement to obtain the same result, and repeatability, whether a single observer obtains the same results when he/she takes the repeated measurement, are types of agreement or reliability. A measurement process should be both accurate and reproducible. It is known from literature that ultrasound, compared to CT or MRI, is an accurate method for visualising kidneys and evaluating renal growth in children.[4, 5] Bakker *et al.* concluded that renal volume calculations obtained by using US with ellipsoid formula resulted is a substantial systematic underestimation of 25% (44.7 mL (95% CI: -51, -38)) compared with MR imaging with the voxel-count method. However, use of the ellipsoid formula with MR imaging also resulted in a systematic underestimation of the renal volume. So, in view of higher costs and increased processing time of MR imaging-based volumetry, ultrasound will remain the modality of choice.[5] However, less is known about the reproducibility of these renal ultrasound measurements. The mean difference between duplicate measurements is an indicator of the amount of systematic difference between the pairs of results. The standard deviation of the differences between duplicated measurements represents the reproducibility of the process. The larger the mean difference, the larger the systematic bias and the lower the accuracy of the process. Similarly, the larger the standard deviation, the larger random errors and the lower the reproducibility.

In this study we used various statistical methods to assess the reproducibility and repeatability of left and right renal dimensions in children measured by ultrasound. A simple plot of the results of one observer against the other showed that the data points are clustered near the line of equality, indicating good agreement and little differences between observers.

Beforehand, we decided that we could consider renal measurements in children as good reproducible and valid in case of ICC over 0.80. Our results showed high ICCs (over 0.80) for almost all renal ultrasound measurements, with low 95% confidence intervals, indicating a high degree of similarity among and between observers and thus good agreement. Only for renal thickness between observers we found ICCs smaller than 0.80, representing a poor reproducibility. Intraobserver ICCs were higher (ranging from 0.93 to 0.99) than interobserver ICCs (ranging from 0.64 to 0.90) for all left and right renal dimensions. This phenomenon, so called interobserver variability, could be expected since two observers measure differently.

The Bland and Altman plots showed good agreement among and between observers. We found for almost all renal ultrasound measurements differences within 10% from the mean. Only for the interobserver measurements of left and right renal thickness the difference from the mean

was more than 10%. The renal thickness measurement is the most difficult measurement of the renal ultrasound measurements performed in this study. This quite large variation in the measurement of renal thickness is probably due a random error, which may result in a poorer precision and a lower power of our study results. Often renal volume is used as an outcome measurement in different studies, but it requires multiple measurements to calculate.[10] Although, renal volume is the best surrogate measure for assessing nephron number in epidemiological studies and measuring renal volume by ultrasound is validated, the lower reproducibility does also affect the accuracy of volume measurements. An alternative for clinical and epidemiological studies, focused on comparisons between groups, would be to use only renal length and width instead of renal volume. This would be appropriate for internal comparisons which are mainly focused on ranking in renal growth characteristics. But it is known from literature that renal volume is a better representation of renal weight and growth than renal length.[13,14] So we think that is it still relevant to measure renal thickness, even if the variation of the measurement is quite large.

A few other studies measured intraobserver and interobserver variations of renal ultrasound measurements in children. The major difference between these previous studies and our study is the aim of the study. Our study was designed to assess renal development in a relatively healthy group of children and to assess whether renal measurements are reproducible and repeatable as an outcome measure for different studies. The aim of the cited papers was rather to evaluate renal growth. Their study population consisted of children referred for renal ultrasound. More in detail, Sargent *et al.* studied sonographic measurements of renal length and volume in children aged 16 years and younger to evaluate renal growth with time.[6,7] They demonstrated an interobserver variation in the sonographic estimation of renal volume of approximately 30-40%. The absolute interobserver variation increased with renal volume, representing approximately 2-3 years' normal growth in children over 2 years.[6] Observer error in the measurement of renal length is equivalent to between 2 and 3 years normal growth for children older than 1 year.[7] They did not present data of intraobserver variation.

Schlesinger *et al.* focused on renal length in children with a median age of 5 years and showed a mean interobserver variation between two imagers ranging from 3.87 to 5.49 mm and an intraobserver variation ranging from 0.87 to 3.61 mm. They concluded that the observed variability in sonographic measurements of renal length is comparable to the expected annual increase in length of the kidneys during childhood. Caution is suggested when using sonography to evaluate renal growth in children during a year's time.[8]

The major strength of our study is the young age at which we studied the intraobserver and interobserver variability of renal ultrasound measurements. To our knowledge, previous studies of reproducibility of renal ultrasound measurements have mostly been performed in older children and adults or focused on one of the renal ultrasound measurements.[15,16] Second, two well trained sonographers performed all the renal ultrasound measurements in our study, so our results are probably to a large extend depended on the experience of these two sonographers. Furthermore,

renal measurements could be measured in 100% of the participating children. Another strength of our study is that we used various statistical methods, which give a useful indication about the reliability of renal ultrasound measurements in children.

A limitation of this study could be the possibility of recall bias. This is an important general issue in intra-observer studies. We tried to avoid recall bias as much as possible by taking 10 minutes between the two measurements of one observer. In this period, the observer left the room and performed other measurements in other children. Additionally, we scheduled many children participating in the study during one session, decreasing the possibility of remembering the results. If present, the effect of recall bias, would only affect the results of the intraobserver variability and not the interobserver variability.

In conclusion, we demonstrated good reproducibility of most left and right renal dimensions in children measured by ultrasound. Only renal thickness has a poor interobserver reproducibility. This information is important for clinical and epidemiological research projects focused on renal dimensions in young children.

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Chapter 3.2

Normal fetal and early childhood kidney growth

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Abstract

Background: 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 and at the postnatal ages of 6 and 24 months. Gender specific reference growth curves were constructed. Analyses were based on more than 2,500 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.

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

Introduction

Assessment of kidney size in children is important for clinical and epidemiological studies. Abnormal early kidney development may have perinatal and neonatal consequences.[1] Smaller fetal kidney size has also been suggested to be related to hypertension and renal disease in adulthood.[2,3] Recently, we showed in the same cohort that small kidney size in fetal life tends to persist in early childhood. Furthermore, maternal anthropometrics and fetal biometrics and blood flow patterns were associated with kidney size in childhood. Higher growth rates in early childhood were positively associated with combined kidney volume.[4] These results suggest that fetal and early postnatal exposures and growth variation might have persistent consequences for kidney size. 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.[5,6] One study showed reference data on postnatal kidney growth from birth to 18 months of age.[7] Previous studies were based on postnatal kidney growth characteristics and mostly focused on kidney volume in relation to weight, height or body surface area. Recently, new reference centiles were generated to assess kidney size of children with 'single kidneys' to identify those patients with unfavorable course and relevant single kidney growth impairment.[8] Currently, no studies have evaluated normal kidney growth from late fetal life until early childhood. This perinatal period may be of importance to identify abnormal kidney size and growth, with subsequent short and long-term clinical consequences.[9,10]

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 postnatal age of 24 months in a population-based cohort.

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.[11,12] Detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of 1,232 mothers and their children.[11,12] 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 behavior 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 than mild pyelectasis over 10 mm (n = 3) were present in our study population. In total, analyses were based on more than 2,500 kidney measurements. The number of available kidney growth measurement for the analyses are shown in Table 1.

Table 1. 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	94.9 (1153)	81.2 (732)	78.2 (669)
Width	95.2 (1157)	77.2 (696)	72.8 (623)
Depth	95.1 (1155)	76.8 (692)	76.9 (624)
Volume	94.4 (1147)	76.6 (690)	72.9 (615)
Right kidney			
Length	94.7 (1151)	82.9 (747)	81.9 (701)
Width	95.4 (1159)	82.4 (742)	80.6 (690)
Depth	95.4 (1159)	82.2 (741)	80.6 (690)
Volume	94.5 (1148)	82.1 (740)	80.4 (688)

Values are percentages (absolute number of children).

Ultrasound measurements

Gestational age was established by fetal ultrasound. Crown-rump length was used for pregnancy dating until a gestational age of 12 weeks and 5 days (crown-rump length smaller than 65 mm), and biparietal diameter was used for pregnancy dating thereafter (gestational age from 12 weeks and 5 days onwards, biparietal diameter larger than 23 mm).[13]

Fetal left and right kidney size were measured in third trimester of pregnancy using an ATL-Philips HDI 5000 (Seattle, Washington, USA) equipped with a 2.0 - 5.0 MHz curved array transducer. 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.[14] 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.[14, 15] The images were sufficiently magnified to ensure optimal measurements.[14] Fetal growth characteristics (head circumference, abdominal circumference, femur length) were measures at the same visit and estimated fetal weight was calculated.[13]

Postnatally, two-dimensional ultrasounds of the kidneys were performed 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.[14, 15] 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 * mean length (cm) * mean width (cm) * mean depth (cm).[15, 16] The infants' anthropometrics, including weight and length, were all measured at the ages of 1.5, 6 and 24 months. Date of birth, birth weight and gender were obtained from midwife and hospital registries.

For the fetal ultrasound measurements, intra- and interobserver studies showed intra-class correlation coefficient (ICC) higher than 0.98 and corresponding coefficients of variation (CV) lower than 6%. Bland and Altman plots to test agreement of measurements demonstrated 95% limits of agreement to be within 10% difference from the mean of measurements, indicating good reproducibility.[17] 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 inter-observer ICCs ranged from 0.64 (right renal thickness) to 0.90 (right renal 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).[18]

Statistical analysis

Differences of fetal and postnatal characteristics between boys and girls were assessed by t-tests and chi-square 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.[19, 20] For reference kidney growth curves, postconceptional age was plotted against kidney length, width, depth and volume. From the original data, measurements of more than two standard deviations (SDS) from the regression line, fitted on our data, were considered to be outliers (n = 10) and were therefore removed. They were probably a result of measurement error or a data entry error. The best fitting curves were determined using second-degree fractional polynominals.[21] 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.[20, 22] The best fitting fractional polynominal curves were chosen by comparing the deviances, by Akaike's Information Criterion, and by visually checking the goodness of fit. Next, regression lines were fitted for the dependency of the residual SD on conceptional age.[23] 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. The median age of 2-year-old children visiting the research center was 25 months (95% range: 23.6 – 28.3). Since only 34 children had measurements beyond the postnatal age of 28 months (160 weeks postconceptional), results are presented until the postnatal age of 28 months. Kidney growth reference curves were constructed for a postconceptional age from 30 to 160 weeks, corresponding to a gestational age of 30 weeks and a postnatal age of 28 months.

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 2). The overall median age at their visit in third trimester of pregnancy was 30 weeks of gestation (total range: 27.1 - 35.1). The overall median age of infants at their 6 months postnatal visit was 6.3 months (total range: 5.1 - 11.0) and at their 24 months postnatal visit 25.1 months (total range: 21.6 - 31.6). Third trimester head circumference and postnatal weight and length at the ages of 6 and 24 months 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 were born with a small size for gestational age (<-2 SDS), 18 children had a low birth weight (<2500 grams) and 23 children were born preterm (gestational age <37 weeks).

Table 2. 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 sample t-tests or X^2 tests.

Table 3 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, these gender differences were only significant for left kidney structures and right kidney length. Both groups showed trends towards smaller left kidney measurements compared to right kidney measurements at all ages (Table 4).

Reference kidney growth curves of individual measurements and fitted centiles are given in Figure 1. 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 values for kidney length, width, depth and volume are given in the Appendix (Tables 1S-4S).

Table 3. Differences between 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 sample t-tests.

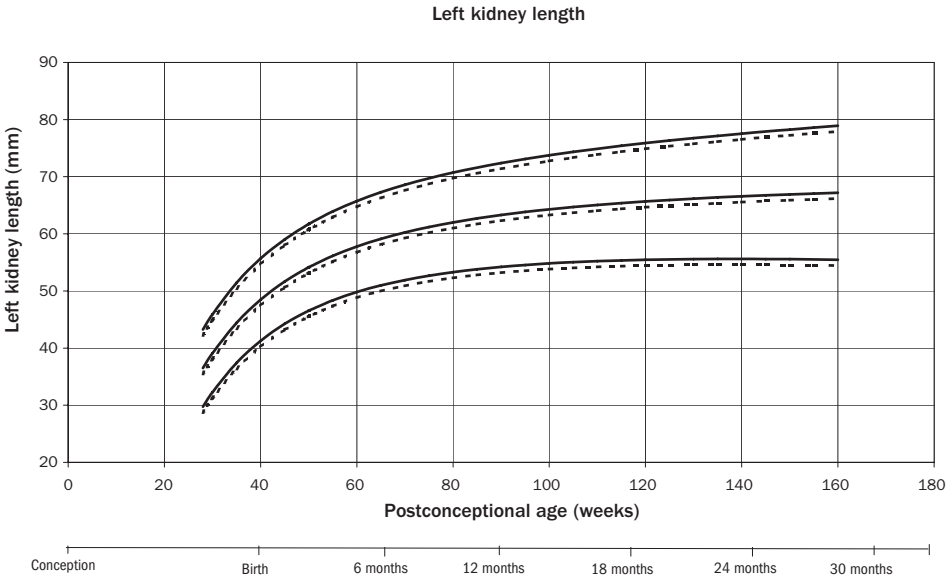
Table 4. Differences between left and right kidney structures stratified for gender

	Boys			Girls		
Kidney measurement	**Left kidney**	**Right kidney**	**P-value**	**Left kidney**	**Right kidney**	**P-value**
Gestational age 30 weeks	**(n = 604)**	**(n = 553)**		**(n = 603)**	**(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

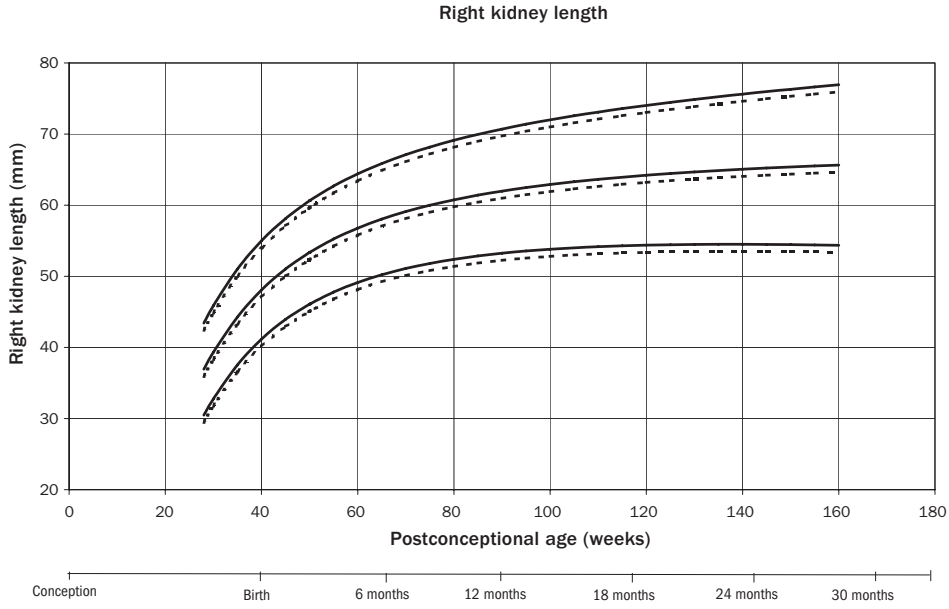
Values are means (95% range). Differences between left and right kidney structures were compared using paired sample t-tests.

Figure 1. Reference growth curves of right and left kidney length, width, depth and volume in boys and girls according to postconceptional age

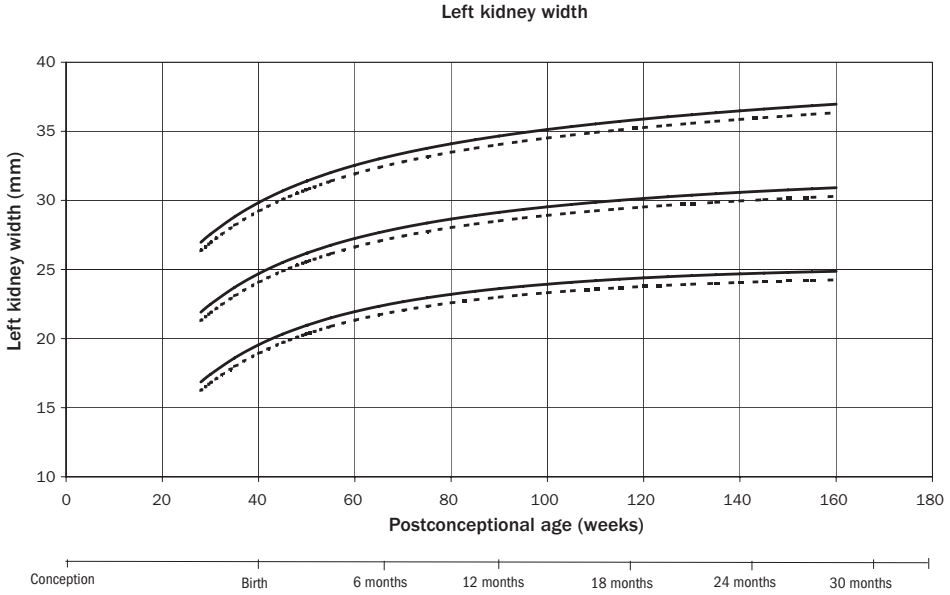
a. Left kidney length



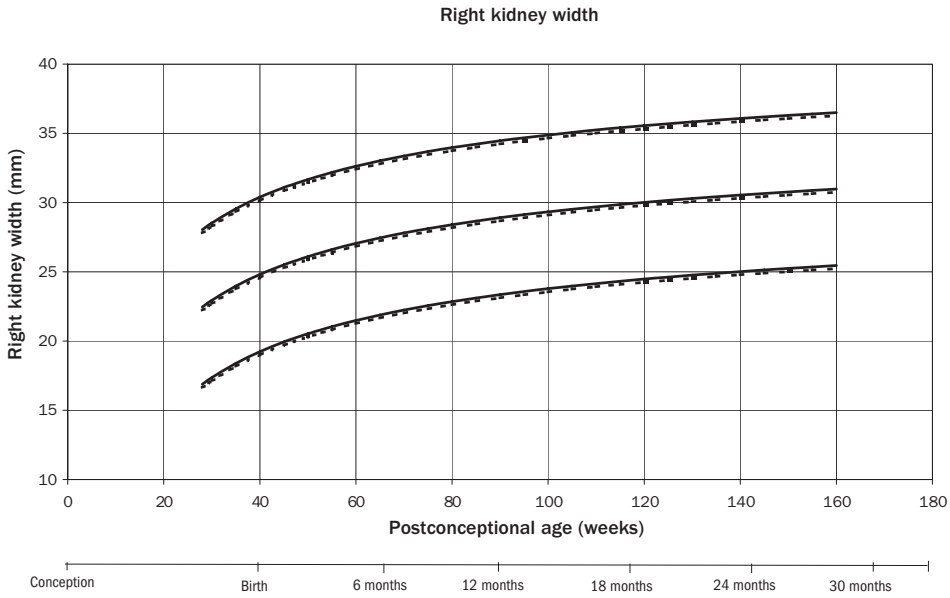
b. Right kidney length



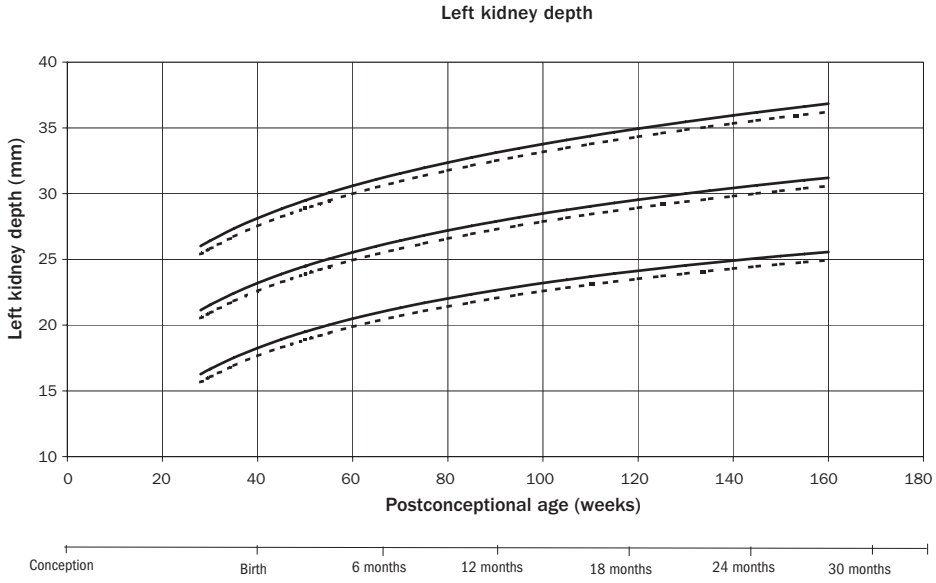
c. Left kidney width



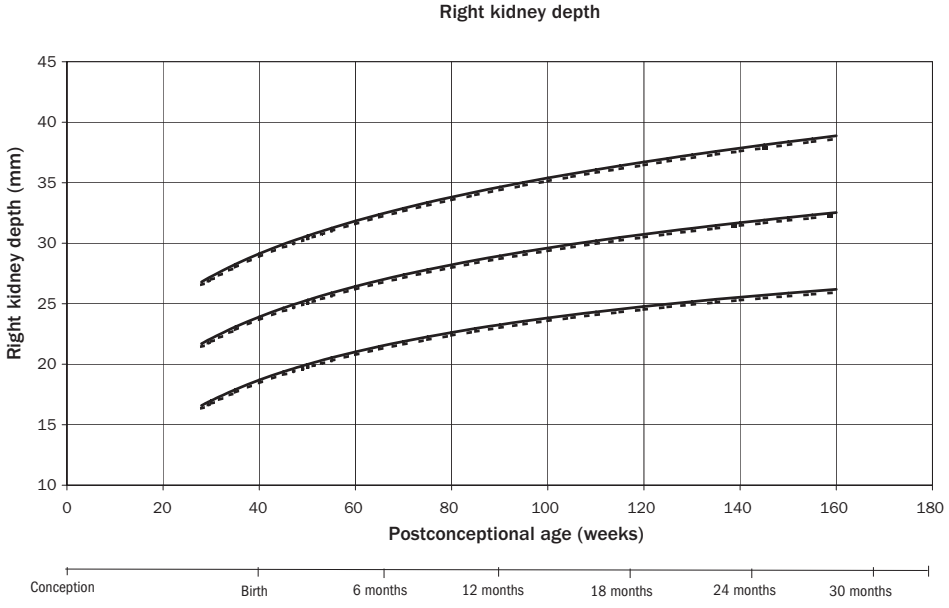
d. Right kidney width



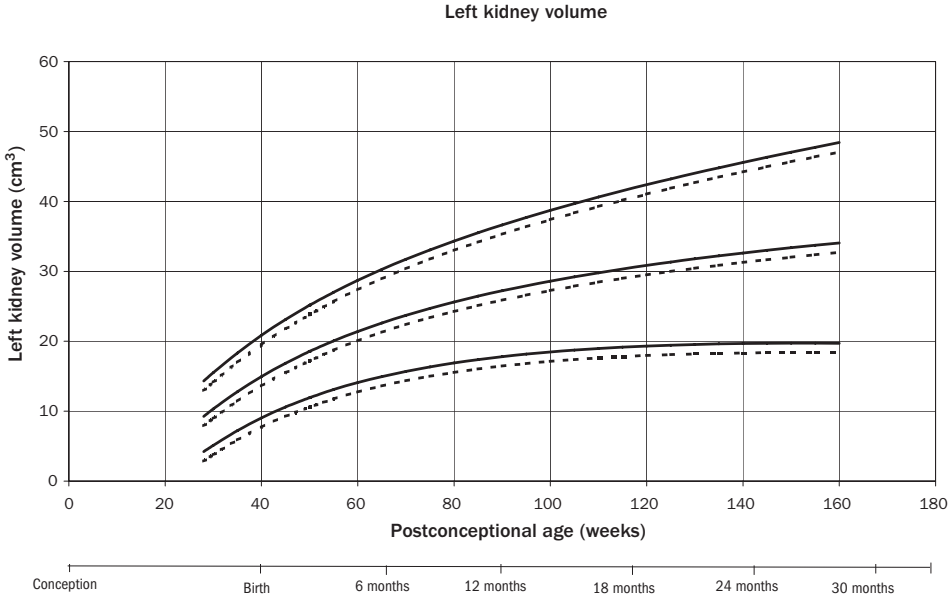
e. Left kidney depth



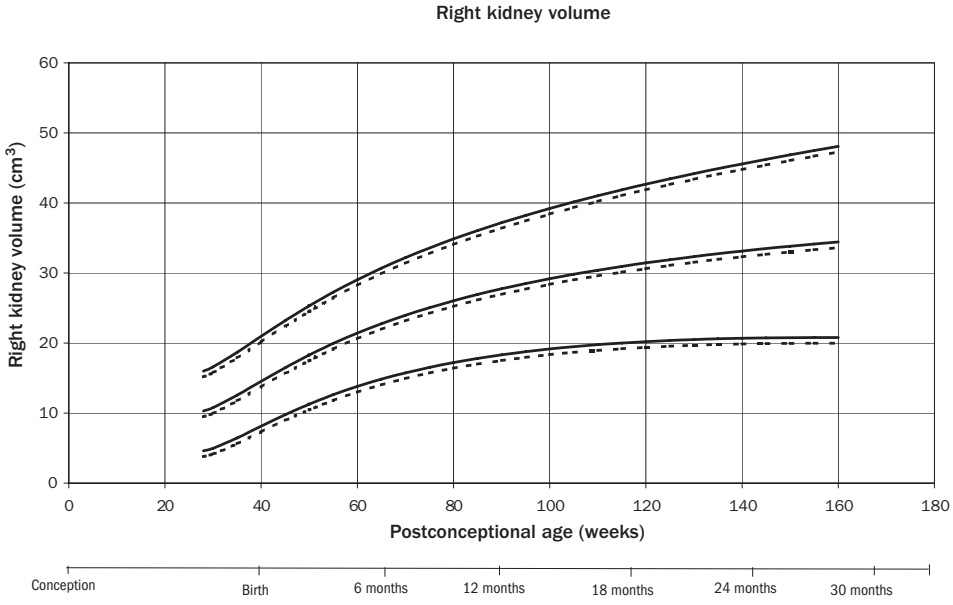
f. Right kidney depth



g. Left kidney volume



h. Right kidney volume



Gender-differentiated growth curves for left and right kidney length, with, depth and volume measurements in relation to post-conceptional age with 3rd and 97th fitted centiles. Postconceptional age was based on last menstrual period. The straight lines represent boys and the dotted lines represent girls.

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

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

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. We observed differences in kidney structures between left and right kidneys and boy and girls.

The major strength of our study is its prospective design from fetal life and the size of the population-based cohort. Our reference curves were based on more than 2,500 kidney measurements. To our knowledge, no previous studies focused on kidney size in early life were based on such large numbers. All fetal ultrasounds were carried out by two sonographers and 86% of all postnatal ultrasounds were performed by one trained sonographer.[24] 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. Our results would be biased if the subject characteristics differ between those included and not included in the present analyses. However, we observed no differences in growth and fetal kidney

characteristics between subjects with and without postnatal kidney measurements. 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 generalizability to preterm children or children with a small size for gestational age at birth children. 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, these gender differences were only significant for left kidney structures and right kidney length. Several studies in healthy neonates and adults have also shown that males have larger kidneys than females.[25, 26] One explanation for this finding may be a growth stimulating effect of androgens or Y-chromosome related genes. Another explanation could be that testosterone levels are significantly higher during fetal life in males compared to females.[27, 28]

Previously published data showed conflicting results concerning differences between left and right kidney size. Some studies found no difference between left and right kidney size[29, 30], whereas others suggested the left kidney to be larger.[31, 32] Most consistent findings have been reported for kidney length, for which left length seems larger that right length.[7, 25, 26, 31, 33] We found that left kidney length was larger in both boys and girls at the postnatal ages of 6 and 24 months. In fetal life we found no significant difference between left and right kidney length.

Kidney growth is fastest during fetal life and early infancy and the rate of increase gradually slows through the remainder of the first year of life and finally stabilizes.[34] In our study, numbers and curves for the 3rd centiles showing decreasing kidney volumes at the older ages from 140 weeks and onwards. The decreasing numbers are due to wider ranges because of the low number of children with visits around 140 weeks and onwards. To deal with non-linear kidney growth, some sonographic standards, including means and standard deviations, for kidney size in relation to age have been published.[34-36] A few other studies created linear or non-linear polynominal regression equations for kidney size during the first year of life.[29, 33] One study created reference materials for kidney size in healthy children beyond the neonatal period.[7] 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.

In conclusion, kidney size differed between boys and girls from the age of 30 weeks of pregnancy until 24 months of age. Left kidney size tend to be smaller than right kidney size. At the age of 24 months, the differences in right kidney size between boys and girls were attenuated. 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.

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Appendix

Table 1S. Reference values for left and right kidney length according to postconceptional age in boys and girls

Boys		Left kidney length (mm)			Right kidney length (mm)		
Postconceptional age (weeks)	Postnatal age (months)	3rd centile	Mean	97th centile	3rd centile	Mean	97th centile
30	-	32.3	39.1	45.9	32.8	39.3	45.9
40	0	41.3	48.5	55.7	41.2	48.1	55.0
50	2.5	46.6	54.1	61.7	46.1	53.4	60.7
60	5	49.8	57.8	65.7	49.1	56.8	64.4
80	9	53.3	62.0	70.7	52.4	60.8	69.1
100	14	54.8	64.3	73.8	53.8	62.9	72.0
120	19	55.5	65.7	75.9	54.4	64.2	74.0
140	23	55.6	66.6	77.5	54.5	65.1	75.6
160	28	55.5	67.2	78.9	54.4	65.7	76.9
Girls		**Left kidney length (mm)**			**Right kidney length (mm)**		
Postconceptional age (weeks)	Postnatal age (months)	3rd centile	Mean	97th centile	3rd centile	Mean	97th centile
30	-	31.2	38.1	44.9	31.8	38.3	44.9
40	0	40.3	47.5	54.7	40.2	47.1	54.0
50	2.5	45.6	53.1	60.7	45.1	52.4	59.6
60	5	48.8	56.8	64.7	48.1	55.8	63.4
80	9	52.3	61.0	69.7	51.4	59.8	68.1
100	14	53.8	63.3	72.7	52.8	61.9	71.0
120	19	54.4	64.7	74.9	53.4	63.2	73.0
140	23	54.6	65.6	76.5	53.5	64.1	74.6
160	28	54.5	66.2	77.9	53.4	64.6	75.9

Table 2S. Reference values for left and right kidney width according to postconceptional age in boys and girls

Boys		Left kidney width (mm)			Right kidney width (mm)		
Postconceptional age (weeks)	Postnatal age (months)	3rd centile	Mean	97th centile	3rd centile	Mean	97th centile
30	-	17.4	22.5	27.6	17.4	22.9	28.5
40	0	19.6	24.7	29.8	19.2	24.8	30.4
50	2.5	21.0	26.2	31.4	20.5	26.1	31.7
60	5	22.0	27.3	32.5	21.5	27.1	32.6
80	9	23.2	28.7	34.1	22.9	28.4	34.0
100	14	23.9	29.5	35.1	23.8	29.3	34.9
120	19	24.4	30.1	35.9	24.5	30.0	35.6
140	23	24.7	30.6	36.5	25.0	30.6	36.1
160	28	24.9	30.9	37.0	25.5	31.0	36.5
Girls		**Left kidney width (mm)**			**Right kidney width (mm)**		
Postconceptional age (weeks)	Postnatal age (months)	3rd centile	Mean	97th centile	3rd centile	Mean	97th centile
30	-	16.8	21.9	26.9	17.1	22.7	28.3
40	0	18.9	24.1	29.2	19.0	24.6	30.2
50	2.5	20.3	25.6	30.8	20.3	25.9	31.5
60	5	21.3	26.6	31.9	21.3	26.8	32.4
80	9	22.6	28.0	33.5	22.6	28.2	33.7
100	14	23.3	28.9	34.5	23.6	29.1	34.7
120	19	23.8	29.5	35.3	24.3	29.8	35.3
140	23	24.1	30.0	35.9	24.8	30.3	35.9
160	28	24.3	30.3	36.4	25.2	30.8	36.3

Table 3S. Reference values for left and right kidney depth according to postconceptional age in boys and girls

Boys		Left kidney depth (mm)			Right kidney depth (mm)		
Postconceptional age (weeks)	Postnatal age (months)	3rd centile	Mean	97th centile	3rd centile	Mean	97th centile
30	-	16.7	21.5	26.4	17.0	22.1	27.3
40	0	18.3	23.2	28.1	18.7	23.9	29.1
50	2.5	19.5	24.5	29.5	20.0	25.3	30.6
60	5	20.5	25.5	30.6	21.0	26.4	31.8
80	9	22.0	27.2	32.4	22.6	28.2	33.8
100	14	23.2	28.5	33.8	23.8	29.6	35.4
120	19	24.1	29.5	34.9	24.8	30.7	36.7
140	23	24.9	30.4	36.0	25.5	31.7	37.9
160	28	25.6	31.2	36.8	26.2	32.5	38.9
Girls		**Left kidney depth (mm)**			**Right kidney depth (mm)**		
Postconceptional age (weeks)	Postnatal age (months)	3rd centile	Mean	97th centile	3rd centile	Mean	97th centile
30	-	16.1	20.9	25.8	16.8	21.9	27.0
40	0	17.7	22.6	27.5	18.5	23.7	28.9
50	2.5	18.9	23.9	28.9	19.7	25.1	30.4
60	5	19.9	24.9	30.0	20.8	26.2	31.6
80	9	21.4	26.6	31.8	22.4	28.0	33.6
100	14	22.6	27.9	33.2	23.6	29.4	35.1
120	19	23.5	28.9	34.3	24.5	30.5	36.5
140	23	24.3	29.8	35.3	25.3	31.5	37.6
160	28	25.0	30.6	36.2	25.9	32.3	38.6

Table 4S. Reference values for left and right kidney volume according to postconceptional age in boys and girls

Boys		Left kidney volume (cm^3)			Right kidney volume (cm^3)		
Postconceptional age (weeks)	Postnatal age (months)	3rd centile	Mean	97th centile	3rd centile	Mean	97th centile
30	-	5.1	10.3	15.6	6.5	10.7	15.0
40	0	8.7	14.6	20.5	10.8	15.1	19.4
50	2.5	11.6	18.2	24.8	14.2	18.6	23.0
60	5	13.9	21.2	28.5	17.0	21.5	26.0
80	9	17.2	25.9	34.7	21.3	26.0	30.6
100	14	19.2	29.3	39.4	24.3	29.2	34.0
120	19	20.1	31.6	43.1	26.5	31.5	36.6
140	23	20.1	33.0	45.9	27.9	33.2	38.4
160	28	19.3	33.6	47.9	28.9	34.3	39.8
Girls		**Left kidney volume (cm^3)**			**Right kidney volume (cm^3)**		
Postconceptional age (weeks)	Postnatal age (months)	3rd centile	Mean	97th centile	3rd centile	Mean	97th centile
30	-	3.8	9.0	14.2	5.7	9.9	14.2
40	0	7.3	13.2	19.1	10.0	14.3	18.6
50	2.5	10.2	16.8	23.4	13.4	17.8	22.2
60	5	12.5	19.8	27.1	16.2	20.7	25.2
80	9	15.9	24.6	33.3	20.5	25.1	29.8
100	14	17.8	28.0	40.0	23.5	28.4	33.2
120	19	18.7	30.3	41.8	25.7	30.7	35.8
140	23	18.7	31.6	44.5	27.1	32.4	37.6
160	28	17.9	32.2	46.5	28.1	33.5	39.0

Chapter 3.3

Fetal kidney volume and growth and blood flow in fetal life

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Adapted from Kidney Int 2007;72:754-761

Abstract

Background: It has been suggested that an adverse fetal environment leads to permanent smaller kidneys and subsequently to hypertension and renal disease in adult life. The aim of this study was to examine whether maternal characteristics, fetal growth, fetal blood flow redistribution and inadequate placental perfusion in different periods of fetal life affect kidney volume in late fetal life. We also examined whether fetal kidney volume was associated with amniotic fluid quantity.
Methods: In a population-based prospective cohort study from early fetal life, fetal growth characteristics and fetal blood flow parameters were assessed with ultrasound and Doppler examinations in 1,215 women in mid- and late pregnancy. Kidney volume was assessed in late pregnancy.
Results: Maternal height and pre-pregnancy weight were associated with kidney volume. After adjustment for the same characteristics in late pregnancy, fetal growth and blood flow parameters in mid-pregnancy were not associated with kidney volume in late pregnancy. In late pregnancy, all fetal growth characteristics were positively associated with kidney volume. The largest effect on kidney volume was found for abdominal circumference (increase of 1.77 cm^3; 95% CI: 1.46 - 2.08, per increase in standard deviation score). Signs of fetal blood flow redistribution and raised placental resistance were associated with reduced kidney volume in late pregnancy. Kidney volume was positively associated with amniotic fluid quantity.
Conclusions: Maternal anthropometrics, fetal growth characteristics, raised placental resistance and fetal blood flow redistribution affect kidney volume. Further research should be aimed at disentangling the causal mechanisms underlying these associations and the long-term consequences.

Introduction

Epidemiological studies have demonstrated low birth weight and fetal growth restriction to be risk factors contributing to renal disease and hypertension in adult life.[1-3] It has been hypothesized that an adverse fetal environment leads to fetal growth restriction and smaller kidneys with a reduced number of nephrons.[4,5] Since nephrogenesis continues until 36 weeks of gestation and the induction of nephron number ceases thereafter, sub-optimal kidney growth and development in fetal life may have life long consequences.[6,7] A permanently reduced number of nephrons would lead to compensatory higher glomerular pressure, progressive glomerular sclerosis and would subsequently predispose the individual to impaired kidney function and hypertension.[4] This hypothesis is supported by studies in animals and humans. Animal studies have shown that low protein intake and reduced placental perfusion lead to fetal growth restriction and a permanent nephron deficit.[8,9] Human studies demonstrated that low birth weight infants and hypertensive subjects have lower kidney weight with a reduced number of nephrons in adult life.[10-13] Thus an adverse environment in utero may lead to fetal growth restriction and impaired kidney development with a nephron deficit, eventually leading to hypertension.[4,14] Fetal kidney weight cannot be measured in utero. Renal volume measured by ultrasound is a valid substitute.[14,15]

The cause of fetal growth restriction and low birth weight is multifactorial. Nutritional deficiencies, smoking and placental insufficiency are causes that might provoke fetal growth restriction and low birth weight infants. Placental insufficiency is the most common and associated with raised placental blood flow resistance.[16] In response to general fetal malnutrition there is a preferential fetal blood flow to the brain and heart, depriving other organs, including the kidneys, from oxygen and nutrients. The increased blood flow to the brain is caused by vasodilatation in the brain resulting in lower peripheral resistance ('brain sparing effect').[17] This is part of the phenomenon known as fetal redistribution, which may be related to disturbed development of the kidneys.

Amniotic fluid is known to represent fetal well-being.[18] An adverse fetal environment as shown by raised placental resistance often results in decreased amniotic fluid indices as well.[19] The main component of amniotic fluid is fetal urinary production, which may therefore be related to kidney volume and reflect kidney function.

The first aim of this population-based prospective cohort study was to evaluate the associations of maternal characteristics and fetal growth with kidney volume during pregnancy. The second aim was to examine the associations of placental resistance indices and fetal blood flow redistribution, as a measure of adverse fetal environment, with kidney volume. Finally, we assessed the relation of fetal kidney volume with amniotic fluid as a measure of fetal urine production. If associations of maternal and fetal growth characteristics with kidney volume exist, further studies designed to identify the causal genetic and environmental mechanisms underlying these associations would be needed.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. This study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail.[20,21] In total, the cohort includes 9,778 mothers and their children living in Rotterdam, the Netherlands. A vast majority (69%) of all mothers were enrolled in the first trimester of pregnancy.[21] Gestational age was determined by ultrasound during the first visit in early pregnancy. Assessments in pregnancy included physical examinations, fetal ultrasounds, biological samples and questionnaires and were planned in early (gestational age <18 weeks), mid- (gestational age 18-25 weeks) and late pregnancy (gestational age ≥25 weeks) to collect information about fetal growth and its main determinants. The children were born between April 2002 and January 2006 and form a prenatally recruited birth-cohort that is currently followed until young adulthood. Of all eligible children born in the study area, 61% participated at birth in the study.[21] Additional more detailed assessments of fetal growth and development were conducted in a subgroup of 1,232 Dutch children and their parents, referred to as the Generation R Focus cohort. For the present study, kidney size was assessed at the fetal ultrasound examination in late pregnancy in this subgroup. This subgroup is ethnic homogeneous to exclude possible confounding or effect modification by ethnicity. Of all approached women, 80% were enrolled in this subgroup study in the third trimester of pregnancy (gestational age of 30 weeks). 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 Generation R Focus Study at a gestational age of 30 weeks. Twin pregnancies (n = 15) and pregnancies leading to perinatal death (n = 2) were excluded from the analysis. No renal or uterovesical anomalies other than mild pyelectasis over 10 mm (n = 3) were present in our study population. Renal ultrasounds were only partially performed in 6 subjects due to unfavorable fetal position or maternal adipositas. The present analysis was performed in a total of 1,215 subjects.

Ultrasound measurements

Fetal biometry

Ultrasound exams were carried out in a research setting at a regional health facility in the centre

of Rotterdam in early, mid- and late pregnancy. These fetal ultrasound procedures were used for both establishing gestational age and assessing fetal growth characteristics. Pregnancy dating curves were constructed on subjects in the study with complete data on gestational age measured by ultrasound and last menstrual period. Crown-rump length was used for pregnancy dating until a gestational age of 12 weeks and 5 days (crown-rump length smaller than 65 mm), and biparietal diameter was used for pregnancy dating thereafter (gestational age from 12 weeks and 5 days onwards, biparietal diameter larger than 23 mm). Fetal biometry including head circumference, abdominal circumference, and femur length was measured during each ultrasound examination using a transabdominal probe. Standard ultrasound planes for fetal measurements were used as described previously.[22-24] Briefly head circumference was measured in a transverse section of the head with a central mid-line echo, interrupted in the anterior third by the cavity of the septum pellucidum with the anterior and posterior horns of the lateral ventricles in view. An ellipse was drawn around the outline of the skull. Abdominal circumference was measured in a symmetrical, transverse, round section through the abdomen, with visualization of the vertebrae on a lateral position in alignment with the ribs. The measurement was taken in a plane with the stomach and the bifurcation of the umbilical and hepatic veins. Femur length was measured with the full length of the bone in view. Gestational age-adjusted standard deviation scores were constructed for these fetal growth measurements. These were based on reference growth curves from the whole study population. Estimated fetal weight was calculated using the formula by Hadlock using head circumference, abdominal circumference and femur length.[25]

Placental and fetal blood flow profiles

Placental resistance as a proxy of placental function was assessed using recorded flow velocity waveforms from the umbilical and uterine arteries, in mid and late pregnancy. Raised umbilical artery pulsatility index (PI) and uterine artery resistance index (RI) indicate increased placental resistance.[16] Umbilical artery PI was measured in a free-floating loop of the umbilical cord. Uterine artery RI was measured in the uterine arteries near the crossover with the external iliac artery. The redistribution of blood flow in favor of the fetal brain was quantified by the middle cerebral artery PI and the cerebro RI / umbilical RI ratio, in late pregnancy. A reduction in middle cerebral artery PI and a decreasing cerebro-umbilical ratio are valid indicators of 'brain sparing effect' due to fetal redistribution.[17, 26] The middle cerebral artery Doppler was performed with color Doppler visualization of the circle of Willis in the fetal brain and the flow velocity wave forms were obtained in the proximal part of the middle cerebral artery.

Kidney measurements

Assessment of fetal kidney size and volume was performed at the scan in late pregnancy. The left and right kidney was measured. 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

and transverse kidney diameter were measured perpendicular to each other, outer to outer, in an axial plane. 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.[27] Kidney volume was calculated using the approximation of an ellipsoid: Volume = length * width * thickness * 0.523.[15] Left and right kidney volume were added for the combined kidney volume (cm^3).[28] Another frequently used measure of the kidney in fetal life is the relative kidney volume. This is the ratio of kidney volume / estimated fetal weight.[15, 29]

Amniotic fluid

Amniotic fluid was assessed using single deepest pocket measurements, the preferable method to give an indication about the quantity of amniotic fluid in clinical practice.[30] All the ultrasound exams were performed using an ATL-Philips® Model HDI 5000 (Seattle, Washington, USA) equipped with a 5.0 MHz, high frequency curved array transducer.

Intra- and interobserver reproducibility

Three well-trained, experienced sonographers performed all measurements. Quality checks were frequently carried out and feedback was provided to minimize interoperator differences. To assess intra- and interobserver reproducibility of the fetal ultrasound measurements, the intraclass correlation coefficient (ICC) and coefficient of variation (CV) between and among observers were calculated in 21 subjects for various ultrasound measurements and Doppler parameters.[31] For fetal ultrasound measurements the ICC was higher than 0.98 and the corresponding 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 the measurements, indicating good reproducibility.[31] Furthermore, for Doppler parameters the results show high ICC (>0.80) with corresponding low CV (<10%) values as well, indicating adequate reproducibility for all Doppler measurements.

Statistical analysis

To establish normal ranges for renal growth parameters with gestation we created scatterplots of the individual measurements and applied the best fitting formula.

The associations of maternal characteristics with combined kidney volume were assessed using multiple linear regression models. The models were adjusted for fetal abdominal circumference in late pregnancy, gestational age and fetal gender. The associations of fetal growth characteristics (head circumference, abdominal circumference and femur length), placental resistance indices, and fetal redistribution parameters in mid- and late pregnancy with combined kidney volume measured in late pregnancy were also assessed using multiple linear regression models. Gestational age-adjusted standard deviation scores (SDS) for the fetal growth measure-

ments were used to compare effect sizes. All models were adjusted for fetal gender. The Doppler measurements were additionally adjusted for gestational age and abdominal circumference. Since fetal size in mid-pregnancy is strongly related to fetal size in late pregnancy, we adjusted the mid-pregnancy models (Model A) for the same growth and Doppler characteristic in late pregnancy (Model B) to estimate the effect size on kidney volume that is explained by fetal growth in mid-pregnancy only.

Furthermore we examined the effect of gestational age-adjusted abdominal circumference in late pregnancy on relative kidney volume (kidney volume / estimated fetal weight). Finally the effect of kidney volume on amniotic fluid was assessed, adjusted for gestational age, gender and abdominal circumference, using linear regression models. All statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Characteristics of the subjects who participated in the study and their mothers for boys and girls in mid- and late pregnancy are presented in Table 1. The percentage of boys was 51%. Median maternal age was 31.9 (95% range: 21.5 - 39.0) years. The median gestational age for the mid-pregnancy visit was 20.5 (95% range: 18.7 - 22.8) weeks and for the late pregnancy visit 30.4 (95% range: 28.4 - 32.6) weeks. Head circumference and abdominal circumference were larger and umbilical artery flow pulsatility index (PI) was lower in boys than in girls at both measurements. Estimated fetal weight was higher for boys in late pregnancy only. No gender differences were observed for femur length and uterine artery flow at both visits. At birth, weight was higher in boys than in girls.

Table 1. Subject characteristics (n = 1,215)

	Boys (n = 629)	**Girls** (n = 586)
Maternal characteristics		
Age (years)	31.8 (21.1-39.2)	32.0 (22.7-39.0)
Height (cm)	170.8 (5.9)	170.9 (6.0)
Pre-pregnancy weight (kg)	68.2 (13.0)	69.2 (12.3)
Pre-pregnancy BMI (kg/m^2)	23.3 (4.2)	23.7 (4.0)
Weight gain until late pregnancy (kg)	8.5 (3.7)	8.4 (3.6)
Systolic blood pressure in late pregnancy (mmHg)	120.4 (11.3)	121 (11.0)
Diastolic blood pressure in late pregnancy (mmHg)	69.8 (9.3)	70.0 (9.5)

Table 1. continued

	Boys (n = 629)	Girls (n = 586)
Hypertension (%)	6.9	8.7
Pre-existent or pregnancy induced diabetes (%)	1.4	1.3
Pre-eclampsia (%)	1.2	2.1
Smoking during pregnancy (%)	15.0	12.6
Mid-pregnancy characteristics		
Gestational age (weeks)	20.6 (18.8-22.8)	20.5 (18.7-22.8)
Head circumference (cm)	18.0 (1.4)	17.7 (1.3)*
Abdominal circumference (cm)	15.8 (1.3)	15.6 (1.3)*
Femur length (cm)	3.3 (0.3)	3.3 (0.3)
Estimated fetal weight (grams)	377 (84)	370 (80)
Umbilical artery, pulsatility index (PI)	1.18 (0.19)	1.21 (0.17)*
Uterine artery, resistance index (RI)	0.54 (0.09)	0.54 (0.09)
Late pregnancy characteristics		
Gestational age (weeks)	30.5 (28.6-32.9)	30.3 (28.3-32.5)
Head circumference (cm)	28.8 (1.2)	28.3 (1.2)*
Abdominal circumference (cm)	26.7 (1.7)	26.5 (1.7)*
Femur length (cm)	5.70 (0.3)	5.73 (0.3)
Estimated fetal weight (kg)	1631 (259)	1599 (259)†
Umbilical artery, pulsatility index (PI)	0.96 (0.16)	0.99 (0.17)*
Uterine artery, resistance index (RI)	0.49 (0.08)	0.49 (0.08)
Postnatal characteristics		
Gestational age (weeks)	40.3 (35.9-42.4)	40.1 (35.6-42.4)
Birth weight (grams)	3549 (547)	3460 (557)*

*Values are means (standard deviation) or medians (95% range). Differences between boys and girls were compared using independent sample t-tests. *p-value <0.05, †p-value <0.01*

Table 2 presents kidney characteristics in late pregnancy for boys and girls. The size of all kidney measurements was larger in boys than in girls. Left and right kidney did not differ in length, but the right kidney had a larger width 0.67 mm difference (95% CI: 0.53, 0.82), depth 0.80 mm difference (95% CI: 0.68, 0.93) and volume 0.72 cm^3 difference (95% CI: 0.60, 0.83) (not shown in Table 2).

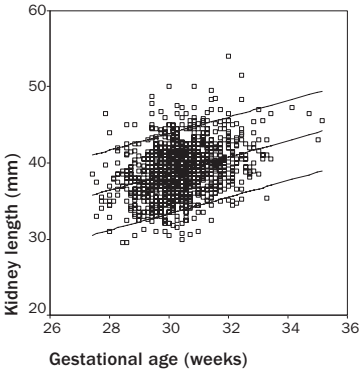
Table 2. Fetal kidney characteristics in late pregnancy

	Boys (n = 629)	**Girls** (n = 586)
Left kidney structures		
Length (mm)	39.5 (3.8)	38.4 (3.5)*
Width (mm)	22.7 (2.9)	22.1 (2.5)*
Depth (mm)	21.6 (2.8)	21.1 (2.5)*
Volume (cm^3)	10.3 (3.0)	9.5 (2.5)*
Right kidney structures		
Length (mm)	39.6 (3.8)	38.5 (3.5)*
Width (mm)	23.2 (3.0)	22.9 (2.7)†
Depth (mm)	22.4 (2.9)	22.0 (2.7)†
Volume (cm^3)	11.0 (3.3)	10.3 (2.8)*
Combined kidney volume (cm^3)	21.3 (5.9)	19.9 (5.0)*

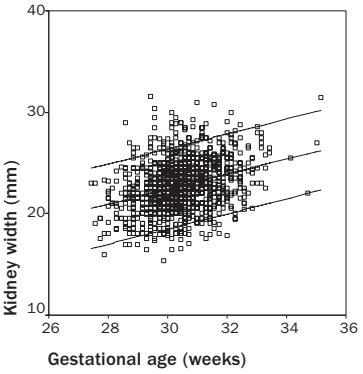
Values are means (standard deviation). Differences between boys and girls were compared using independent sample t-tests.
**p-value <0.05, †p-value <0.01*

Figure 1 shows individual measurements for kidney structures in late pregnancy with the 5th and 95th percentiles. Formulas for normal ranges for mean fetal kidney size and volume between 28-34 weeks of gestational age are listed beneath the figures.

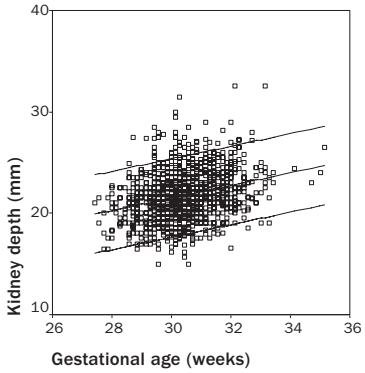
Figure 1. Individual measurements of mean kidney length, width, depth and volume with fitted median, 5th and 95th centiles with gestational age and formulas for normal values

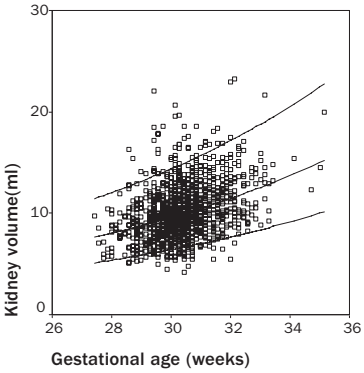


Mean length = 6 + (1.086*GA)
SD = 3.2



Mean width = 0.19 + (0.74*GA)
SD = 2.39





Mean depth = 2.91 + (0.62*GA)
SD = 2.35

Mean volume = exp(-0.406 + 0.089*GA)
SD = exp 0.245

GA, gestational age in exact weeks between 28 and 34 weeks; SD, standard deviation.

Table 3 gives the associations of maternal characteristics with combined (left plus right) kidney volume. Maternal pre-pregnancy weight and height were positively associated with kidney volume. Other maternal characteristics like obesity, blood pressure, pre-eclampsia, diabetes or smoking were not associated with kidney volume.

Table 3. Associations of maternal characteristics with combined fetal kidney volume in late pregnancy

Maternal characteristics	**Difference in total kidney volume (cm^3) (95% confidence interval)**
Age (years)	-0.01 (-0.07, 0.06)
Height (cm)	0.07 (0.02, 0.11)
Pre-pregnancy weight (kg)	0.03 (0.01, 0.05)
Pre-pregnancy BMI (kg/m^2)	0.04 (-0.03, 0.11)
Weight gain during pregnancy (kg)	0.05 (-0.04, 0.13)
Systolic blood pressure in late pregnancy (mmHg)	-0.01 (-0.03, 0.02)
Diastolic blood pressure in late pregnancy (mmHg)	-0.01 (-0.04, 0.02)
Hypertension (yes vs. no)	-0.03 (-1.28, 1.33)
Pre-existent or pregnancy induced diabetes (yes vs. no)	-0.60 (-3.49, 2.30)
Pre-eclampsia (yes vs. no)	2.14 (-0.25, 4.53)
Smoking during pregnancy (yes vs. no)	-0.06 (-0.88, 0.77)

Values are regression coefficients (95% confidence interval) and reflect the difference in kidney volume per unit increase in maternal characteristics or lifestyle measure.
Models adjusted for fetal abdominal circumference in late pregnancy, gestational age and fetal gender. BMI, body mass index.

Table 4 presents the associations of fetal growth characteristics and placental resistance indices in mid-pregnancy with combined kidney volume measured in late pregnancy. In Model A, adjusted for gestational age and fetal gender only, all fetal growth characteristics were positively associated with combined kidney volume and umbilical artery PI negatively associated (Model A). After additional adjustment for the same fetal growth characteristic or blood flow parameter in late pregnancy, measured at the same time as kidney volume, associations were no longer significant (Model B). These results suggest that the associations of mid-pregnancy growth characteristics and placental resistance indices with late pregnancy kidney volume are largely explained by the same characteristics in late pregnancy.

Table 4. Associations of fetal growth characteristics and placental resistance indices in mid-pregnancy with combined fetal kidney volume in late pregnancy

Measurements in mid-pregnancy	**Difference in combined kidney volume (cm^3) (95% confidence interval)**	
	Model A	**Model B**
Growth characteristic		
Head circumference (SDS)	0.49 (0.16, 0.82)	0.30 (-0.03, 0.64)
Abdominal circumference (SDS)	0.80 (0.46, 1.14)	0.20 (-0.13, 0.53)
Femur length (SDS)	0.48 (0.14, 0.82)	0.34 (-0.02, 0.69)
Ratio abdominal circumference/ head circumference (SDS)	0.59 (0.18, 1.00)	0.18 (-0.22, 0.58)
Estimated fetal weight (SDS)	0.84 (0.50, 1.19)	0.04 (-0.14, 0.56)
Placental resistance indices		
Umbilical artery, pulsatility index (PI)	-2.23 (-4.09, -0.37)	-1.38 (-3.22, 0.53)
Uterine artery, resistance index (RI)	-2.24 (-6.52, 2.03)	-1.30 (-5.91, 3.31)

Values are regression coefficients (95% confidence interval) and reflect the difference in kidney volume per unit increase in fetal growth and placental perfusion blood flow characteristic.
SDS: gestational age-adjusted standard deviation score.
Model A: adjusted for gestational age and fetal gender.
Model B: additionally adjusted for the same parameter and gestational age in late pregnancy.

Table 5 shows that in late pregnancy, all growth characteristics were positively associated with combined kidney volume. The largest effects on combined kidney volume were found for estimated fetal weight and abdominal circumference (combined kidney volume increased 1.77 (95% CI: 1.46, 2.08) cm^3 and 1.76 (95% CI: 1.47, 2.05) cm^3 for each standard deviation score increase in estimated fetal weight and abdominal circumference, respectively). Placental resistance indices were inversely associated with combined kidney volume, indicating that signs of increased placental resistance reduced kidney volume. Signs of fetal redistribution as quantified by the cerebro-umbilical ratio were associated with reduced kidney volume. No associations were found for middle cerebral artery PI.

Table 5. Associations of fetal growth and blood flow characteristics with combined fetal kidney volume in late pregnancy

Measurements in late pregnancy	**Difference in combined kidney volume (cm^3) (95% confidence interval)**
Growth characteristic	
Head circumference (SDS)	0.91 (0.57, 1.23)
Abdominal circumference (SDS)	1.76 (1.47, 2.05)
Femur length (SDS)	1.03 (0.71, 1.35)
Ratio head circumference/ abdominal circumference (SDS)	1.71 (1.34, 2.09)
Estimated fetal weight (SDS)	1.77 (1.46, 2.08)
Placental resistance indices	
Umbilical artery, pulsatility index (PI)	-2.74 (-4.55, -0.92)
Uterine artery, resistance index (RI)	-6.40 (-10.4, -2.43)
Redistribution parameters	
Middle cerebral artery (PI)	0.46 (-0.41, 1.33)
Cerebro-umbilical (C/U) ratio	0.87 (0.22, 1.51)

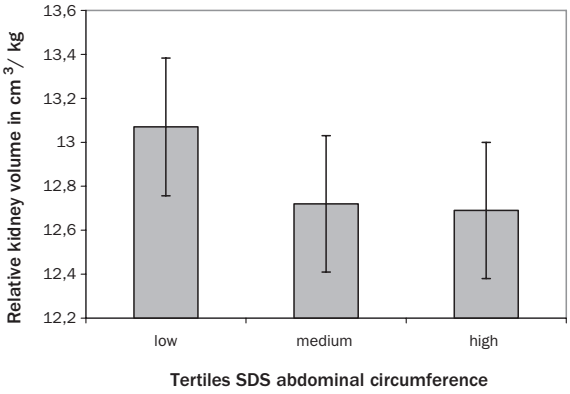
Values are regression coefficients (95% confidence interval) and reflect the difference in kidney volume per unit increase in fetal growth and fetal blood flow characteristic.

SDS, standard deviation score; PI, pulsatility index.

Models adjusted for gestational age and fetal gender. Blood flow parameters additionally adjusted for abdominal circumference.

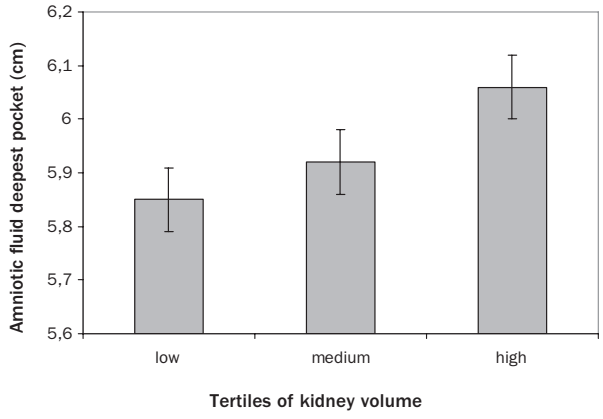
Figure 2 shows that in late pregnancy, there is a tendency to larger relative kidney volume in subjects in the smallest tertile of sds abdominal circumference. This suggests that small for gestational age fetuses have a larger kidney volume per kg fetal weight. Figure 3 shows that in late pregnancy, kidney volume is positively associated with amniotic fluid deepest pocket.

Figure 2. The association between fetal abdominal circumference and fetal weight adjusted combined fetal kidney volume in late pregnancy



SDS, gestational age-adjusted standard deviation score.
P = 0.07 (P-value for trend using linear regression models).

Figure 3. Relation of fetal kidney volume with amniotic fluid deepest pocket



Model adjusted for gestational age, abdominal circumference and fetal gender.
P <0.05 (P-value for trend using linear regression models).

Discussion

This population-based prospective cohort study from early fetal life showed that maternal pre-pregnancy anthropometrics, fetal growth characteristics and indices of placental resistance as well as fetal blood flow redistribution parameters were associated with kidney volume. Larger kidneys yielded a deeper amniotic fluid pocket.

The main strength of our study is the prospective design from fetal life with serial growth measurements within a large population-based cohort. Of all mothers who were approached for the detailed subgroup, 80% participated in the focus study. Non-participation was mainly due to lack of time. No differences in offspring birth weight were found between mothers participating and not participating in the present study. Thus, we do not assume major health related differences between these groups. To our knowledge, this is the largest population-based cohort in which kidney size in late pregnancy was established. The population-based setting enabled us to assess kidney size and volume over the whole range of normal fetal size rather than in fetuses with growth restriction or other complications only.

Both environmental and genetic factors are important determinants of fetal growth.[32-34] We examined the association of maternal characteristics with fetal kidney volume in late pregnancy. Maternal weight and height were positively associated with kidney volume. This association may be explained by both environmental (maternal nutritional status) and common genetic factors that are important in the determination of kidney volume during pregnancy. Maternal smoking, obesity, blood pressure and diabetes did not considerably influence fetal kidney size in this study. Even though these factors have a known influence on overall fetal size.[32, 33]

We found positive associations of fetal growth characteristics in mid-pregnancy with kidney volume in late pregnancy. But after adjustment for the same growth parameter in late pregnancy these effects are no longer present, suggesting that the main influence of fetal growth on kidney volume in late pregnancy exerts after mid-pregnancy.

In late pregnancy, all fetal growth characteristics were positively associated with kidney volume. Abdominal circumference and the characteristics that included abdominal circumference were most strongly associated with kidney volume. The positive association for the ratio of abdominal circumference / head circumference suggests that asymmetrical fetal growth restriction reduced kidney volume more than symmetrical growth restriction although this effect might be partially explained by abdominal circumference only.

Our results showed the largest effect of fetal growth on kidney volume in late pregnancy, this is in line with previous studies that found the period of maximum kidney growth to occur between 26-34 weeks of gestation.[14] Growth restriction in this period most likely affects kidney size and volume considerably.

Inadequate placental perfusion leads to an adverse fetal environment by decreased supply of nutrients and oxygen and is one of the most important causes of fetal growth retardation in

Western countries.[16] Increased placental vascular resistance and signs of blood flow redistribution with decreased cerebral resistance is known to be associated with reduced fetal growth. In our study measures of placental vascular resistance were associated with estimated fetal weight (decrease in estimated fetal weight per unit increase in umbilical artery PI: 151 (95% CI: 90, 212) grams and per unit increase in uterine artery RI: 273 (95% CI: 141, 405) grams). Also, signs of fetal blood flow redistribution were associated with estimated fetal weight (estimated fetal weight decrease per unit decrease in middle cerebral artery PI: 16 (95% CI: -5, 57) grams and per unit decrease in cerebro-umbilical artery ratio: 45 (95% CI: 24, 67) grams). We showed that in late pregnancy, adverse blood flow resistance patterns of the umbilical and uterine artery were associated with reduced kidney volume, independent of fetal abdominal circumference at the time of the kidney measurement. This implies that kidney volume did not solely depend on abdominal circumference and overall fetal size but to some extend directly on placental vascular resistance or blood flow redistribution. So signs of increased placental resistance and fetal blood flow redistribution to protect the developing central nervous system impaired fetal growth and are sufficiently deleterious to reduce fetal kidney volume as well.

A hypothesis for a decrease in renal size in growth restricted fetuses is alteration in renal perfusion caused by a preferential blood flow to the brain.[35] In our study we did not find any relation with middle cerebral artery PI and kidney volume. Another parameter is the cerebro-umbilical ratio, which did show a relation with kidney size. It is not unlikely that redistribution with decrease in middle cerebral artery PI is a later sign in fetal growth restriction what is not yet eminent in this population-based study. A direct measure of renal blood flow would be renal artery PI, which we did not measure. A previous study showed that renal artery blood flow was not altered in growth restricted fetuses.[5] However, abnormal renal artery Doppler flow velocity waveforms were demonstrated in hypoxic growth restricted fetuses in another study.[35] Altered renal artery flow velocity seems to be a late effect that is not present in growth restricted fetuses that do not yet show signs of redistribution. We think that the reduced kidney volume in our study is not solely explained by redistribution because it is already present in smaller fetuses when signs of redistribution are absent.

This study showed that fetuses in the lowest tertile of gestational age-adjusted abdominal circumference had a tendency towards larger relative kidney volume, suggesting an organ or kidney sparing effect in small for gestational age fetuses. Thus, smaller fetal body size is associated with smaller kidneys, but these kidneys are relatively large for that body size. Previous studies suggested that the ratio of kidney volume with estimated fetal weight or abdominal circumference is constant in fetuses with different size and age.[15, 29] This inconsistency with our results may be due to different and smaller study populations. Therefore, further studies are needed focused on the effects of various fetal growth characteristics on relative kidney volume.

The main component of amniotic fluid is fetal urinary production. In our study the amniotic fluid deepest pool was decreased in fetuses with smaller kidneys, after adjustment for abdominal

circumference and gestational age. This indicates that the reduction of amniotic fluid is not solely attributable to growth restriction but to kidney volume as well. It is possible that the association we found between kidney volume and amniotic fluid is a reflection of the number of nephrons and kidney function.

Our study underlines the importance of fetal growth and growth characteristics for determination of kidney size. Since we know that the number of nephrons is largely determined in prenatal life, sub-optimal kidney growth and development in fetal life may have life long consequences.[10-13]

In conclusion, our findings suggest that reduced fetal growth, signs of raised placental resistance and fetal blood flow redistribution result in a decreased kidney volume in late fetal life. Further research to disentangle the causal mechanisms underlying the demonstrated associations is needed. Impaired fetal development results in smaller kidneys and may result in increased risk of hypertension and renal disease in later life. Follow-up studies in our children are currently performed to examine whether and to what extend changes in fetal kidney size persist during childhood and whether they are related to renal function and blood pressure development in postnatal life.

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Chapter 3.4

Maternal smoking during pregnancy and fetal kidney size

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Abstract

Background: An adverse fetal environment may lead to smaller kidneys, which predispose the individual to subsequent development of kidney disease and hypertension in adult life. We examined whether smoking during pregnancy, as adverse fetal exposure, directly influences third trimester fetal kidney development.

Methods: In a prospective cohort study among 1,031 children followed from early fetal life onwards, we assessed maternal smoking during pregnancy (non-smoking, first trimester only, continued) using questionnaires. Fetal kidney characteristics and amniotic fluid deepest pocket, as measure of fetal urine production, were measured by ultrasound at 30 weeks of gestation. Analyses were adjusted for various potential confounders.

Results: Of all mothers, 9.4% smoked in first trimester only, and 15.9% continued smoking during pregnancy. Compared to non-smoking, first trimester only smoking was not associated with any fetal kidney characteristic. We observed a curved-shaped association between the number of cigarettes smoked during pregnancy with fetal kidney size. Smoking less than 5 cigarettes per day was associated with larger combined kidney volume (difference 2.04 cm^3 (95% confidence interval: 0.73, 3.36)), while smoking more than 10 cigarettes per day was associated with smaller combined kidney volume (difference -1.99 cm^3 (95% confidence interval: -3.86, -0.11)). We did not find any associations of maternal smoking and amniotic fluid deepest pocket.

Conclusions: This study showed for the first time that smoking during pregnancy affects kidney development in fetal life with a curved shaped relationship. Further studies are needed to assess the underlying mechanisms and whether these developmental adaptations have postnatal consequences for kidney function and blood pressure.

Introduction

The developmental plasticity hypothesis suggests that various adverse intrauterine 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 hyperfiltration and results in glomerular sclerosis.[2-4] These adaptations may predispose the individual to kidney damage and subsequent development of higher blood pressure, impaired kidney function and end stage kidney disease in adulthood. This hypothesis is supported by many studies showing associations of low birth weight with cardiovascular disease and end stage kidney disease.[5-7] Thus far, the specific adverse exposures and mechanisms underlying these associations are not known.

Maternal smoking is the most important modifiable adverse fetal exposure in Western countries and leads to a decrease of 150 to 200 grams in offspring birth weight.[8] Maternal smoking during pregnancy may also have both direct and indirect adverse effects on fetal kidney development. Low birth weight, of which maternal smoking is an important determinant, is associated with impaired kidney growth, raised blood pressure, and impaired kidney function.[7, 9-14] Several studies suggested that maternal smoking during pregnancy is also associated with higher blood pressure in the offspring, independent of birth weight.[15, 16] This association might be explained by an adverse kidney development with a smaller nephron number.[17, 18]

For the present study, we hypothesized that maternal smoking during pregnancy, as specific adverse fetal exposure, affects fetal kidney size. We evaluated in a population-based prospective cohort study among 1,031 mothers and children followed from early fetal life onwards, the associations of maternal smoking during pregnancy, both first trimester only and continued smoking, with third trimester fetal kidney characteristics and amniotic fluid deepest pocket as measure of urine production.

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.[19, 20] Assessments during pregnancy included physical and fetal ultrasound examinations, biological samples and questionnaires, and were performed in each trimester of pregnancy to collect information about fetal growth and its main determinants. Additional, more detailed assessments were conducted in a subgroup of Dutch children and their parents. Of all approached women, 80% were enrolled in this subgroup in the third trimester of pregnancy (gestational age of 30 weeks). For the present study, kidney biometrics were assessed in third trimester of pregnancy. 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. The present analysis was restricted to singleton live births (n = 1,215). Twin pregnancies (n = 15) and pregnancies leading to perinatal death (n = 2) were excluded from the analysis. No renal or uterovesical anomalies other than mild pyelectasis over 10 mm (n = 3) were present in our study. Information about smoking during pregnancy was not available in 110 subjects. In the remaining 1,105 mothers, kidney characteristics could not completely be measured in 74 subjects due to fetal position or maternal adiposity, leading to a total of 1,031 subjects available for the present analyses. Since two subjects did not report the number of cigarettes smoked, analyses focused on the numbers of cigarettes smoked were based on a total of 1,029 subjects.

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. Active maternal smoking at enrolment was assessed in the first questionnaire by asking whether mother smoked in pregnancy (no, first trimester only, continued). 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 previously used categories: non-smoking; less than five cigarettes per day; five to nine cigarettes per day; and ten or more cigarettes per day. To examine the dose-response relationship of maternal smoking during pregnancy more extensively we also used the following categories: non-smoking, less than one cigarette per day, one to two cigarettes per day, three to four cigarettes per day, five to nine cigarettes per day; and ten or more cigarettes per day.

Third trimester fetal ultrasound measurements

Fetal biometry

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 biometrics including head circumference (HC), abdominal circumference (AC), and femur length (FL) were measured using standardized ultrasound procedures. Estimated fetal weight (EFW) was calculated using the formula:

EFW (grams) = 10 ** (1.326 – 0.00326 * AC * FL + 0.0107 * HC + 0.0438 * AC + 0.158 * FL). Gestational age-adjusted SD scores were constructed for these fetal growth characteristics.

Kidney measurements

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, outer tot outer, 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.[21] Kidney volume was calculated using the approximation of an ellipsoid: volume (cm^3) = 0.523 * length (cm) * width (cm) * depth (cm). Left and right kidney volume were added for the combined kidney volume (cm^3).

Amniotic fluid

Amniotic fluid was assessed using single deepest pocket measurements, the preferable indication about the quantity of amniotic fluid in clinical practice.[22]

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. The interclass correlation coefficients were higher than 0.98 and the corresponding coefficient of variations lower than 6%. Bland and Altman plots to test agreement of measurements, demonstrated 95% limits of agreement in proportions to be within 10% difference from the mean of the measurements, indicating good reproducibility.

Covariates

Exact gestational age was established using fetal biometry measured in first trimester of pregnancy.[23] Estimated fetal weight was calculated from fetal biometry measured in third trimester ultrasound. Maternal height was measured at first visit. Information on maternal pre-pregnancy weight, educational level and alcohol use during pregnancy was assessed using self reported questionnaires. Date of birth, birth weight and sex were obtained from midwife and hospital registries.

Statistical analysis

Differences in maternal and fetal characteristics between the categories 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 fetal kidney structures and combined kidney volume were assessed using multiple linear regression models. The models were adjusted for estimated fetal weight in third trimester of pregnancy (30 weeks), gestational age at visit, fetal sex, alcohol use during pregnancy, maternal educational level, maternal height and maternal weight before pregnancy. Subsequently, using similar models we examined the associations of the number of cigarettes smoked with fetal kidney characteristics and combined kidney volume. Tests for trends were performed using multiple linear and non-linear multiple regression analyses. All measures of association are presented with their 95% confidence intervals (95% CI). 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 52.2%. The overall median maternal age was 31.8 years (95% range: 21.7 - 38.9). The median gestational age for the third trimester visit was 30.2 weeks (95% range: 28.5 - 32.5). The study group comprised 30 children who were born with a small size for gestational age (birth weight less than -2 SDS for gestational age), 36 children with a birth weight less than 2,500 grams and 41 children who were born preterm. Table 2 shows all fetal kidney characteristics and amniotic fluid deepest pocket measured in third trimester of pregnancy.

Table 1. Subject characteristics (n = 1,031)

	Non-smoking (n = 769)	**First trimester only** (n = 98)	**Continued** (n = 164)
Maternal characteristics			
Age (years)	31.9 (23.2-39.0)	31.3 (20.4-38.8)	31.4 (19.3-41.2)*
Height (cm)	171.3 (6.3)	170.6 (6.8)	169.7 (6.2)†
Pre pregnancy weight (kg)	69.3 (12.7)	66.4 (10.4)†	67.8 (13.3)
Parity ≥1 (%)	39.2	27.8	44.5
Highest educational level >secondary school (%)	68.8	58.8†	36.2†
Alcohol use during pregnancy (%)	52.3	55.1	51.9

Table 1. continued

	Non-smoking (n = 769)	First trimester only (n = 98)	Continued (n = 164)
Third trimester pregnancy characteristics			
Gestational age (weeks)	30.4 (28.5-32.6)	30.2 (28.4-32.2)	30.2 (28.3-32.0)
Estimated fetal weight (grams)	1639 (265)	1634 (272)	1564 (272)†
Birth characteristics			
Gender (% boys)	52.1	43.3	57.9
Gestational age (weeks)	40.3 (36.0-42.4)	40.1 (33.2-42.3)	40.1 (34.8-42.4)
Gestational age <37 weeks (%)	3.6	5.2	4.9
Birth weight (grams)	3561 (524)	3542 (611)	3355 (556)†
Birth weight <2,500 grams (%)	2.8	4.2	6.1*
Small for gestational age (%)	2.3	2.1	6.7

*Values are means (standard deviation) or medians (95% range) for continuous variables and percentages for categorical variables. Small for gestational age was defined as children with birth weight below –2 standard deviation for gestational age. Differences in maternal and fetal characteristics (compared with the non-smoking category) were compared using ANOVA for continuous variables and X^2 tests for categorical variables. *p-value <0.05, †p-value <0.01*

Table 2. Third trimester fetal kidney characteristics and amniotic fluid deepest pocket (n=1,031)

	Mean	SD	95% range
Left kidney structures			
Length (mm)	39.0	3.77	32.0-47.0
Width (mm)	21.4	2.67	16.9-26.9
Depth (mm)	22.4	2.73	17.1-28.8
Volume (cm^3)	9.9	2.81	5.4-16.0
Right kidney structures			
Length (mm)	39.0	3.72	32.0-46.0
Width (mm)	22.2	2.81	17.0-28.0
Depth (mm)	23.1	2.89	18.0-29.0
Volume (cm^3)	10.7	3.11	5.8-17.6
Combined kidney volume (cm^3)	20.6	5.58	11.5-33.5
Amniotic fluid deepest pocket (cm)			
(n = 1,009)	5.94	1.28	3.7-8.7

SD, standard deviation

Table 3 gives the associations of maternal smoking with third trimester fetal kidney characteristics and combined kidney volume. Compared to non-smoking, no associations of smoking, either first trimester only or continued during pregnancy, with fetal kidney characteristics were observed. However, we observed an association between the number of cigarettes smoked with fetal kidney characteristics in third trimester of pregnancy (Table 4). Smoking less than five cigarettes per day was positively associated with most kidney structures and was associated with larger combined kidney volume (difference 2.04 cm^3 (95% CI: 0.73, 3.36)) whereas smoking ten or more cigarettes per day showed tendencies towards smaller fetal kidney characteristics and a smaller combined kidney volume (difference -1.99 cm^3 (95% CI: -3.86, -0.11)). Figure 1 shows the associations of number of cigarettes smoked with fetal combined kidney volume using a more extensive classification of the number of cigarettes smoked per day. We found a curved shaped dose-response relationship of the number of cigarettes smoked and combined kidney volume (p-value for trend = 0.001). Smoking less than five cigarettes leads to a larger combined kidney volume, whereas smoking more cigarettes leads to smaller kidneys.

Table 3. Maternal smoking during pregnancy and third trimester fetal kidney structures (n = 1,031)

	Left kidney structures				Right kidney structures			
Smoking during pregnancy	Length (mm)	Width (mm)	Depth (mm)	Volume (cm^3)	Length (mm)	Width (mm)	Depth (mm)	Volume (cm^3)
Non-smoking (n = 769)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
First-trimester only (n = 98)	0.41 (-0.36, 1.18)	0.00 (-0.56, 0.56)	0.34 (-0.22, 0.87)	0.28 (-0.27, 0.82)	0.02 (-0.75, 0.79)	0.09 (-0.49, 0.68)	-0.04 (-0.63, 0.55)	-0.03 (-0.64, 0.59)
Continued (n = 164)	0.22 (-0.42, 0.85)	0.31 (-0.15, 0.77)	0.15 (-0.30, 0.61)	0.30 (-0.15, 0.75)	0.20 (-0.44, 0.83)	0.04 (-0.44, 0.53)	0.02 (-0.47, 0.52)	0.12 (-0.39, 0.63)
P_{trend}	0.454	0.674	0.421	0.662	0.904	0.765	0.903	0.875

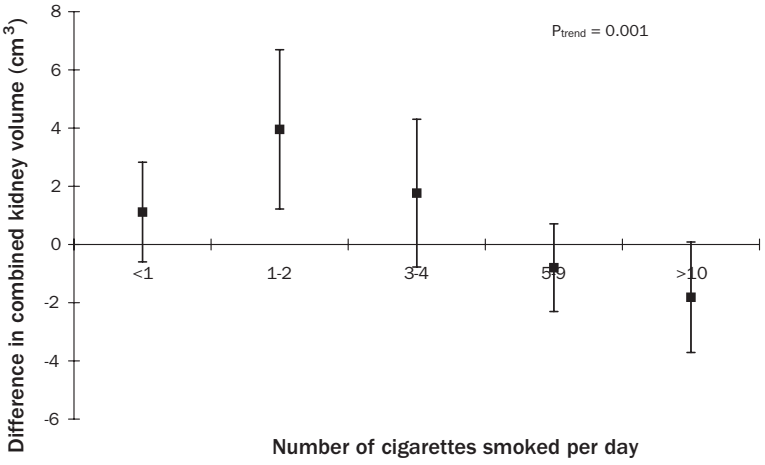
Values are regression coefficients (95% CI) and reflect the difference in kidney size for different categories of maternal smoking during pregnancy. Regression analysis was adjusted for estimated fetal weight, fetal sex, maternal height, weight before pregnancy, alcohol use during pregnancy and educational level and gestational age.

Table 4. Number of cigarettes smoked and fetal kidney structures in third trimester of pregnancy (n = 1,029)

Number of cigarettes smoked	Left kidney structures				Right kidney structures				Combined
	Length (mm)	Width (mm)	Depth (mm)	Volume (cm^3)	Length (mm)	Width (mm)	Depth (mm)	Volume (cm^3)	Volume (cm^3)
Non-smoking	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
(n = 769)									
<5/day	1.09	1.14	0.80	1.20	0.78	0.51	0.74	0.84	2.04
(n = 76)	(0.14, 2.03)*	(0.45, 1.84)†	(0.12, 1.48)*	(0.53, 1.86)†	(-0.16, 1.72)	(-0.21, 1.23)	(0.03, 1.46)*	(0.09, 1.60)*	(0.73, 3.36)†
5-10/day	-0.33	-0.28	-0.21	-0.30	-0.63	-0.42	-0.25	-0.49	-0.78
(n = 55)	(-1.43, 0.76)	(-1.08, 0.52)	(-0.99, 0.58)	(-1.07, 0.47)	(-1.72, 0.46)	(-1.25, 0.42)	(-1.09, 0.58)	(-1.36, 0.39)	(-2.31, 0.74)
≥10/day	-0.92	-0.60	-0.74	-0.80	-0.60	-0.75	-1.53	-1.18	-1.97
(n = 32)	(-2.26, 0.43)	(-1.59, 0.39)	(-1.70, 0.23)	(-1.75, 0.15)	(-1.94, 0.74)	(-1.73, 0.28)	(-2.56, -0.51)†	(-2.26, 0.11)*	(-3.86, -0.11)*
P_{trend}	0.018	0.004	0.04	0.001	0.19	0.06	0.001	0.005	0.001

*Values are regression coefficients (95% CI) and reflect the difference in kidney size for different numbers of cigarettes smoked during pregnancy. Regression analysis was adjusted for estimated fetal weight, fetal sex, maternal height, weight before pregnancy, alcohol use during pregnancy and educational level and gestational age. *p-value <0.05, †p-value <0.01*

Figure 1. Maternal smoking and combined kidney volumes in third trimester of pregnancy



The associations between maternal smoking during pregnancy and amnion fluid deepest pocket are presented in Table 5. We found no associations between the number of cigarettes smoked and deepest pocket of amniotic fluid.

Table 5. Number of cigarettes smoked and amniotic fluid deepest pocket in third trimester of pregnancy (n = 1,009)

Number of cigarettes smoked	Amniotic fluid deepest pocket (cm)
Non-smoking (n = 753)	Reference
<5/day (n = 76)	-0.01 (-0.35, 0.32)
5-10/day (n = 54)	-0.09 (-0.48, 0.31)
≥10/day (n = 32)	-0.12 (-0.60, 0.36)
P_{trend}	0.53

Values are regression coefficients (95% CI) and reflect the difference in amniotic fluid deepest pocket for different categories of smoking during pregnancy and different number of cigarettes smoked continuously during pregnancy. Regression analysis was adjusted for estimated fetal weight, fetal sex, maternal height, weight before pregnancy, alcohol use during pregnancy and educational level and gestational age.

Discussion

This population-based prospective cohort study from early fetal life onwards showed for the first time that smoking during pregnancy affects fetal kidney size. The size and direction of this effect depend on the number of cigarettes smoked. Smoking less than five cigarettes per day was associated with larger combined kidney volume, whereas smoking larger numbers of cigarettes was associated with smaller kidney volume. First trimester only smoking was not associated with third trimester fetal kidney size. We found no associations between maternal smoking and amniotic fluid deepest pocket.

The main strength of our study is the prospective design from early fetal life and the size of the population-based cohort. Our analyses were based on 1,031 complete fetal kidney ultrasounds. To our knowledge, this study group is the largest population-based cohort in which these associations have been studied. The size of the cohort enabled us to assess the associations between the number of cigarettes smoked during pregnancy and kidney size. The ultrasound

measurements were carried out by two sonographers with good reproducibility. Fetal kidney characteristics could be measured completely in 93% of all subjects participating in the third trimester assessments. The effect estimates would be biased if the associations of maternal smoking during pregnancy and fetal kidney size differed between those included and not included in the present analyses. This seems unlikely. A 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.

The associations of low birth weight with hypertension and impaired renal function and end stage renal disease in adulthood are well established.[5, 9, 14, 24-27] Smaller kidneys with a reduced number of nephrons in low birth weight children might lead to hyperfiltration resulting in glomerular sclerosis.[2-4, 28, 29] 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, relative vitamin A deficiency, decreased placenta perfusion, and administration of steroids, lead to fetal growth retardation and smaller kidney size with a lower nephron number.[30-33] Post mortem studies in humans showed that a lower nephron number is associated with both low birth weight and hypertension.[28, 29, 34-38] Since nephron number varies between 250,000 and 2,000,000 per kidney and nephron development ceases after birth[17, 35, 38], these findings suggest that early kidney development may be an underling mechanism for the associations between adverse fetal exposures and hypertension in later life.

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.[39, 40] We showed an association between continued smoking during pregnancy and third trimester kidney size. The effect of continued smoking during pregnancy was dependent on the number of cigarettes smoked. Severe smoking (more than ten cigarettes per day) was associated with smaller kidney size. In addition, moderate smoking (less than five cigarettes per day) was associated with larger kidney size. These associations were independent of estimated fetal weight. Our results suggest a differential effect of maternal smoking during pregnancy, depending on the specific period and number of cigarettes smoked. Smaller kidneys may predispose an individual to subsequent development of kidney disease and hypertension in adult life. However, it remains unclear whether larger kidneys in third trimester of pregnancy would be harmful or beneficial in later life.

One other study among 34 subjects showed that maternal smoking during pregnancy leads to a different fetal kidney growth pattern compared to non-smoked exposed subjects. This study suggested that reduction in kidney growth is present in third trimester of pregnancy, resulting in relatively thinner kidneys.[41] This is in line with our finding that smoking more than 10 cigarettes in third trimester negatively affects fetal kidney size, whereas first trimester only smoking is not associated with altered kidney size. No differential effects were presented in this study on

the number of cigarettes smoked in third trimester. In rats, it was shown that prenatal nicotine exposure leads to smaller kidneys only in genetically susceptible rats, indicating a gene-environment interaction.[42] This animal study also showed that the effect of intrauterine exposure to nicotine on offspring kidney size is stronger in hypertensive mothers. They have significantly reduced kidney weight and higher blood pressure compared to offspring of normotensive mothers.[42] To our knowledge, no other studies examined the associations between maternal smoking during pregnancy and kidney size in a large cohort.

The mechanisms underlying the associations between smoking during pregnancy and fetal kidney size are not known. 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 nicotine in pre-constricted kidney vasculature. [43, 44] However, differential effects on vascular structures are reported.[45-48] It has also been suggested that nicotine has both vasodilatatory and vasoconstrictive effects, depending on the dose.[47] This could partly explain our findings of smaller kidney size when smoking more than five cigarettes. Further studies are needed to elucidate the vasoactive mechanisms and effect of nicotine in different tissues and at different doses.

The main component of amniotic fluid is fetal urinary production. In our study, maternal smoking during pregnancy was not associated with amniotic fluid deepest pocket. We have shown before that kidney size in third trimester of pregnancy is associated with amniotic fluid deepest pocket.[49] The absence of associations between maternal smoking and amniotic fluid deepest pocket could indicate that the adaptations causing differences in kidney size does not alter fetal urine production, as reflected in the amniotic fluid production. Whether the changes in kidney size in response to maternal smoking also alter kidney function remains unclear.

In conclusion, we found that maternal smoking during pregnancy is associated with an altered kidney size in late pregnancy. The direction and size of the effect depends on the number of cigarettes smoked. Further studies are needed to identify the underlying mechanisms and to assess whether these changes in fetal kidney are related to renal function and blood pressure development in later life.

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Chapter 3.5

Tracking and determinants of kidney size from fetal life until early childhood

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Abstract

Background: An adverse fetal environment may lead to smaller kidneys and subsequently renal disease and hypertension in adulthood. The aims of this study were to examine whether kidney size tracks from fetal life to childhood and to examine whether maternal and fetal characteristics are associated with kidney size at the age of 2 years.

Methods: This study was embedded in a population-based prospective cohort study from fetal life onwards. Maternal characteristics, age, height and pre-pregnancy weight were measured in early pregnancy. Fetal growth, head circumference, abdominal circumference, femur length and estimated fetal weight, and placental characteristics were assessed in second and third trimester. Kidney size, defined as length, width, depth and volume, was measured in third trimester of pregnancy and at the postnatal ages of 6 and 24 months. Analyses were based on 683 children.

Results: Children tended to remain in the lowest and highest quartiles of kidney volume from third trimester to the age of 2 years (OR 2.05 (95% CI: 1.38, 3.06) and 3.29 (95% CI: 2.22, 4.87)). Maternal height and pre-pregnancy weight were positively associated with kidney volume at the age of 2 years. Third trimester fetal head circumference, abdominal circumference and estimated weight and postnatal length were positively associated with kidney volume at the age of 2 years. Preferential fetal blood flow to the brain was associated with smaller kidneys.

Conclusions: Small kidney size in fetal life tends to persist in early childhood. Maternal anthropometrics and fetal biometrics and blood flow patterns are associated with kidney size in childhood. Follow-up studies are needed to examine whether these variations in kidney size are related to renal function and blood pressure in later life.

Introduction

Low birth weight is associated with hypertension and cardiovascular mortality in adult life.[1, 2] It has also been demonstrated that low birth weight is related to kidney diseases in childhood and adulthood.[3-8] The underlying mechanisms for these associations are unknown. It has been hypothesized that low birth weight infants have smaller kidneys with a reduced number of nephrons, which leads to compensatory higher glomerular pressure, progressive glomerular sclerosis and subsequently predispose the individual to impaired kidney function and hypertension.[9]

This hypothesis is supported by several studies. Nephrogenesis continues until 36 weeks of gestation and the induction of nephron number ceases thereafter.[10, 11] Animal studies have shown that low protein intake, relative vitamin A deficiency, reduced placenta perfusion or administration of steroids in late pregnancy lead to fetal growth restriction and a permanent nephron deficit.[12-15] Human studies demonstrated that low birth weight infants have lower kidney weight with a reduced number of nephrons.[16-18] It was also demonstrated that hypertensive subjects have lower nephron numbers.[19] The best surrogate measure for assessing nephron number in epidemiological studies appears to be kidney weight or size measured by ultrasound.[11] We have previously demonstrated in a population-based prospective cohort study from early fetal life onwards, that maternal anthropometrics, fetal abdominal circumference, fetal blood flow redistribution, and raised placental resistance are associated with third trimester fetal kidney volume.[20]

In this prospective cohort study, we examined whether kidney volume tracks from fetal life to the age of 2 years. We also studied whether maternal characteristics, as a measure of fetal nutrition, and fetal growth and blood flow characteristics measured in second and third trimester of pregnancy are associated with kidney size at the age of 2 years.

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.[21, 22] Assessments in pregnancy included physical examinations, fetal ultrasounds, biological samples and questionnaires and were planned three times in pregnancy to collect information about fetal growth and its main determinants. More detailed assessments of fetal and postnatal growth and development are conducted in a subgroup, the Focus cohort.[21, 22] In this subgroup, postnatal renal ultrasounds were performed in infants at the ages of 6 months and 2 years. The study has been approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam. Written informed consent was obtained from all participants. All analyses, including the current study, in the Generation R Study are performed and reported according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) recommendations.[23]

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). Twin pregnancies (n = 15) and pregnancies leading to perinatal death (n = 2) were excluded from the analysis. Of these singleton live births, 70% (n = 856) participated in the postnatal assessments at the age of 2 years. Kidney ultrasounds were successfully performed in 80% (n = 683) of these infants. Infants who had a postnatal renal ultrasound at the age of 2 years did not differ from the postnatal non-responders in fetal characteristics (difference in birth weight 18 grams (95% CI: -48, 83)). No renal or ureterovesical anomalies other than mild pyelectasis over 10 mm (n = 3) were present in our study population. Missing values were mainly due to crying behavior or unavailability of equipment or radiographer.

Maternal characteristics

Maternal weight (kg) and height (cm) were measured light clothed in early pregnancy (gestational age <18 weeks), second trimester (gestational age 18-25 weeks) and third trimester (gestational age ≥25 weeks). We used maternal weight and height at intake in the analyses for the present study. Maternal weight gain during pregnancy was defined as the difference in pre-pregnancy and in third trimester weight.

Ultrasound measurements

Fetal growth characteristics

Fetal ultrasound examinations were carried out during visits to the research centers. The median (95% range) gestational age for these visits was 12.6 (9.6 – 16.9) weeks, 20.4 (18.6 - 22.5) weeks and 30.2 (28.5 – 32.5) weeks, respectively. The latter two visits were considered as second and third trimester measurements. These fetal ultrasounds were used for both establishing gestational age and assessing fetal growth characteristics.[24] Fetal growth measurements used for the present study included head circumference (HC), abdominal circumference (AC) and femur length (FL) in second and third trimester measured to the nearest millimeter using standardized ultrasound procedures.[25-27] Estimated fetal weight was calculated using the formula by Hadlock using head circumference, abdominal circumference and femur length: EFW (grams) = 10**(1.326 – 0.00326*AC*FL + 0.0107*HC + 0.0438*AC + 0.158*FL).[28] Growth measures in early pregnancy were not included since these fetal ultrasound examinations were primarily performed to establish gestational age. Gestational age-adjusted standard deviation scores were constructed for these fetal growth measurements.

Placental and fetal blood flow profiles

Placental resistance as a proxy of placental function was assessed using recorded flow velocity waveforms from the umbilical and uterine arteries, in mid and late pregnancy. Raised umbilical artery pulsatility index (PI) and uterine artery resistance index (RI) indicate increased placental resistance.[29] The redistribution of blood flow in favor of the fetal brain was quantified by the middle cerebral artery PI and the cerebro RI / umbilical RI ratio, in late pregnancy. A reduction in middle cerebral artery PI and a decreasing cerebro-umbilical ratio are valid indicators of 'brain sparing effect' due to fetal redistribution.[30, 31]

Kidney size

The left and right kidney were measured in third trimester of pregnancy. 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 and transverse kidney diameter were measured perpendicular to each other, outer to outer, in an axial plane.[32] Postnatally, two-dimensional ultrasounds of the kidneys were performed on Kretz Voluson 530D equipment in children at the ages of 6 months and 2 years. 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.[32, 33] One radiographer performed the vast majority (86%) of these measurements. Two other radiographers performed the measurements in the remaining children. 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.[32, 34] Mean length, width and depth were calculated as the average of three measurements and used for data analysis. Prenatal and postnatal kidney volume were both calculated in cubic centimeters using the equation of an ellipsoid: volume (cm^3) = 0.523 * mean length (cm) * mean width (cm) * mean depth (cm).[33, 34] Left and right kidney volume were summed to establish combined kidney volume (cm^3).[35]

For the ultrasound measurements the intra class 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.[36]

Covariates

All anthropometrics in the infants were measured without clothes at the same visits as the kidney ultrasounds at the ages of 6 and 24 months. Weight was measured to the nearest gram using electronic scales. Length was measured in supine position to the nearest millimeter using a

neonatometer. Date of birth, birth weight and gender were obtained from midwife and hospital registries.

Statistical analysis

Differences in maternal and fetal characteristics between boys and girls were assessed by t-tests and non-parametric tests for independent samples. We created scatterplots to assess the correlations of weight and height with combined kidney volume at each age. The associations between third trimester combined kidney volume and combined kidney volume at the ages of 6 months and 2 years were first assessed using scatterplots and Pearson correlation coefficients. Additionally, third trimester combined kidney volume as well as combined kidney volume at 6 months and 2 years was categorized in quartiles to assess whether children tend to remain in the same quartile from fetal to postnatal life. For these analyses, odds ratios were calculated.

Subsequently, the associations of maternal characteristics and fetal growth and blood flow characteristics (standard deviation scores of head circumference, abdominal circumference and femur length in mid- and late pregnancy, umbilical artery pulsatility index, uterine artery resistance index, middle cerebral artery pulsatility index and the cerebral/umbilical ratio) with combined kidney size were assessed using multiple linear regression models. We examined the associations of fetal growth and blood flow characteristics in second and third trimester of pregnancy with combined left and right kidney volume at the age of 2 years. Furthermore we analysed the associations of weight gain until two years of age with combined kidney volume at each visit. We entered all main determinants seperately into the model. Variables were included in these models when they changed the effects estimates of interest on combined kidney volume substantially (>10%), or when they were strongly associated with the outcome of interest in previous studies. Therefore, all models were adjusted for gender and age at measurement of the kidney volume (Model A). Subsequently, to take account for current anthropometrics, we adjusted for current height (Model B). All measures of association are presented with their 95% confidence intervals (CI).

Results

The percentage of boys was 51% (Table 1). The overall median age of infants at their 6 months visit was 6.3 months (95% range: 5.4 - 8.1) and at their 2 years visit 25.1 months (95% range: 23.6 - 28.2). We defined small for gestational age as children with birth weight below –2 SD for gestational age. In total, only 15 children in our study group fullfilled this criterium for small for gestational age. Furthermore, we reported only 18 children with birth weight <2500 grams and 23 children with gestational age <37 weeks. Table 2 shows that at all ages, kidney length and volume were larger in boys than in girls.

Table 1. Subject characteristics (n = 688)

	Boys (n = 351)	Girls (n = 332)	P-value
Maternal characteristics			
Age (years)	32.3 (22.5-39.7)	32.4 (23.4-39.6)	0.1
Height (cm)	170.7 (6.1)	171.0 (6.7)	0.4
Pre-pregnancy weight (kg)	67.8 (12.5)	70.1 (12.6)	0.01
Pre-pregnancy BMI (kg/m^2)	23.2 (4.0)	23.9 (4.2)	0.01
Weight gain until third trimester of pregnancy (kg)	7.9 (3.6)	7.8 (3.8)	0.6
Second trimester fetal characteristics			
Gestational age (weeks)	20.5 (18.6-22.8)	20.5 (18.7-22.8)	0.02
Head circumference (cm)	18.0 (1.3)	17.6 (1.2)	<0.001
Abdominal circumference (cm)	15.7 (1.4)	15.6 (1.3)	0.02
Femur length (cm)	3.3 (0.3)	3.3 (0.3)	0.6
Estimated fetal weight (grams)	375 (85)	364 (75)	0.09
Umbilical artery, pulsatility index (PI)	1.18 (0.19)	1.20 (0.17)	0.07
Uterine artery, resistance index (RI)	0.54 (0.09)	0.54 (0.09)	0.5
Third trimester fetal characteristics			
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
Umbilical artery, pulsatility index (PI)	0.96 (0.16)	0.99 (0.18)	0.01
Uterine artery, resistance index (RI)	0.49 (0.08)	0.49 (0.08)	0.9
Middle cerebral artery, pulsatility index (PI)	1.98 (0.33)	1.99 (0.34)	0.6
Cerebral-umbilical (C/U) ratio	2.05 (0.48)	2.08 (0.48)	0.2
Characteristics at birth			
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 (2.3)	10 (3.0)	0.6
Small for gestational age (%)	6 (1.7)	9 (2.7)	0.4
Characteristics at 6 months			
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			
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) or medians (95% range).

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

Table 2. Kidney structures (n = 688)

	Boys (n = 351)	Girls (n = 332)	P-value
Third trimester			
Left kidney			
Length (mm)	39.5 (31.1-47.0)	38.5 (32.9-45.2)	<0.001
Width (mm)	22.6 (17.4-29.0)	22.0 (17.0-28.0)	<0.001
Depth (mm)	21.6 (17.0-28.0)	21.1 (16.0-26.0)	0.04
Volume (cm^3)	10.3 (5.4-17.0)	9.5 (5.3-14.8)	<0.001
Right kidney			
Length (mm)	39.6 (31.8-46.7)	38.7 (32.9-49.3)	<0.001
Width (mm)	23.1 (17.6-29.7)	22.9 (17.8-29.0)	0.3
Depth (mm)	22.2 (17.0-28.0)	22.0 (17.3-28.8)	0.5
Volume (cm^3)	10.9 (5.8-17.9)	10.4 (6.1-16.9)	0.05
Combined kidney volume (cm^3)	21.2 (11.5-33.5)	19.9 (11.9-32.2)	<0.001
Age 6 months			
Left kidney			
Length (mm)	60.0 (49.6-70.7)	58.7 (49.4-68.0)	<0.001
Width (mm)	28.1 (22.4-35.7)	27.5 (21.4-35.6)	0.03
Depth (mm)	26.4 (19.5-32.1)	25.5 (20.1-32.1)	<0.001
Volume (cm^3)	23.6 (14.1-34.4)	21.5 (13.4-31.8)	<0.001
Right kidney			
Length (mm)	58.9 (49.7-69.1)	57.4 (49.3-68.4)	<0.001
Width (mm)	27.9 (22.0-35.2)	27.5 (20.9-36.3)	0.2
Depth (mm)	27.6 (21.2-35.4)	27.0 (20.9-34.1)	0.07
Volume (cm^3)	23.7 (15.7-35.2)	22.3 (13.9-34.9)	<0.001
Combined kidney volume (cm^3)	47.3 (32.8-68.0)	43.8 (29.4-61.8)	<0.001
Age 24 months			
Left kidney			
Length (mm)	66.8 (56.1-80.3)	65.9 (53.8-78.1)	0.04
Width (mm)	30.8 (25.6-37.1)	30.2 (23.7-37.8)	0.6
Depth (mm)	31.0 (25.4-38.9)	30.1 (24.2-38.1)	0.7
Volume (cm^3)	33.6 (22.8-51.6)	31.8 (19.5-52.3)	<0.001
Right kidney			
Length (mm)	65.2 (54.8-76.6)	64.3 (53.2-76.5)	0.06
Width (mm)	30.8 (25.6-38.1)	30.7 (25.5-37.1)	0.01
Depth (mm)	32.0 (25.8-39.4)	31.9 (25.9-39.6)	<0.001
Volume (cm^3)	33.8 (22.9-53.3)	33.1 (22.1-49.2)	0.2
Combined kidney volume (cm^3)	67.4 (48.4-102.2)	64.9 (42.9-97.8)	0.03

Values are means (95% range). Differences between boys and girls were compared using independent sample t-tests.

We found that children tend to remain in the lowest and highest quartiles of combined kidney volume from third trimester of pregnancy to the age of 6 months (OR 2.99 (95% CI: 1.96, 4.55) and 2.61 (95% CI: 1.71, 4.00), respectively) and to the age of 2 years (OR 2.05 (95% CI: 1.38, 3.06) and 3.29 (95% CI: 2.22, 4.87), respectively) (Table 3). In total 212 children were in the lowest two quartiles in third trimester. Of these 212 children, 135 children (63.4%) stayed within the lowest two quartiles until the age of two years.

Table 3. Tracking analysis of combined kidney volume in quartiles from third trimester until 6 and 24 months of age

	Combined kidney volume 6 months quartile				
Third trimester combined kidney volume quartile	**1**	**2**	**3**	**4**	**Total**
1	2.99 (1.96, 4.55) (50)	0.96 (0.60, 1.54) (26)	0.58 (0.34, 0.98) (19)	0.26 (0.13, 1.54) (10)	(105)
2	1.33 (0.85, 2.09) (32)	0.98 (0.61, 1.57) (27)	1.29 (0.82, 2.03) (30)	0.60 (0.35, 1.01) (19)	(108)
3	0.47 (0.27, 0.82) (16)	1.08 (0.68, 1.71) (29)	0.86 (0.53, 1.38) (26)	1.74 (1.13, 2.70) (38)	(109)
4	0.30 (0.16, 0.57) (11)	0.68 (0.41, 1.12) (23)	1.36 (0.87, 2.13) (32)	2.61 (1.71, 4.00) (46)	(112)
Total	(109)	(105)	(107)	(113)	(434)
	Combined kidney volume 24 months quartile				
Third trimester combined kidney volume quartile	**1**	**2**	**3**	**4**	**Total**
1	2.05 (1.38, 3.06) (51)	1.07 (0.70, 1.63) (36)	1.05 (0.69, 1.61) (36)	0.36 (0.21, 0.61) (17)	(140)
2	1.31 (0.86, 1.98) (39)	1.20 (0.79, 1.82) (38)	0.76 (0.48, 1.19) (29)	0.69 (0.44, 1.09) (26)	(132)
3	0.68 (0.43, 1.07) (27)	0.92 (0.60, 1.43) (33)	1.45 (0.96, 2.18) (43)	1.17 (0.77, 1.78) (39)	(142)
4	0.34 (0.19, 0.59) (15)	0.69 (0.44, 1.09) (29)	0.84 (0.54, 1.31) (29)	3.29 (2.22, 4.87) (63)	(136)
Total	(132)	(136)	(137)	(145)	(550)

Values are unadjusted odds ratios (95% CI) estimated by logistic regression for quartiles of combined kidney volume at the ages of 6 months and 24 months.

Table 4 demonstrates that maternal height and pre-pregnancy weight were positively associated with combined kidney volume (increase in combined kidney volume of 0.35 cm^3 (95% CI: 0.18, 0.51) and of 0.14 cm^3 (95% CI: 0.06, 0.23) per cm increase in maternal height and kg increase in pre-pregnancy weight, respectively). Other maternal characteristics were not associated with combined kidney volume.

Table 4. Associations of maternal characteristics with combined kidney volume at the age of 24 months

	Difference in combined kidney volume (cm^3) at the age of 24 months (95% confidence interval)
Maternal characteristics	
Age (years)	0.09 (-0.19, 0.36)
Height (cm)	0.35 (0.18, 0.51)
Pre-pregnancy weight (kg)	0.14 (0.06, 0.23)
Pre-pregnancy BMI (kg/m^2)	0.23 (-0.04, 0.49)
Weight gain until late pregnancy (kg)	0.07 (-0.24, 0.38)

Values are regression coefficients (95% confidence interval) and reflect the difference in combined kidney volume per unit increase in maternal characteristic or lifestyle measure. Models are adjusted for age and fetal gender at 24 months of age. BMI, body mass index.

No associations were found for second trimester fetal growth and blood flow characteristics with combined kidney volume at the age of 2 years (Table 5). Third trimester fetal head circumference and abdominal circumference were at 2 years of age positively related to both absolute combined kidney volume (increase of 1.68 cm^3 (95% CI: 0.56, 2.80) and 1.76 cm^3 (95% CI: 0.68, 2.84) per increase in standard deviation score, respectively) and to relative combined kidney volume (increase of 1.30 cm^3/m (95% CI: 0.11, 2.49) and 1.30 cm^3/m (95% CI: 0.17, 2.44) per increase in standard deviation score, respectively). Estimated fetal weight and birth weight were only positively associated with absolute combined kidney volume at the age of 2 years (increase of 1.19 cm^3 (95% CI: 0.17, 2.26) and 1.58 cm^3 (95% CI: 0.50, 2.67) per standard deviation score, respectively). Cerebro-umbilical (C/U) blood flow ratio was positively related to abosolute combined kidney volume. Placental resistance indices were inversely associated with absolute and relative combined kidney volume, indicating that signs of increased placental resistance reduce kidney volume. We further analysed whether growth in various periods in early life was associated with combined kidney volume. Results show that weight gain in early life is strongly associated with combined kidney volume at the age of 2 years. Table 6 and 7 show an increase of 3.01 cm^3/m (95% CI: 1.42, 4.59) and 3.37 cm^3/m (95% CI: -1.01, 7.76) per standard deviation score increase in weight gain from age 6 months to age 24 months, respectively.

Table 5. Associations of fetal growth characteristics and placental indices in mid- and late pregnancy with postnatal combined kidney volume at the age of 24 months

	Difference in absolute combined kidney volume (cm^3) at the age of 24 months (95% confidence interval)	**Difference in relative combined kidney volume (cm^3/m) at the age of 24 months (95% confidence interval)**
	Model A	**Model B**
Second trimester		
Fetal growth characteristics		
Head circumference (SDS)	0.44 (-0.65, 1.53)	0.36 (-0.79, 1.52)
Abdominal circumference (SDS)	0.56 (-0.56, 1.68)	0.43 (-0.75, 1.62)
Femur length (SDS)	0.01 (-1.08, 1.10)	-0.36 (-1.52, 0.79)
Estimated fetal weight (SDS)	0.47 (-0.67, 1.60)	0.19 (-1.01, 1.39)
Placental resistance indices		
Umbilical artery, pulsatility index (PI)	-4.14 (-10.4, 2.14)	-0.02 (-0.09, 0.04)
Uterine artery, resistance index (RI)	-10.7 (-23.8, 2.39)	-0.14 (-0.27, 0.01)
Third trimester		
Fetal growth characteristics		
Head circumference (SDS)	1.68 (0.56, 2.80)	1.30 (0.11, 2.49)
Abdominal circumference (SDS)	1.76 (0.68, 2.84)	1.30 (0.17, 2.44)
Femur length (SDS)	-0.17 (-1.28, 0.95)	-0.90 (-2.09, 0.29)
Estimated fetal weight (SDS)	1.19 (0.17, 2.26)	0.59 (-0.56, 1.74)
Placental resistance indices		
Umbilical artery, pulsatility index (PI)	-4.51 (-10.7, 1.71)	-3.83 (-10.4, 2.76)
Uterine artery, resistance index (RI)	-13.5 (-27.7, 0.63)	-11.0 (-26.0, 3.96)
Redistribution parameters		
Middle cerebral artery (PI)	2.48 (-0.74, 5.69)	2.15 (-1.25, 5.55)
Cerebro-umbilical (C/U) ratio	2.45 (0.23, 4.67)	2.30 (-0.05, 4.65)
Birth weight (SDS)	1.58 (0.50, 2.67)	1.12 (-0.03, 2.27)
Gestational age at birth (weeks)	0.40 (-0.30, 1.10)	0.43 (-0.33, 1.19)
Weight gain (SDS)		
Third trimester – age 24 months	2.52 (1.61, 3.43)	2.16 (1.19, 3.13)
Birth – age 24 months	2.62 (1.63, 3.61)	2.20 (1.14, 3.25)
Age 6 months – age 24 months	3.24 (1.76, 4.72)	3.01 (1.42, 4.59)

Values are regression coefficients (95% confidence interval) and reflect the difference in combined kidney volume per unit increase in fetal growth and fetal blood flow characteristic. SDS, gestational age-adjusted standard deviation score; PI, pulsatility index. Model A: adjusted for age and gender. Model B: additionally adjusted for current length.

Table 6. Associations of growth in various periods in early life and combined kidney volume at birth and at 6 and 24 months of age

	Difference in combined kidney volume (cm^3/m) (95% confidence interval)		
Weight gain (SDS)	**Birth**	**Age 6 months**	**Age 24 months**
Third trimester - birth	0.40 (-0.76, 1.56)	1.06 (0.05, 2.08)	2.16 (1.19, 3.13)
Birth - age 6 months	-	1.07 (-0.08, 2.23)	2.20 (1.14, 3.25)
Age 6 months - age 24 months	-	-	3.01 (1.42, 4.59)

Values are regression coefficients (95% confidence interval) and reflect the difference in combined kidney volume per unit increase in weight gain. Models are adjusted for age, fetal gender and current length.

Table 7. Associations of growth in various periods in early life and combined kidney volume at birth and at 6 and 24 months of age

	Catch-down	**Reference**	**Catch-up**
Weight (SDS)			
Second - third trimester	0.88 (-1.97, 3.72)	Reference	1.54 (-0.89, 3.97)
Third trimester – birth	-1.01 (-3.60, 1.58)	Reference	0.22 (-2.45, 2.89)
Birth – age 6 months	-0.79 (-3.57, 2.00)	Reference	1.97(-0.80, 4.73)
Age 6 months – age 24 months	-3.06 (-5.52, -0.60)	Reference	3.37 (-1.01, 7.76)

Values are regression coefficients (95% confidence interval) and reflect the difference in growth pattern per unit increase in weight gain. Models are adjusted for age and fetal gender. Catch-up = >0.67 SD increase in weight; catch-down = <-0.67 SD increase in weight.

Discussion

Our population-based prospective cohort study showed for the first time that kidney size tracks from third trimester of pregnancy to early childhood. Maternal anthropometrics before pregnancy and fetal growth characteristics in third trimester of pregnancy were positively associated with kidney volume at the age of 2 years. Preferential fetal blood flow to the brain was also associated with smaller kidneys.

The major strength of our study is its prospective design from early fetal life and the size of the population-based cohort. Our analyses were based on almost 2,000 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] Furthermore, our study is the first that examines the associations of maternal anthropometrics during pregnancy and longitudinally measured fetal growth characteristics with kidney size until the age of 2 years. A limitation could be that of all children participating in the Generation R measurements at the age of 2 years, kidney measurements were successfully performed in 80% of these infants. Missing values were mainly due to crying behavior or unavailability of equipment or radiographer. The effect estimates would be biased if the associations of maternal anthropometrics and fetal growth characteristics with kidney structures differ between those included and not included in the present analyses. This seems unlikely. Another limitation could be that the current study was perfomed in a healthy, population based cohort study. In total, we had only 15 children with a birth weight below –2 SD for gestational age, which is the definition for small size for getstational age. Furthermore, we reported only 18 children with birth weight <2500 grams and 23 children with gestational age <37 weeks. 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 to small to be assessed in further detail.

The correlations between early and late kidney measurements, although statistical significant, are weak. These correlations just show that the kidney measurements are related to each other, but it doesn't mean that these relations have to be causative. Our findings suggest that impaired growth in fetal life has consequences for kidney size in postnatal life. Children with smaller kidneys in fetal life tend to keep their relatively smaller kidneys in early childhood. The risks of remaining in the same quartile of kidney size between third trimester and early childhood varied between two and three.

The association of maternal anthropometrics and fetal growth with kidney size until the age of 2 years may be explained by various factors. Animal studies have shown that various determinants of fetal nutrition including low protein intake, relative vitamin A deficiency, reduced placenta perfusion and administration of steroids in late pregnancy cause fetal growth restriction, smaller kidneys and a permanent reduced nephron number.[12-15] Maternal anthropometrics are major determinants of fetal nutrition, fetal growth and birth weight.[37] We showed that maternal height and weight are associated with kidney volume in their offspring. This association may be explained by both genetic and environmental determinants. Further studies focused on potential genetic variants and detailed maternal dietary habits during pregnancy are needed to identify the underlying mechanisms. Unfortunately, for the present study no data about maternal dietary habits were available. According to the Brenner hypothesis, IUGR leads to both a low nephron en-

dowment and glomerular hyperfiltration. Prenatal programming can be expected to be of importance to the kidney.[38] We showed that there are associations between measures of body size and kidney size. Therefore we examined the effect on both absolute and relative kidney size. Third trimester fetal head circumference and abdominal circumference were associated with absolute and relative kidney size in infancy. The largest effect was seen for fetal abdominal circumference. This is in line with our previous study which showed the largest effect on third trimester kidney volume for fetal abdominal circumference.[20] Estimated fetal weight and birth weight only showed possitive associations with absolute kidney volume. We found that fetal blood flow distribution, measured as cerebro-umbilical blood flow ratio was positively related to combined kidney volume. This means that relatively increased blood flow to the brain at expense of the extremities is associated with persistent smaller kidneys. Smaller growth rates in various periods in early life were associated with combined kidney volume, showing that weight gain in early life is strongly associated with combined kidney volume at the age of 2 years.

The size of the effect estimates of the association of maternal anthropometrics and fetal growth with kidney size until the age of 2 years was rather small. For example, an increase of 1 standard deviation score in third trimester estimated fetal weight leads to an increase of 1.19 cm^3 (95% confidence interval: 0.17, 2.26) increase in combined kidney volume at 2 years of age. Furthermore, our results suggest that part of the effect is explained by length or other current anthropometrics. Further studies in humans and animals are needed to examine the underlying causal mechanisms and whether these small changes in kidney size have clinical consequences.

A few other studies investigated renal length or volume in early life. Schmidt *et al.* compared pre- and postmature children with mature children. Identical to our analyses, they studied both absolute and relative combined kidney volume and concluded that being small for gestational age (SGA) is associated with small kidneys at birth and impaired kidney growth in early childhood.[35] Hotoura *et al.* compared children with a gestational age of 31-36 weeks with a control group of appropriate for gestational age (AGA) children. They concluded that kidney length in preterm SGA infants follows closely the other anthropometric parameters during the first year of life.[39] Renal growth during the first 2 years of life in SGA children and AGA children was also studied by Giapros *et al.*[40] They reported that SGA term infants had shorter kidney length at birth compared to AGA infants, but a similar length from 3rd to 24th month of life. Therefore, they concluded that this observation may represent either an accelerated renal maturation or early compensatory kidney hypertrophy in the SGA infants.

Our study suggests that adverse fetal growth patterns lead to smaller kidneys postnatally. Smaller kidneys are related to the number of nephrons. Since the number of nephrons is largely determined in prenatal life, sub-optimal kidney growth and development in fetal life leads to a smaller number of nephrons.[10, 11] The kidneys respond to this reduced number of nephrons by hyperperfusion and remodeling.[9] This process may be in favor of short-term renal function but may

eventually lead to glomerular hypertrophy and damage.[9] Finally, this might result in renal failure and hypertension. It has been shown that low birth weight is associated with early onset chronic renal failure. In subjects aged less than 50 years, those who weighted less than 2.5 kg at birth had a higher risk for end-stage renal disease than people who weighted 3-3.5 kg at birth.[5] This association was shown in all groups of primary causes of end stage renal failure in adults (hypertension, diabetes and other causes). Studies in younger subjects have focused on urine albumin excretion, a predictor of cardiovascular and renal disease in diabetic and non-diabetic subjects.[41] Low birth weight is associated with microalbuminuria in children and adults independent of blood pressure and measures of insulin resistance.[3, 4, 6] The pathway leading from small kidneys to hypertension may include the renin-angiotensin system, which has been demonstrated to be altered in the early phase of primary hypertension.[42] An increased activity of the renin-angiotensin system could be a compensatory mechanism in a decreased number of nephrons in order to maintain normal renal filtration. It has been demonstrated that renin activity in umbilical cord blood is inversely related with the size of the kidney at birth.[43]

In conclusion, our study showed for the first time in a large population-based cohort that renal size at the age of two years may be partially affected by several perinatal or maternal factors. Whether these relations are causal, persist during later life and are related to renal function and blood pressure needs further investigation.

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Cardiovascular growth and development

4

Chapter 4.1

Reliability of echocardiographic measurements of left cardiac structures in children

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Abstract

Background: Echocardiographic measurements are widely used as outcome of different studies. The aim of this study was to assess intraobserver and interobserver reliability of echocardiographic measurements in healthy children.

Methods: The study comprised of 28 children (median age 7.5 years; inter-quartile range: 3.0 – 11.0). Intraobserver and interobserver reliability were assessed by repeated measurements of aortic root diameter, left atrial diameter, left ventricular end diastolic diameter, interventricular end-diastolic septal thickness and left ventricular end-diastolic posterior wall thickness. Intraclass correlation coefficients with corresponding 95% confidence intervals were calculated and Bland and Altman plots were computed. Limits of agreement plus or minus 2 standard deviations for the mean differences in cardiac measurements were derived.

Results: High intraobserver and interobserver intraclass correlation coefficients were found, ranging from 0.91 for interventricular septal thickness (95% confidence interval: 0.78, 0.96) to 0.99 for aortic root diameter (95% confidence interval: 0.97, 1.00). Limits of agreement in the Bland and Altman plots ranged from 0 millimeter (0%) (left ventricular end-diastolic posterior wall thickness) to 1.60 millimeter (6.3%) (left atrial diameter).

Conclusions: This study demonstrated good repeatability and reproducibility of left cardiac structures in children measured by ultrasound. Left cardiac structures can be measured in both clinical and epidemiological research projects.

Introduction

Ultrasound is widely recognized as method of choice for visualizing organs in children. M-mode and cross-sectional echocardiography is a noninvasive, cost-effective and efficient method. Many epidemiological studies use echocardiographic measurements to examine the associations of various determinants with left ventricular structures in children.[1] This information may be relevant for identifying critical periods in life for left ventricular growth and development. To compare echocardiographic measurements as an outcome of different studies, it is necessary to minimize intraobserver and interobserver variability.[2] Although the clinical relevance and research importance of echocardiographic measurements in children is well known, little has been published about the reliability of these measurements. Lipschultz *et al.* assessed the reliability of pediatric echocardiographic measurements in a group children of mothers infected with the Human Immunodeficiency Virus and demonstrated that measurements of left ventricular wall thickness and fractional shortening made in a local facility differed from those made in a central echocardiography laboratory.[3] In this study intraobserver and interobserver reliability were not assessed directly. Results from Project Heartbeat! showed that echocardiographic measurements in healthy children aged 8 years and older can be performed accurately with acceptable reproducibility.[4] However, less is known about the reliability of echocardiographic measurements in younger children.

The aim of the present study was to quantify intraobserver and interobserver reliability of echocardiographic measurements in young children.

Methods

Design

The children were selected from the outpatient clinic of the Erasmus Medical Center – Sophia Children's Hospital, a university hospital in Rotterdam, the Netherlands. In total, 28 subjects, resulting in 56 paired measurements, participated in this study to validate echocardiographic measurements. None of them had congenital heart disease or other cardiovascular disease influencing the circulation. Written informed consent was obtained for enrollment. All children in this study were participants in two ongoing follow-up studies (Intra-Uterine Growth Retardation follow-up study, the Generation R Study[5,6]) and had given informed consent for echocardiographic measurements.

Two experienced sonographers (SPE Snijders, VE Kleyburg) performed all examinations at the same visit. Every single cardiac ultrasound measurement was measured twice by the two sonographers. The first observer scanned the ultrasound measurements in no specific order. Subsequently, the other observer did the same, after which the first examiner repeated the process. The sonographers left the ultrasound room during each other's assessment. The time interval for the first observer to rescan the patient depended on the time the second observer

took for the measurements, being about 10 minutes. Measurement results were blinded to both operators; the caliper read out on the screen was hidden. Printouts of the measurements were not seen by the observers and were saved on hard disc for later analyses.

Ultrasound measurements

The examination was carried out in a quiet room with the child quietly awake in a supine position. To measure left cardiac structures, M-mode, cross-sectional echocardiography was performed in all the children. Left ventricular measurements included left ventricular end diastolic diameter, interventricular end-diastolic septal thickness and left ventricular end-diastolic posterior wall thickness were measured according to the recommendations of the American Society of Echocardiography.[7] Other assessments of left cardiac structures were aortic root diameter and left atrial diameter. All examinations were performed using an ATL-Philips Model HDI 5000 (Seattle, Washington, USA) equipped with a 5.0 megahertz, high frequency, curved array transducer.

Statistical analysis

We used the methods to compare observers in detail described by Bland and Altman.[8,9] The first step was to plot the data and draw the line of equality. This visualizes the degree of agreement.[8] Second, the consensus between and among observers was analyzed using the intraclass correlation coefficient for all cardiac measurements. The intraclass correlation coefficient is defined as the ratio of the variance between subjects to total variance. The intraclass correlation coefficient measures the strength of the agreement of the variables, independent of the dimension of the variable considered. Additionally, the 95% confidence interval was calculated for all cardiac measurements.

Finally, agreement was tested to investigate intra- and interobserver differences and reproducibility.[8] For assessing intraobserver agreement, main interest was in the mean difference between repeated measures of one observer. For assessing the interobserver agreement, the difference between the first measurements of each separate observer was used in the analyses. We created Bland and Altman plots by plotting the differences of all the measurements against their mean with the 2 standard deviations of the mean to see the distribution and to find any possible differences from the mean within or between the observers.[8] The average differences between duplicate measures were tested using the paired sample t-test to see if there was systemic bias. If the differences are Gaussian distributed, 95% of the differences will lie between the mean plus or minus 2 standard deviation limits. These are the limits of agreement, and the measures between and among observers can be assumed to be interchangeable within these limits. An advantage of the Bland and Altman plots over the statistical testing is that one can visualize between which boundaries a measurement is interchangeable and how large the measurement differences

between operators are in proportions. How small the limits of agreement should be is a clinical, not a statistical, decision that should be made in advance of the analysis. We decided that we could confidently say that cardiac measurements in children would be reliable and valid in case of intraclass correlation coefficient over 0.80 and mean difference within 10% from the mean of two different measurements. Statistical analysis was performed using Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Median age of the children was 7.5 years (interquartile range: 3.0 – 11.0) and 17 (61%) were male. A total of 14 children (50%) was younger than 8 years of age. Table 1 presents the descriptive statistics of the echocardiographic measurements of left cardiac structures assessed in this study. A comparison of the means and standard deviation of the different echocardiographic measurements among and between observers is shown. None of the differences between the means on repeated observation and between observers were statistically significant.

Table 1. Descriptive statistics for echocardiographic measurements in children

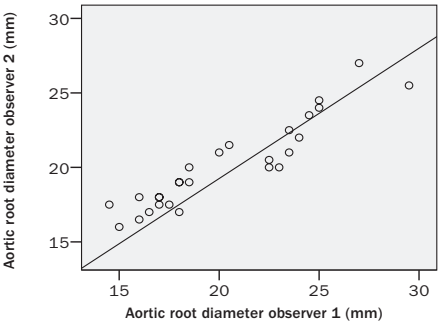
	Sonographer 1				Sonographer 2			
	First observation		Second observation		First observation		Second observation	
Cardiac measurements, end diastolic (millimeters)	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Aortic root diameter	20.36	3.99	20.81	4.04	19.89	2.94	20.33	3.04
Left atrial diameter	26.07	4.40	26.46	3.94	25.07	4.53	24.44	3.87
Interventricular septal thickness	5.89	1.07	5.81	0.90	6.11	1.10	6.11	1.28
Left ventricular diameter	36.93	5.27	37.73	5.06	37.96	4.98	38.64	5.07
Left ventricular posterior wall thickness	5.82	1.09	5.85	0.97	6.43	1.73	6.36	1.64

Analyses were based on a group of 28 children.

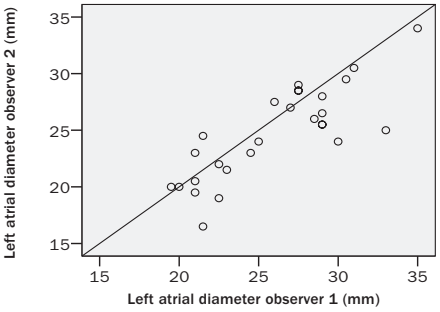
Figure 1 shows plots of left cardiac structure measurements between observers against the line of equality. All points seem to lie randomly around this line, meaning that there seems to be no bias. The variables are close to the line of equality as well, indicating good agreement and suggesting small differences between observers.

Figure 1. Echocardiographic measurements of observers with the line of equality

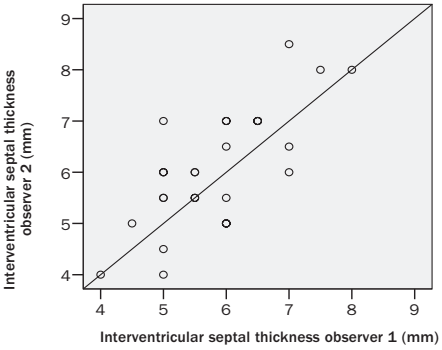
a. Aortic root diameter measurements



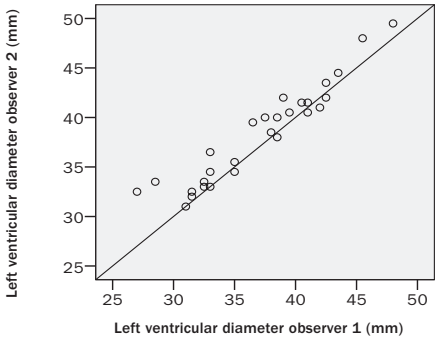
b. Left atrial diameter measurements



c. Interventricular septal thickness measurements



d. Left ventricular diameter measurements



e. Left ventricular posterior wall thickness measurements

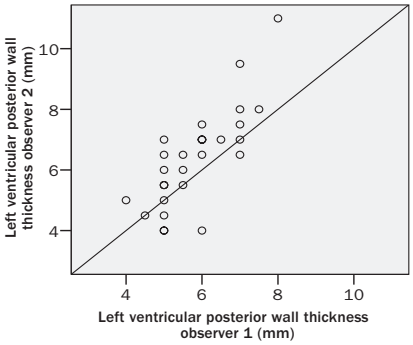


Table 2 presents the intraclass correlation coefficients with their 95% confidence interval. Intraobserver intraclass correlation coefficient ranged from 0.91 (interventricular septal thickness) to 0.99 (aortic root diameter) and interobserver intraclass correlation coefficient ranged from 0.78 (left ventricular posterior wall thickness) to 0.96 (left ventricular diameter).

Table 2. Intra- and interobserver intraclass correlation coefficient and 95% confidence interval for left cardiac structures in childhood

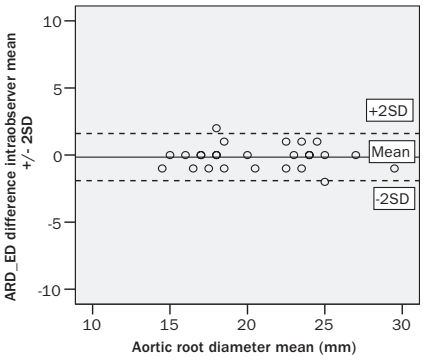
	Intraobserver intraclass correlation coefficient (95% confidence interval)	**Interobserver intraclass correlation coefficient (95% confidence interval)**
Cardiac measurements, end-diastolic		
Aortic root diameter	0.99 (0.97, 1.00)	0.93 (0.83, 0.97)
Left atrial diameter	0.96 (0.91, 0.98)	0.84 (0.65, 0.93)
Interventricular septal thickness	0.91 (0.78, 0.96)	0.81 (0.58, 0.91)
Left ventricular diameter	0.98 (0.96, 0.99)	0.96 (0.92, 0.98)
Left ventricular posterior wall thickness	0.94 (0.87, 0.97)	0.78 (0.53, 0.90)

Analyses were based on a group of 28 children.

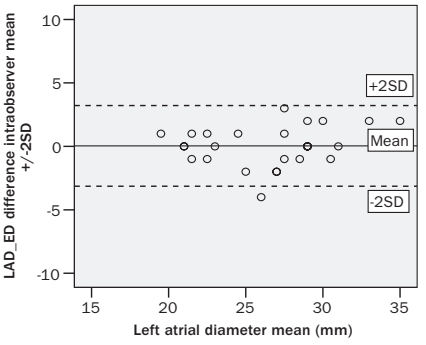
Figure 2 shows Bland and Altman plots for the differences in left cardiac measurements among or between observers against the mean. The limits of agreement (plus or minus 2 standard deviations) are plotted in the figures. Most differences lie between the limits of agreement.

Figure 2. Intra- and interobserver Bland and Altman plots of variation in echocardiographic measurements among and between observers

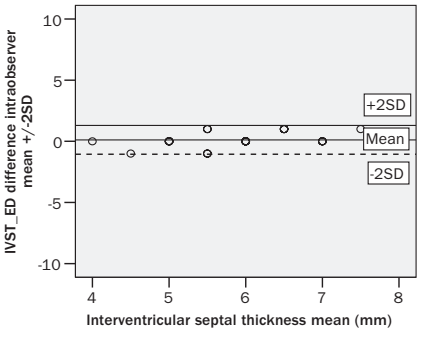
a. Intraobserver agreement aortic root diameter



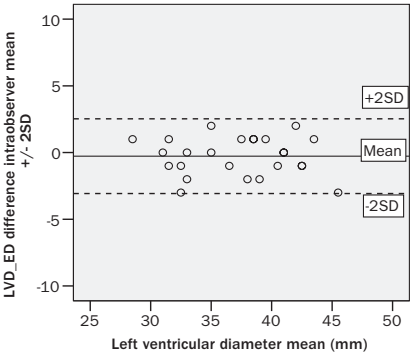
b. Intraobserver agreement left atrial diameter



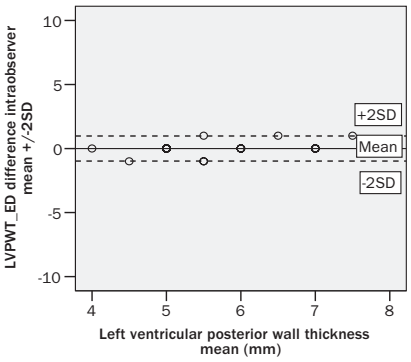
c. Intraobserver agreement interventricular septal thickness



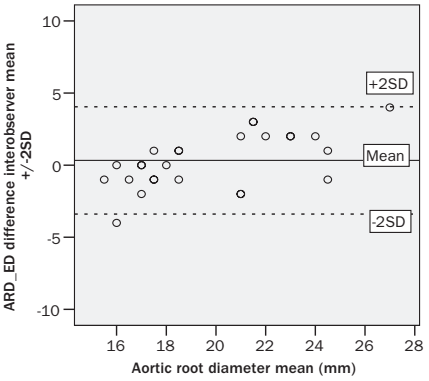
d. Intraobserver agreement left ventricular diameter



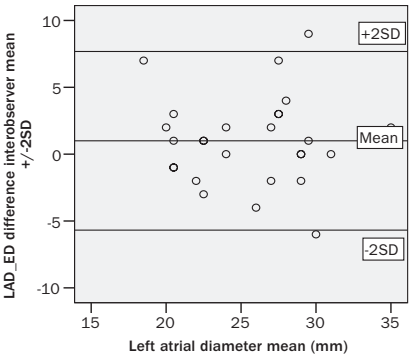
e. Intraobserver agreement left ventricular posterior wall thickness



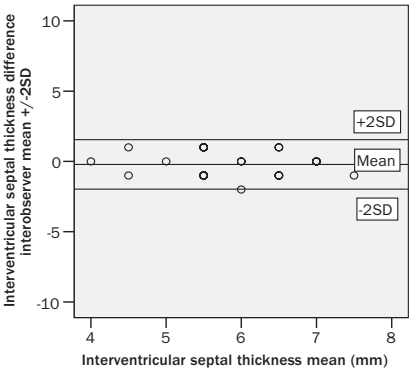
f. Interobserver agreement aortic root diameter



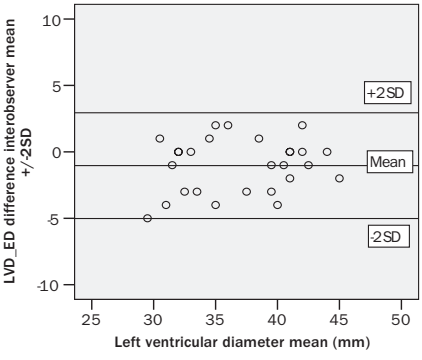
g. Interobserver agreement left atrial diameter



h. Interobserver agreement interventricular septal thickness



i. Interobserver agreement left ventricular diameter



j. Interobserver agreement left ventricular posterior wall thickness

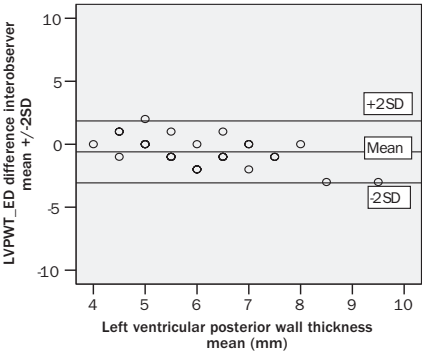


Table 3 shows the mean differences of the left cardiac structures among and between observers and the 95% limits of agreement (mean difference plus or minus 2 standard deviations of the difference). The observed difference from the mean for left cardiac structures ranged from 0 millimeter (0%) (left ventricular end-diastolic posterior wall thickness) to 1.60 millimeter (6.3%) (left atrial diameter). The mean difference between duplicate measures was not significantly different from zero. This suggest that no systematic difference between the pairs of results. The limits of agreement ranged from –0.96 to 0.96 millimeter (left ventricular end-diastolic posterior wall thickness) to the widest limit from –5.55 to 7.55 millimeters (left atrial diameter).

Table 3. Intra- and interobserver measurement variation in absolute number and in proportions of the mean with 95% limits of agreement

Intraobserver agreement - observer 1	**Mean difference**		**95% limits of agreement**	
Cardiac measurements, end-diastolic (millimeters)	**Millimeters**	**%**	**Lower limit**	**Upper limit**
Aortic root diameter	-0.15	0.7	-1.87	1.57
Left atrial diameter	0.04	0.2	-3.08	3.16
Interventricular septal thickness	0.12	2.0	-1.04	1.28
Left ventricular diameter	-0.27	0.7	-3.01	2.47
Left ventricular posterior wall thickness	0.00	0.0	-0.96	0.96

Intraobserver agreement - observer 2	**Mean difference**		**95% limits of agreement**	
Cardiac measurements, end-diastolic (millimeters)	**Millimeters**	**%**	**Lower limit**	**Upper limit**
Aortic root diameter	-0.44	2.2	-2.89	2.01
Left atrial diameter	0.63	2.5	-3.41	4.67
Interventricular septal thickness	0.11	1.8	-1.01	1.23
Left ventricular diameter	-0.68	1.8	-4.23	2.87
Left ventricular posterior wall thickness	0.07	1.1	-1.62	1.76

Interobserver agreement – observation 1	**Mean difference**		**95% limits of agreement**	
Cardiac measurements, end-diastolic (millimeters)	**Millimeters**	**%**	**Lower limit**	**Upper limit**
Aortic root diameter	0.33	1.6	-3.32	3.98
Left atrial diameter	1.00	3.9	-5.55	7.55
Interventricular septal thickness	-0.21	3.5	-1.93	1.51
Left ventricular diameter	-1.04	2.8	-4.94	2.86
Left ventricular posterior wall thickness	-0.61	9.9	-3.02	1.80

Interobserver agreement – observation 2	**Mean difference**		**Limits of agreement**	
Cardiac measurements, end-diastolic (millimeters)	**Millimeters**	**%**	**Lower limit**	**Upper limit**
Aortic root diameter	0.12	0.5	-3.82	4.06
Left atrial diameter	1.60	6.3	-2.93	6.13
Interventricular septal thickness	-0.31	5.3	-2.13	1.51
Left ventricular diameter	-1.35	3.6	-4.39	1.69
Left ventricular posterior wall thickness	-0.69	11.4	-2.51	1.13

T-tests were performed to compare the difference from the mean, none of the measurements was significantly different from zero.

Discussion

Echocardiography is widely used to visualize organs in children. Left cardiac structures can be measured in children with M-mode, cross-sectional echocardiography. The aim of this study was to assess the reliability of left cardiac structures, aortic root diameter, left atrial diameter, left ventricular end diastolic diameter, interventricular end-diastolic septal thickness and left ventricular end-diastolic posterior wall thickness, in young children without congenital heart disease.

Both reproducibility, whether two observers use the same measurement to obtain the same result, and repeatability, whether a single observer obtains the same results when he/she takes the repeated measurement, are types of agreement or reliability. A measurement process should be both accurate and reproducible. The mean difference between duplicate measurements is an indicator of the amount of systematic difference (relative bias) between the pairs of results. The standard deviation of the differences between duplicated measurements represents the reproducibility of the process. The larger the mean difference, the larger the systematic bias and the lower the accuracy of the process. Similarly, the larger the standard deviation, the larger random errors and the lower the reproducibility.

In this study we used various statistical methods to assess the reproducibility and repeatability of left cardiac structures in children measured by ultrasound. A simple plot of the results of one observer against the other showed that the data points are clustered near the line of equality, indicating good agreement and little differences between observers.

Beforehand, we decided that we could consider cardiac measurements in children as good reliability and valid in case of intraclass correlation coefficient over 0.80. Our results showed high intraclass correlation coefficients (over 0.80) for almost all echocardiographic measurements, with low 95% confidence intervals, indicating a high degree of similarity among and between observers and thus good agreement. Intraobserver intraclass correlation coefficients were higher (ranging from 0.99 to 0.91) than interobserver intraclass correlation coefficients (ranging from 0.96 to 0.78) for all left cardiac structures. This phenomenon, so called interobserver variability, could be expected since two observers measure differently.

The Bland and Altman plots showed good agreement among and between observers. We found for almost all echocardiographic measurements differences within 10% from the mean, so the differences are unlikely to be of clinical significance. Only for the measurement of left ventricular posterior wall thickness the difference from the mean was more than 10%. The differences were similar regardless of the mean size of the measurement, meaning that differences did not vary in any systemic way across the range of the measurement.

A few other studies measured intraobserver and interobserver variations of echocardiographic measurements in children. The reliability of pediatric echocardiographic measurements in a group children of mothers infected with the Human Immunodeficiency Virus is studied by Lipschultz *et al.*[3] They showed in a group children of mothers infected with the Human Immunodeficiency

Virus substantial variability between pediatric echocardiographic measurements made in a central echocardiography laboratory versus a local facility, although left ventricular dimension was reliably measured. Intraclass correlation coefficients ranged from 0.64 (fractional shortening) to 0.97 (left ventricular dimension).

The Project Heartbeat! showed that echocardiographic measurements in healthy children aged 8, 11 and 14 years can be performed accurately with acceptable reproducibility.[4] They presented mean differences in left ventricular mass for intraobserver variability of –1.82 (standard deviation 18.79) grams and for interobserver variability of 4.50 (standard deviation 24.16) grams. Although the children in the Project Heartbeat were older than in our study, the mean differences and standard deviations for the left cardiac structures reported were similar to the measurements in our study.

The major strength of our study is the young age at which we studied the intraobserver and interobserver variability of echocardiographic measurements. To our knowledge, previous studies of reliability of echocradiographic measurements have only been performed in older children[3, 4] and adults[10-12]. Second, two well trained sonographers performed all the echocardiographic measurements in our study, so our results are probably to a large extend dependend on the experience of these two sonographers. Currently, these sonographers work together, but they were trained in different echocardiographic laboratories according to current recommendations.[7] We expect that the reproducibility would have been lower if the ultrasound measurements were performed by sonographers working in different research centers. However, this difference should be small since all measurements were performed using strict protocols, which are used by many centers to maximize uniformity of performance. Furthermore, cardiac measurements could be measured in 100% of the participating children. None of the children started crying or wanted to quit before the measurements were finished. The sonographers put major efforts to get high quality measurements. Therefore, all echocardiograms we performed, including septal and free wall left ventricular endocardium measurements, had quality enough for tracing. Another strength of our study is that we used various statistical methods, which give a useful indication about the reliability of echocardiographic measurements in children.

A limitation could be that we only used one probe to measure the left cardiac structures at all different ages. Furthermore, the amount of children in our study is relatively low. However, other studies assessing reliability of cardiac and other ultrasound measurements were performed in similar groups and demonstrated that a total number of 30 children is large enough to evaluate reliability.[10, 12-14] Especially, in studies that are designed to assess whether ultrasound measurements are reproducible and repeatable as an outcome measure for different studies.

In conclusion, we demonstrated good repeatability and reproducibility of most left cardiac structures in children measured by ultrasound. This information is important for clinical and epidemiological research projects focused on left cardiac structures in young children.

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Chapter 4.2

Tracking and determinants of cardiac structures during early childhood

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Abstract

Background: To examine whether cardiac size and function track in early childhood and are associated with fetal and early postnatal growth and blood flow characteristics.

Methods: This study was embedded in a population-based prospective cohort study from fetal life onwards. Fetal growth and fetal and placental blood flow parameters in second and third trimester of pregnancy were measured by ultrasound and Doppler. Left cardiac structures and shortening fraction were measured postnatally at the ages of 1.5, 6 and 24 months. Analyses were based on 1,001 children.

Results: Left ventricular mass tended to remain in the lowest and highest quartiles from the age of 1.5 to 24 months (OR 1.70 (95% CI: 1.10, 2.63) and 2.15 (95% CI: 1.41, 3.30), respectively). Similar results were found for aortic root diameter and left atrial diameter. Birth weight was positively associated with aortic root diameter (0.08 mm (95% CI: 0.01, 0.17) per SD increase) and left ventricular mass (0.65 grams (95% CI: 0.09, 1.21) per SD increase). Resistance indices of the umbilical and uterine arteries showed weak tendencies towards inverse associations with left cardiac structures. Fetal cardiac output was positively associated with both left atrial diameter (increase of 1.96 mm (95% CI: 1.28, 2.64) per ml/min increase) and left ventricular mass (increase of 1.79 grams (95% CI: 0.35, 3.22) per ml/min increase).

Conclusions: This study suggest moderate tracking of left cardiac structures during the first two years and that small size and hemodynamic variations in fetal life have consequences for postnatal cardiac size and function.

Introduction

Left ventricular hypertrophy is a strong and independent risk factor of cardiovascular morbidity and mortality in adulthood.[1] Studies in children showed that left ventricular mass tracks from childhood to adulthood.[2-4] This tracking phenomenon has been demonstrated from the age of 6 years onwards.[2, 4] These studies suggest that cardiac structure and function have their origins at least partly in early life. Tracking studies of cardiac structures have not been performed in infants and preschool children.

It is not well-known which factors in early life affect left cardiac structures and function. Studies in children aged 4 to 17 years showed that overweight or obesity is associated with an increased left ventricular mass.[5] Furthermore, inverse associations between birth weight and left ventricular mass in adolescents and adults have been demonstrated.[6, 7] These findings have not been replicated in prospective cohort studies. Recently, positive associations of birth weight with left ventricular outflow structures and total coronary diameters were reported.[8] Also, maternal anthropometrics during pregnancy were positively associated with left ventricular mass in infants.[9]

Since the human heart has its highest growth and development rate in fetal and early postnatal life, cardiac size and function may be permanently affected by various factors in fetal and early postnatal life.[10] Recently, we showed that decreased fetal growth is associated with fetal adaptations in cardiac function in the whole range of fetal growth.[11] Thus far, it is not known whether such fetal cardiovascular adaptations have postnatal consequences.

We examined in 1,001 healthy children participating in a population-based prospective cohort study from early fetal life onwards whether left cardiac structures track and are associated with fetal growth and blood flow characteristics until the age of two years.

Methods

Design

This study was embedded in the Generation R Study, a population-based, prospective cohort study from fetal life until young adulthood.[12, 13] The cohort comprises 9,778 mothers and their children living in Rotterdam, The Netherlands. More detailed assessments of fetal and postnatal growth and development are conducted in a subgroup, the Focus cohort.[12, 13] In this subgroup, blood pressure and postnatal cardiac ultrasounds were performed until the age of 2 years. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants.

Population for analysis

A total of 1,232 women were enrolled in the Focus cohort. The present analysis was limited to singleton live births (n = 1,215). Twin pregnancies (n = 15) and pregnancies leading to perinatal death (n = 2) were excluded from the analysis. None of the remaining 1,198 children had congenital heart disease. A total of 74% (n = 901) participated in the postnatal assessments at the ages of 1.5 and 6 months, and 70% (n = 856) in the postnatal assessments at 24 months of age. Echocardiograms were successfully performed in 91%, 95% and 85% at the ages of 1.5, 6 and 24 months. Missing echocardiograms were mainly due to crying or unavailability of equipment or echocardiographer.

Ultrasound measurements

Fetal growth characteristics

Fetal ultrasound examinations were carried out at the research centers. The respective median (95% range) gestational ages for these visits were 12.6 weeks (9.6 – 16.9), 20.4 weeks (18.6 – 22.5) and 30.2 weeks (28.5 – 32.5). These fetal ultrasounds were used both to establish gestational age and to assess fetal growth characteristics.[14] Fetal growth measurements comprised head circumference (HC), abdominal circumference (AC) and femur length (FL) in the second and third trimester. Estimated fetal weight was calculated using the formula by Hadlock.[15] Growth measurements in early pregnancy (gestational age <18 weeks) were not included, since these fetal ultrasound examinations were performed primarily to establish gestational age. Gestational age-adjusted standard deviation scores were constructed for these fetal growth measurements.

Placental and fetal blood flow characteristics

Placental resistance was measured in second and third trimester using flow-velocity waveforms from the umbilical and uterine arteries. Increased placental resistance is indicated by higher umbilical artery pulsatility index (PI) and uterine artery resistance index (RI).[16] Cardiac flow velocity waveforms at the level of the mitral valve leaflets were recorded from the apical 4-chamber view of the fetal heart, with the sample volume placed just below the mitral valves. Peak velocities of the E wave and the A wave were recorded. The E/A ratio, which is an index for ventricular diastolic function and expresses both cardiac compliance and preload conditions, was calculated.[11] Cardiac outflow flow velocity waveforms were recorded from the aorta and pulmonary artery from the five-chamber view and the short axis view of the fetal heart just above the semilunar valves, respectively. Left- and right-sided cardiac output were calculated in milliliter per minutes by multiplying the vessel area by the time-velocity integral by fetal heart rate.[11]

Left cardiac structures

Two-dimensional M-mode and Doppler echocardiograms were performed using an ATL-Philips Model HDI 5000 (Seattle, Washington, USA) equipment when the children were aged 1.5, 6 and 24 months. The vast majority (86%) of the measurements was performed by a single echocardiographer; the other measurements were performed by two other echocardiographers. These echocardiographers were supervised by a pediatric cardiologist. Left ventricular end diastolic diameter (LVEDD), interventricular septal thickness (IVST), left ventricular posterior wall thickness (LVPWT) and shortening fraction were measured using methods recommended by the American Society of Echocardiography.[17] Left ventricular mass was computed using the formula derived by Devereux *et al.*:

Left ventricular mass = $0.80 * 1.04 ((IVST + LVEDD + LVPWT)^3 – (LVEDD)^3) + 0.6$.[18]

Other measurements of left cardiac structures were aortic root diameter and left atrial diameter. We used shortening fraction as a quantification of cardiac function.

Covariates

Weight and length were measured at the ages of 1.5, 6 and 24 months. Body surface area (BSA) was computed by use of the formula derived by Dubois *et al.*[19] Blood pressure was measured at the age of 24 months. Diastolic blood pressure was taken at the 5th Korotkoff phase. The mean of two consecutive measurements was used in the analyses. Date of birth, birth weight and gender were obtained from midwife and hospital registries.

Statistical analysis

Differences in cardiac characteristics between boys and girls were quantified using t-tests. Left cardiac structures and shortening fraction at 1.5 and 24 months of age were categorized in quartiles to assess whether children tend to remain in the same quartile during the first two years of life. For these analyses, odds ratios (OR) were calculated.

Associations of fetal growth (standard deviation scores of estimated fetal weight in second and third trimester of pregnancy and birth weight), fetal and placental blood flow characteristics (umbilical artery PI, uterine artery RI, cardiac output and mitral valve waveforms) and systolic and diastolic blood pressure with left cardiac structures and shortening fraction repeatedly measured at the ages of 1,5, 6 and 24 months were assessed using repeated measures regression analysis using the Proc Mixed module of SAS.[20] We also analysed the associations of height, weight and weight gain until two years of age with left cardiac structures and shortening fraction at each visit. Models were adjusted for gender and age at measurement. Subsequently, to take account for current anthropometrics, we adjusted for current body surface area (BSA). 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

Table 1 presents the characteristics of infants who participated in the postnatal echocardiogram studies. The percentage of boys was 52%. The overall median ages (95% range) at their visits were 1.5 months (1.0 – 2.9), 6.3 months (5.5 – 8.3) and 25.1 months (23.6 – 28.3). Table 2 shows the means and mid-95% ranges for the left cardiac dimensions at all ages. Aortic root diameter and left ventricular mass were larger in boys than in girls.

To assess reproducibility of ultrasound measurements, the intraclass correlation coefficient between and among observers were calculated in 30 subjects and varied between 0.85 and 0.99. The intraobserver intraclass correlation coefficients for the echocardiographic measurements in the present study were similar to those previously demonstrated in large-scale multicenter studies in young children.[21, 22] Bland and Altman plots showed that the difference from the mean for left cardiac structures ranged from 0 mm (0%) (left ventricular end-diastolic posterior wall thickness) to 1.60 mm (6.3%) (left atrial diameter). The limits of agreement ranged from –0.96 to 0.96 mm (left ventricular end-diastolic posterior wall thickness) to the widest limit from –5.55 to 7.55 mm (left atrial diameter).[21, 22]

Both systolic blood pressure and diastolic blood pressure at 24 months of age were not significantly associated with left cardiac structures and shortening fraction. Correlation coefficients ranged from r = 0.01 to r = 0.10. Adjustment of our models for blood pressure did not materially affect the effect estimates.

Table 1. Subject characteristics (n = 1,001)

	Boys (n = 514)	**Girls** (n = 487)	**P-value**
Second trimester fetal characteristics			
Gestational age (weeks)	20.5 (18.8-22.8)	20.4 (18.7-22.8)	0.02
Estimated fetal weight (grams)	378 (83)	370 (78)	0.12
Umbilical artery, pulsatility index (PI)	1.2 (0.2)	1.2 (0.2)	0.43
Uterine artery, resistance index (RI)	0.54 (0.1)	0.53 (0.1)	0.03
Third trimester fetal characteristics			
Gestational age (weeks)	30.5 (28.7-32.6)	30.3 (28.2-32.5)	0.05
Estimated fetal weight (grams)	1641 (259)	1624 (262)	0.32
Umbilical artery, pulsatility index (PI)	0.96 (0.2)	0.99 (0.2)	0.03
Uterine artery, resistance index (RI)	0.49 (0.1)	0.49 (0.1)	0.91
Cardiac measurements			
Left cardiac output (ml/min)	613 (172)	606 (236)	0.58
Right cardiac output (ml/min)	836 (257)	841 (236)	0.78
Mitral valve E wave	39.6 (6.3)	40.7 (6.3)	0.01
Mitral valve A wave	51.5 (7.7)	52.1 (8.2)	0.25
Mitral valve E/A ratio	0.77 (0.09)	0.79 (0.09)	0.02
Characteristics at birth			
Gestational age at birth (weeks)	40.3 (35.9-42.4)	40.3 (35.6-42.4)	0.95
Gestational age <37 weeks (%)	23 (4.5)	19 (3.9)	0.65
Birth weight (grams)	3552 (541)	3466 (540)	0.01
Birth weight <2500 grams (%)	18 (3.5)	19 (3.9)	0.11
Small for gestational age (%)	16 (3.1)	11 (2.3)	0.40
Characteristics at 1.5 weeks			
Age at visit (months)	1.5 (1.0-3.0)	1.5 (1.0-2.8)	0.60
Weight at visit (grams)	5080 (714)	4792 (649)	<0.01
Length at visit (cm)	57.4 (2.5)	56.4 (2.5)	<0.01
Characteristics at 6 months			
Age at visit (months)	6.3 (5.5-8.1)	6.3 (5.5-8.4)	0.51
Weight at visit (grams)	8202 (859)	7648 (824)	<0.01
Length at visit (cm)	69.5 (2.4)	67.8 (2.5)	<0.01
Characteristics at 24 months			
Age at visit (months)	25.1 (23.7-28.2)	25.1 (23.4-28.3)	0.42
Weight at visit (grams)	12299 (1380)	12412 (1350)	<0.01
Length at visit (cm)	89.6 (3.1)	88.4 (3.3)	<0.01
Diastolic blood pressure (mmHg)	100.8 (9.2)	102.0 (9.7)	0.13
Systolic blood pressure (mmHg)	61.5 (9.3)	62.8 (10.4)	0.10

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

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

Table 2. Cardiovascular measurements (n = 1,001)

	Boys (n = 514)	**Girls** (n = 487)	**P-value**
Age 1.5 months (n = 800)			
Heart beat (beats/min)	145 (19)	146 (20)	0.89
Left atrial diameter (mm)	16.9 (2.0)	16.5 (1.9)	0.03
Aortic root diameter (mm)	12.0 (1.3)	11.6 (1.2)	<0.01
Left ventricular mass (grams)	15.1 (3.1)	13.7 (2.8)	<0.01
Shortening fraction (%)	35.3 (4.9)	35.2 (5.1)	0.83
Age 6 months (n = 835)			
Heart beat (beats/min)	132 (16)	133 (16)	0.33
Left atrial diameter (mm)	18.0 (1.9)	17.9 (1.9)	0.57
Aortic root diameter (mm)	13.9 (1.2)	13.4 (1.2)	<0.01
Left ventricular mass (grams)	19.9 (3.9)	18.3 (3.8)	<0.01
Shortening fraction (%)	36.9 (5.3)	37.1 (4.8)	0.57
Age 24 months (n = 709)			
Heart beat (beats/min)	105 (11)	106 (10)	0.12
Left atrial diameter (mm)	20.7 (2.5)	20.5 (2.4)	0.27
Aortic root diameter (mm)	16.6 (1.5)	16.0 (1.4)	<0.01
Left ventricular mass (grams)	32.4 (5.8)	30.0 (5.1)	<0.01
Shortening fraction (%)	35.6 (5.4)	35.9 (6.8)	0.51

Values are means (standard deviation).
Differences between boys and girls were compared using independent sample t-tests.

In Table 3, odds ratios (OR) for staying in the same quartiles of left cardiac structure and shortening fraction from the age of 1.5 month to 24 months are presented. For aortic root diameter, children tend to remain in the lowest and highest quartiles from the age of 1.5 to 24 months (OR 2.49 (95% CI: 1.66, 3.73) and 3.37 (95% CI: 2.19, 5.19), respectively). We found the same trends for quartiles of left atrial diameter, left ventricular mass and shortening fraction. Our results indicate that around 60% of the subjects in the upper or lower half of the distribution tend to stay in the same part of the distribution.

Table 3. Tracking analysis of left cardiac structures and shortening fraction in quartiles from 6 weeks until 24 months of age

Aortic root diameter	Aortic root diameter 24 months				
1.5 months	1	2	3	4	Total no.
1	2.49 (1.66, 3.73) (58)	1.40 (0.92, 2.11) (39)	0.56 (0.33, 0.93) (20)	0.30 (0.16, 0.55) (39)	156
2	0.97 (0.63, 1.49) (48)	1.43 (0.96, 2.12) (47)	1.22 (0.80, 1.86) (38)	0.54 (0.33, 0.89) (55)	188
3	0.65 (0.41, 1.04) (26)	0.83 (0.54, 1.28) (35)	1.37 (0.90, 2.10) (39)	1.34 (0.89, 2.04) (54)	154
4	0.30 (0.16, 0.58) (35)	0.77 (0.47, 1.24) (39)	0.84 (0.51, 1.40) (48)	3.37 (2.19, 5.19) (97)	219
Total no.	167	160	145	245	717
Left atrial diameter	**Left atrial diameter 24 months**				
1.5 months	1	2	3	4	Total no.
1	1.47 (0.99, 2.19) (49)	1.18 (0.78, 1.79) (43)	0.85 (0.55, 1.29) (36)	0.65 (0.41, 1.03) (45)	173
2	0.58 (0.35, 0.95) (26)	1.13 (0.72, 1.78) (35)	1.33 (0.86, 2.04) (37)	1.07 (0.68, 1.69) (49)	147
3	0.95 (0.62, 1.46) (38)	0.87 (0.55, 1.36) (33)	0.92 (0.60, 1.41) (33)	1.35 (0.88, 2.05) (63)	167
4	0.83 (0.51, 1.35) (44)	0.47 (0.26, 0.83) (41)	1.0 (0.63, 1.60) (43)	2.07 (1.33, 3.21) (102)	230
Total no.	157	152	149	259	717
Left ventricular mass	**Left ventricular mass 24 months**				
1.5 months	1	2	3	4	Total no.
1	1.70 (1.10, 2.63) (40)	1.52 (0.99, 2.36) (39)	0.81 (0.50, 1.29) (20)	0.37 (0.21, 0.67) (13)	112
2	1.22 (0.78, 1.91) (35)	0.82 (0.51, 1.33) (25)	1.39 (0.90, 2.13) (31)	0.67 (0.41, 1.10) (22)	113
3	0.86 (0.54, 1.38) (30)	0.96 (0.61, 1.53) (32)	1.37 (0.89, 2.10) (32)	0.85 (0.53, 1.36) (26)	120
4	0.35 (0.19, 0.64) (14)	0.81 (0.50, 1.31) (28)	1.19 (0.70, 1.85) (28)	2.15 (1.41, 3.30) (43)	113
Total no.	119	124	111	104	458
Shortening fraction	**Shortening fraction 24 months**				
1.5 months	1	2	3	4	Total no.
1	2.02 (1.37, 2.99) (54)	1.18 (0.79, 1.75) (46)	0.52 (0.31, 0.86) (20)	0.62 (0.39, 0.99) (59)	179
2	0.94 (0.59, 1.52) (28)	1.26 (0.81, 1.95) (35)	0.91 (0.55, 1.51) (24)	0.87 (0.53, 1.41) (48)	135
3	0.87 (0.54, 1.40) (27)	1.05 (0.67, 1.63) (33)	1.01(0.62, 1.65) (25)	1.13 (0.71, 1.80) (52)	137
4	0.78 (0.48, 1.29) (46)	1.09 (0.69, 1.71) (57)	0.72 (0.42, 1.24) (46)	1.54 (0.98, 2.43) (117)	266
Total no.	155	171	115	276	717

Values are unadjusted odds ratios (95% confidence interval) (number of children in quartile) estimated by logistic regression for quartiles of left cardiac structures and shortening fraction at the ages of 1.5 months and 24 months.

Table 4 presents regression coefficients of fetal growth and blood flow characteristics with repeatedly measured left cardiac structures until the age of 24 months. Birth weight was positively associated with aortic root diameter (increase of 0.08 mm (95% CI: 0.01, 0.17) per SD increase; a change of 2.1%) and left ventricular mass (increase of 0.65 grams (95% CI: 0.09, 1.21) per SD increase, a change of 0.5%). Almost all placental hemodynamics showed weak tendencies towards inverse associations with left cardiac structures. No associations were found with shortening fraction. After additional adjustment for birth weight, we found the same effect estimates for these associations.

Fetal hemodynamics were positively related to left cardiac structures. Left cardiac output showed the strongest relation and was associated with left atrial diameter and left ventricular mass (increase of 1.96 mm (95% CI: 1.28, 2.64), a change of 9.5%; and 1.79 grams (95% CI: 0.35, 3.22), a change of 5.7%, per unit increase in left cardiac output). Right cardiac output was also associated with left atrial diameter and left ventricular mass. Mitral valve waveforms tended to be inversely associated with aortic root diameter. No associations were found with left atrial diameter, left ventricular mass and shortening fraction.

Each standard deviation score increase in weight from the age of 6 months to 24 months resulted in an increase of 0.22 mm (95% CI: 0.07, 0.38) in left atrial diameter and 0.40 grams (95% CI: 0.08, 0.72) in left ventricular mass, respectively (data not shown).

Table 4. Associations of fetal growth characteristics and placental indices in second and third trimester of pregnancy with repeatedly measured left ventricular structures until the age of 2 years

	Difference in aortic root diameter (mm)	Difference in left atrial diameter (mm)	Difference in left ventricular mass (mm)
Fetal weight (SDS)			
Second trimester	0.08 (-0.01, 0.16)	0.15 (0.02, 0.28)	0.04 (-0.57, 0.65)
Third trimester	0.06 (-0.01, 0.14)	0.02 (-0.11, 0.14)	0.16 (-0.40, 0.71)
Birth	0.08 (0.01, 0.17)	-0.03 (-0.16, 0.10)	0.65 (0.09, 1.21)
Placental hemodynamics			
Second trimester			
Umbilical artery PI	-0.62 (-1.05, -0.20)	-0.13 (-0.82, 0.56)	-0.62 (-3.92, 2.68)
Uterine artery RI	0.35 (-0.63, 1.33)	-1.43 (-2.98, 0.12)	-3.64 (-10.89, 3.61)
Third trimester			
Umbilical artery PI	-0.56 (-0.99, -0.13)	-0.52 (-1.22, 0.19)	-0.83 (-4.22, 2.55)
Uterine artery RI	-0.77 (-1.75, 0.22)	-1.69 (-3.30, -0.08)	-4.97 (-12.59, 2.65)
Fetal hemodynamics third trimester			
Cardiac measurements			
Left cardiac output (L/min)	0.31 (-0.13, 0.76)	1.96 (1.28, 2.64)	1.79 (0.35, 3.22)
Right cardiac output (L/min)	0.01 (-0.30, 0.33)	1.56 (1.08, 2.05)	1.23 (0.21, 2.25)
Mitral valve E wave	-0.02 (-0.03, -0.01)	0.01 (-0.01, 0.03)	-0.02 (-0.11, 0.07)
Mitral valve A wave	0.01 (-0.01, 0.02)	0.01 (-0.01, 0.02)	-0.02 (-0.09, 0.06)
Mitral valve E/A ratio	-1.24 (-2.00, -0.49)	0.41 (-0.82, 1.63)	0.27 (-5.60, 6.15)

Values are regression coefficients (95% confidence interval) and reflect the difference in left cardiac structure per unit increase in fetal growth and fetal blood flow characteristic. SDS, gestational age-adjusted standard deviation score; PI, Pulsatility Index. Models are adjusted for age, gender and body surface area.

Discussion

Our population-based prospective cohort study showed that cardiac size and function track during the first 2 years of life. Furthermore, birth weight and measures of placental vascular resistance and fetal cardiac output were associated with left cardiac structures.

The major strengths of our study are its prospective design from early fetal life and the size of the population-based cohort. Our analyses were based on more than 2,000 cardiac measurements. Furthermore, during the first two years of life, the effect of adverse postnatal exposures of left cardiac structures is expected to be very limited.

A limitation could be that of all children participating in the Generation R measurements at the age of 24 months, cardiac measurements could not be measured in 14% of these infants. Missing values were due mainly to crying behavior or to the unavailability of equipment or a radiographer. The effect estimates would be biased if the associations of fetal growth and blood flow characteristics with left cardiac structures differed between those included and not included in the present analyses. However, this seems unlikely.

Our study was designed to assess heart development in a relatively healthy group of children. Mean birth weight and weight at the age of two years did not differ from the normal population norms in the Netherlands. Only 37 children were born with low birth weight (lower than 2,500 grams) and 27 children did have a small size for gestational age at birth. These groups were too small for specific analyses. We expect that including more children with more variation in growth and left cardiac structures would have led to stronger effect estimates. Our findings were in line with a previous study in this population showing fetal cardiovascular adaptations in the whole range of fetal growth and not only in children with overt or clinically relevant fetal growth restriction.[11] Our findings may be not clinically relevant in childhood, but are relevant for insight in the early etiology of adult cardiovascular disease.

Children with smaller left cardiac structures in early postnatal life tend to keep their relatively smaller left cardiac structures in childhood (risks varied between 1.5 and 3.5). Tracking of left ventricular mass has well been described previously in older children.[2, 4] However, to our knowledge, our study is the first that shows tendencies for tracking of left cardiac structures from birth until the age of 2 years in such a large group of healthy children. The small but significant tracking coefficients suggest that at least part of left cardiac structures and function is established in fetal and early postnatal life.

Our study was not designed to test the “fetal nutrition hypothesis”. This hypothesis is based on inverse associations between birth weight and risk factors for cardiovascular disease.[6, 7] However, the number of studies showing inverse associations of birth with left ventricular mass is small and this association could not be replicated in a recent prospective cohort study in children.[8] Our results showed a positive association of birth weight with left ventricular mass and aortic root diameter. These findings are in line with a recent prospective cohort study showing positive associations of birth weight with total coronary artery diameter, aortic root diameter and left ventricular outflow tract diameter in children aged 9 years.[8] Recently, it has been suggested that maternal smoking in pregnancy is associated with changes in fetal cardiac dimensions and volumes.[23, 24] These studies suggested that undernutrition in third trimester of pregnancy leads to persisting increase of left ventricular structures, mediated through cardiovascular adaptive changes in utero.

All placental hemodynamics, umbilical artery pulsatility index (PI) and uterine artery resistance index (RI), tended to be inversely associated with left cardiac structures. Reduced end-diastolic flow velocities in fetal umbilical and maternal uteroplacental arteries have been

associated with increased peripheral vascular resistance.[25] This increase in peripheral arterial resistance and subsequently in fetal cardiac afterload may lead to an increase in fetal cardiac performance.[26] These fetal changes in hemodynamic stimuli may lead to persistent structural left ventricular changes. Previous studies demonstrated that during fetal growth changes occur in left ventricular diastolic filling patterns, which may be attributed to increased ventricular stiffness.[26] Our results suggest that fetal cardiac performance was positively associated with left cardiac structures in the present study. This finding may reflect both larger cardiac size in fetal and postnatal life, which may lead to higher stroke volume and cardiac output.

In conclusion, our study showed in a large population-based cohort that cardiac size and function track until the age of two years and may be partially affected by several fetal and early postnatal factors. It should be investigated whether these relations are persist during later life and are related to the development of cardiovascular disease in adulthood.

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Chapter 4.3

Maternal smoking in pregnancy, fetal arterial resistance and cardiovascular adaptations

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Abstract

Background: To unravel the mechanisms underlying the previously demonstrated associations between low birth weight and cardiovascular disease in adulthood, we examined whether maternal smoking during pregnancy leads to fetal arterial resistance adaptations and subsequently to fetal growth retardation and changes in postnatal blood pressure and cardiac development.

Methods: We performed a prospective cohort study from early fetal life onwards. Maternal smoking during pregnancy (non-smoking, first trimester smoking, continued smoking (<5 and ≥5 cigarettes/day)) was assessed by questionnaires. Third trimester placental and fetal arterial resistance indices and fetal growth were assessed by ultrasound and Doppler. Postnatal blood pressure and cardiac structures were measured at two years of age. Analyses were based on 1,120 children.

Results: First trimester smoking was not associated with third trimester placental and fetal blood flow adaptations. Continued smoking of ≥5 cigarettes/day was associated with increased resistance in the uterine, umbilical, and middle cerebral arteries and a decreased flow and diameter of the ascending aorta. Among mothers who continued smoking, estimated fetal weight at 30 weeks and birth weight were most affected in children with the highest umbilical artery resistance. Fetal arterial resistance indices were also associated with aortic root diameter and left atrial diameter.

Conclusions: Our findings suggest that fetal arterial resistance adaptations may be involved in the pathways leading from maternal smoking during pregnancy to both low birth weight and cardiovascular developmental changes in childhood in the offspring. Future studies are needed to examine whether these adaptations have cardiovascular consequences in later life.

Introduction

The developmental plasticity hypothesis suggests that various adverse intrauterine exposures lead to persistent fetal developmental adaptations. These adaptations may be beneficial on short term but have adverse consequences at birth and in postnatal life, leading to both low birth weight and cardiovascular diseases in adulthood.[1] Thus, cardiovascular disease may at least partly originate in early fetal life. This hypothesis is supported by consistent associations between low birth weight and an increased risk of hypertension and coronary heart disease in adulthood.[2-4] However, the effect size of low birth weight on blood pressure in adulthood seems to be small and the specific adverse exposures and underlying mechanisms are not known.[5]

Maternal smoking during pregnancy is the most important modifiable adverse fetal exposure in Western countries. Continued smoking during pregnancy is strongly associated with fetal growth restriction.[6, 7] This association is partly mediated by restricted blood flow in the vascular beds of the placenta, due to increased resistance of the umbilical-placental circulation.[8, 9] Fetal hemodynamic adaptations are also present across the whole range of fetal growth, even without clinical overt growth restriction.[10] Recent studies showed that maternal smoking is also associated with high blood pressure in childhood and adulthood, suggesting that fetal exposure to maternal smoking adversely affects both fetal growth and cardiovascular development.[11, 12] The associations of maternal smoking with blood pressure in the offspring may also be confounded by several environmental exposures.[13]

The present study was designed to unravel the mechanisms underlying the associations between low birth weight and the development of cardiovascular risk factors. We hypothesized that maternal smoking during pregnancy leads to increased fetal arterial resistance and subsequently to both fetal growth retardation and postnatal cardiovascular developmental changes. We examined whether maternal smoking during pregnancy leads to placental and fetal arterial resistance adaptations and whether these adaptations subsequently affect fetal growth characteristics and postnatal blood pressure and left cardiac structures in early childhood.

Methods

Design

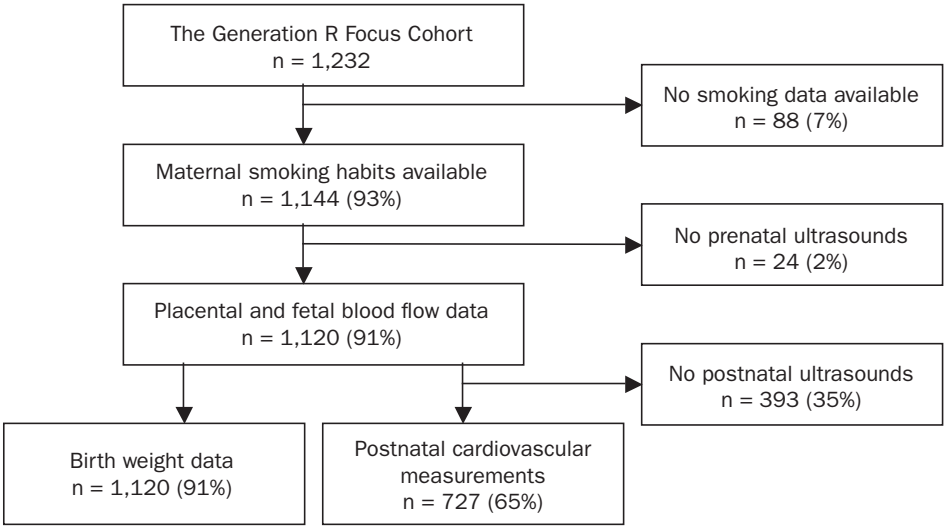
This study was embedded in the Generation R Study, a population-based, prospective cohort study from fetal life until young adulthood in Rotterdam, the Netherlands.[14] Measurements were planned in first (median 12.7 (95% range: 10.7 – 17.0) weeks of gestation), second (median 20.5 (95% range: 18.7 – 22.8) weeks of gestation), and third trimester (median 30.3 (95% range: 28.4 – 32.6) weeks of gestation) of pregnancy. Detailed assessments of fetal growth and development were conducted in a subgroup, the Focus cohort.[14] The study has been approved by

the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants.

Population for analysis

A total of 1,232 women were enrolled in the Focus cohort. Twin pregnancies (n = 15) and pregnancies leading to perinatal death (n = 2) were excluded from the analysis. None of the remaining children had major cardiac anomalies other than small ventricular septum defects (n = 3). Information on maternal smoking habits in pregnancy was available for 1,144 subjects. Ultrasound and Doppler measurements were successfully performed in 1,120 of these subjects in late pregnancy. Analyses focused on fetal arterial resistance indices were performed on these subjects. Of these 1,120 subjects, 856 subjects participated in the postnatal phase of the study of which cardiovascular measurements were available for 727 (85%) subjects at the age of two years (Figure 1).

Figure 1. Flow diagram indicating number of subject in the study



Maternal smoking during pregnancy

For the present study, information about maternal smoking was obtained by postal questionnaires sent in early and late pregnancy. Response rates for these questionnaires were 91% and 77%, respectively. At enrollment, maternal smoking was assessed in the first questionnaire by asking the mother whether she smoked during pregnancy. In the third questionnaire, the mothers were

asked whether they smoked in the past three months. According to these questionnaires, maternal smoking during pregnancy was categorized in the following three categories: non-smoking; first trimester smoking and continued smoking. Among mothers who continued smoking, the number of cigarettes smoked was categorized according to the highest amount reported in late pregnancy: non-smoking; <5 cigarettes/day; and ≥5 cigarettes/day.

Third trimester fetal arterial resistance and biometry measurements

For each measurement, three consecutive uniform waveforms were recorded by pulsed Doppler ultrasound.[10] The mean of three measurements was used for further analysis. To entirely appreciate all aspects of the fetal circulation, we integrated Doppler measurements in different vascular beds.[15] For the present study, main interest was in placental, cardiac and cerebral blood flow adaptations.

Placental vascular resistance was evaluated with recorded flow-velocity waveforms from the umbilical and uterine arteries. A raised umbilical and uterine artery pulsatility index (PI) indicates increased feto-placental and maternal-placental resistance, respectively.[16] Umbilical artery PI was measured in a free-floating loop of the umbilical cord. Uterine artery PI was measured in the uterine arteries near the crossover with the external iliac artery.

Fetal cerebral blood flow was quantified by the middle cerebral artery PI. A reduction in the middle cerebral artery PI is a valid indicator of the brain-sparing effect and fetal redistribution.[17] Middle cerebral artery was measured with colour Doppler visualization of the circle of Willis, and flow-velocity waveforms were obtained in the proximal part of the cerebral arteries.

Cardiac flow-velocity waveforms at the level of the mitral valve were recorded from the apical 4-chamber view of the fetal heart, with the sample volume placed just below the atrioventricular valve. Colour Doppler visualization of the blood flow allowed us to align the Doppler beam in the direction of the blood flow. Peak velocities of the E wave and the A wave were recorded. The E/A ratio, which is an index for ventricular diastolic function and expresses both cardiac compliance and preload conditions, was calculated. Cardiac outflow flow-velocity waveforms from the aorta were recorded from the 5-chamber view and the short-axis view of the fetal heart just above the semilunar valves. Peak systolic velocity (PSV) and the inner diameter during systole of the aorta were recorded. Left-sided cardiac output was calculated in milliliters per minutes by multiplying the vessel area by the time-velocity integral by fetal heart rate.

Fetal biometrics were measured at the same visit as the Doppler measurements and comprised head circumference (HC), abdominal circumference (AC) and femur length (FL) measured to the nearest millimeter using standardized ultrasound procedures.[18] Estimated fetal weight was calculated using the formula by Hadlock.[19]

High intraclass correlation coefficient values (>0.80) with corresponding low coefficients of variation (<10%), indicating adequate reproducibility for all Doppler measurements, have been

reported.[10] All fetal and postnatal ultrasound and Doppler examinations were performed with an ATL-Philips model HDI 5000 (Seattle, Wash) equipped with a 5.0-MHz high-frequency, curved-array transducer.

Blood pressure and cardiac measurements at the age of two years

Systolic and diastolic blood pressure were measured twice to the nearest mmHg at the left upper arm by using an automatic sphygmomanometer (Vital Signs Monitor CAS 740, CAS Medical Systems, Inc., Branford, Connecticut, USA).[20] The mean of the two readings was used for data analysis. Two-dimensional M-mode and Doppler echocardiograms were performed to measure left ventricular end diastolic diameter (LVEDD), interventricular septal thickness (IVST) and left ventricular posterior wall thickness (LVPWT) using methods recommended by the American Society of Echocardiography.[21] Left ventricular mass was computed using the formula derived by Devereux *et al.*[22] We also measured aortic root diameter and left atrial diameter. To assess reproducibility of ultrasound measurements, the intraclass correlation coefficients between and among observers were calculated in 30 subjects and varied between 0.85 and 0.99 for the main outcome measures. This is similar as previously demonstrated in large-scale multicenter studies in young children.[23,24]

Covariates

Information on birth weight and fetal sex were obtained from community midwifery and hospital registries at birth. Weight and height of the infants were measured at the age of two years.

Statistical analysis

Differences in baseline maternal and infant birth characteristics were compared between the different smoking categories. Second, overall means (standard deviation) and mid-95% ranges were computed for all third trimester fetal resistance characteristics. Associations of maternal smoking during pregnancy (non-smoking, first trimester smoking, continued smoking) with placental, cerebral and cardiac resistance characteristics in fetal life were assessed using multiple linear regression models. We also studied the effects of the number of cigarettes smoked in third trimester of pregnancy. These models were adjusted for fetal sex and gestational age at visit. Subsequently, to assess whether the effect of maternal smoking on estimated fetal weight and birth weight was mediated through fetal circulatory adaptations, we examined the associations of tertiles of umbilical artery PI with fetal and birth weight in each third trimester smoking category (no, <5 cigarettes/day, ≥5 cigarettes/day). Tests for trends were performed using linear regression models within these strata. Next, we examined whether the fetal arterial resistance characteristics, which were associated with maternal smoking in pregnancy, were also associated with blood pressure and

left cardiac structures at the age of two years. The effect estimates are presented as difference per change in standard deviation. These analyses were adjusted for age at visit, sex and current weight and height of the children. All statistical analyses were performed using the Statistical Package for the Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Table 1 presents the maternal and infant birth characteristics per category of maternal smoking during pregnancy. Mothers who continued smoking during pregnancy had children with significant smaller third trimester fetal growth characteristics and lower birth weight and more children with a birth weight <2500 grams or small size for gestational age. Table 2 shows the means (standard deviation) and mid-95% ranges for all placental and fetal cerebral and cardiac flow characteristics in third trimester of pregnancy.

Table 1. Subject characteristics (n = 1,120)

	Non-smoking (n = 843)	**First trimester smoking** (n = 104)	**Continued smoking** (n = 173)
Maternal characteristics			
Age (years)	31.7 (3.8)	30.8 (4.4)	30.4 (5.2)†
Height (cm)	171.1 (6.3)	170.7 (6.7)	169.5 (5.7)†
Weight (kg)	69.0 (12.9)	66.3 (10.3)	67.2 (10.5)
Body mass index (km/m^2)	24.4 (4.2)	24.0 (3.7)	24.6 (4.0)
Parity ≥1 (%)	39.6	27.7*	42.6
Alcohol consumption (%)			
Not in pregnancy	33.8	18.3†	32.3
Until pregnancy was known	14.7	26.0†	16.1
Continued alcohol use	51.5	55.8	51.6
Education (%)			
Lower education	32.3	41.7	33.0
Higher education	67.7	58.4	67.0
Third trimester characteristics			
Gestational age (weeks)	30.4 (28.3 – 32.8)	30.3 (28.5 – 32.6)	30.3 (28.3 – 32.5)
Head circumference (mm)	286.2 (12.1)	286.1 (11.6)	282.3 (13.0)†
Abdominal circumference (mm)	265.8 (16.7)	266.3 (17.3)	261.9 (56.1)†
Femur length (mm)	57.4 (3.1)	56.9 (2.9)	56.1 (3.0)†
Estimated fetal weight (grams)	1636 (266)	1628 (270)	1560 (256)†

Table 1. continued

	Non-smoking (n = 843)	First trimester smoking (n = 104)	Continued smoking (n = 173)
Birth outcomes			
Gender (% boys)	52.1	43.6	56.5
Gestational age (weeks)	40.3 (35.9 – 42.4)	40.1 (33.3 – 42.3)	40.1 (34.7 – 42.4)
Gestational age <37 weeks (%)	4.5	7.7	5.2
Birth weight (grams)	3550 (535)	3524 (632)	3340 (558)†
Birth weight <2500 grams (%)	3.2	4.8	6.4*
Small for gestational age (%)	2.2	2.9	6.9†

*Values are means (standard deviation) or medians (95% range) for continuous variables and percentages for categorical variables. Small for gestational age was defined as children with birth weight below –2 standard deviation for gestational age. Differences in maternal and fetal characteristics (compared with the non-smoking category) were compared using ANOVA for continuous variables and X^2 tests for categorical variables. *p-value <0.05, †p-value <0.01*

Table 2. Fetal circulation characteristics

	Number	Mean (SD)	95% range
Placental flow characteristics			
Uterine artery PI	1,033	0.75 (0.21)	0.48 – 1.31
Umbilical artery PI	1,071	0.97 (0.17)	0.68 – 1.32
Cerebral flow characteristics			
Middle cerebral artery PI	1,063	1.98 (0.33)	1.33 – 2.65
Middle cerebral artery PSV (cm/s)	1,064	42.9 (8.3)	27.7 – 60.2
Cardiac flow characteristics			
Ascending aorta diameter (mm)	984	6.4 (0.7)	5.20 – 7.90
Ascending aorta PSV (cm/s)	968	91.2 (12.4)	66.4 – 114.0
Left cardiac output (mL/min)	944	606 (174)	326 – 981
Mitral valve E wave	1,052	39.9 (6.3)	28.7 – 53.3
Mitral valve A wave	1,052	51.6 (8.0)	37.4 – 68.4
Mitral valve E/A ratio	1,060	0.78 (0.09)	0.61 – 0.99

SD, standard deviation; PI, Pulsatility Index; PSV, Peak Systolic Velocity.

First trimester smoking was not associated with third trimester placental or fetal arterial resistance characteristics. Also when first trimester smoking was categorized in two different groups (<5 cigarettes/day and ≥5 cigarettes/day), we did not find any association with arterial resistance characteristics (data not shown). Table 3 presents the associations for continued smoking with placental and fetal arterial resistance characteristics. We did not find associations with arterial resistance characteristics in the offspring of mothers who smoked <5 cigarettes/day. The offspring of mothers who smoked ≥5 cigarettes/day showed an increased umbilical artery PI (0.09 (95% CI: 0.05, 0.12)), an increased uterine artery PI (0.07 (95% CI: 0.02, 0.12)) and an increased middle cerebral artery PI (0.08 (95% CI: 0.01, 0.16)) compared to non-smokers. Furthermore, we found a decreased ascending aorta diameter and ascending aorta PSV (-0.23 mm (95% CI: -0.39, -0.07) and -3.60 cm/s (95% CI: -6.92, -0.28), respectively) and a decreased left cardiac output (-45.7 mL/min (95% CI: -87.7, -3.76)). When we applied the Bonferroni approach to adjust for multiple comparisons, all presented associations were still significant.

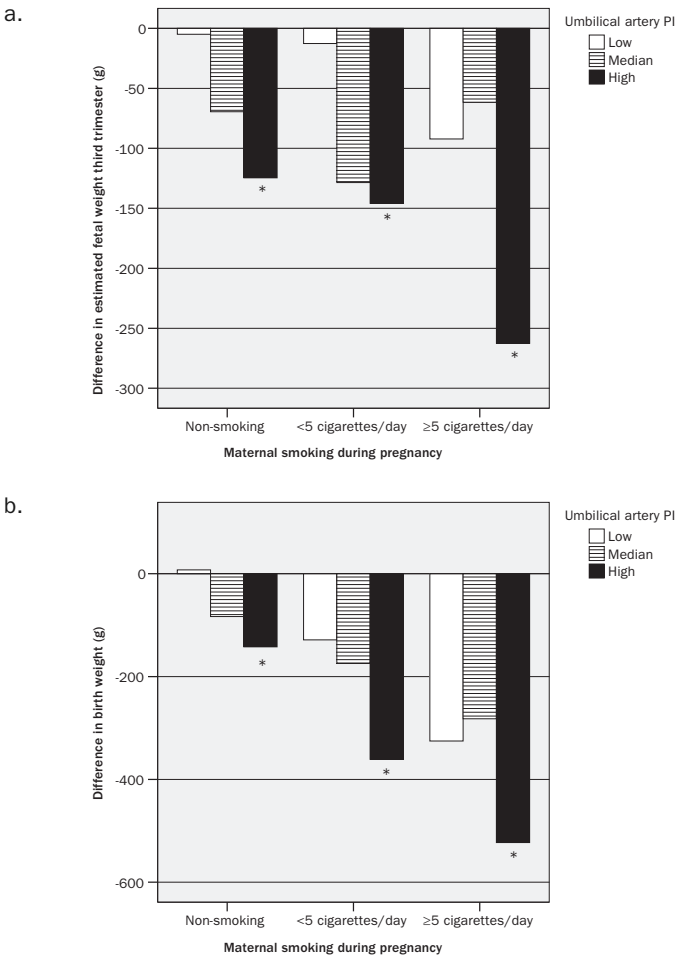
Table 3. Associations of number of cigarettes per day smoked in third trimester of pregnancy and placental and fetal cerebral and cardiac flow characteristics

	No smoking	**Continued smoking during pregnancy (95% CI)**			
		<5 cigarettes/day		**≥5 cigarettes/day**	
	(n = 843)	(n = 68)		(n = 72)	
Placental flow characteristic			(%)		(%)
Uterine artery PI	Reference	0.01 (-0.04, 0.07)	1.3	0.07 (0.02, 0.12)	9.3
Umbilical artery PI	Reference	0.01 (-0.04, 0.04)	1.0	0.09 (0.05, 0.12)	9.3
Cerebral flow characteristic					
Middle cerebral artery PI	Reference	0.03 (-0.05, 0.12)	1.5	0.08 (0.01, 0.16)	4.0
Middle cerebral artery PSV (cm/s)	Reference	1.37 (-0.55, 3.29)	3.2	1.72 (-0.03, 3.47)	4.0
Cardiac flow characteristic					
Ascending aorta diameter (mm)	Reference	-0.10 (-0.26, 0.07)	1.6	-0.23 (-0.39, -0.07)	3.6
Ascending aorta PSV (cm/s)	Reference	0.01 (-3.16, 3.18)	0.01	-3.60 (-6.92, -0.28)	3.9
Left cardiac output (mL/min)	Reference	-2.65 (-46.7, 41.4)	0.4	-45.7 (-87.7, -3.76)	7.5
Mitral valve E wave	Reference	-0.78 (-2.31, 0.75)	1.9	-1.11 (-2.57, 0.34)	2.8
Mitral valve A wave	Reference	-0.85 (-2.83, 1.14)	1.6	-1.67 (-3.55, 0.22)	3.2
Mitral valve E/A ratio	Reference	-0.01 (-0.03, 0.02)	1.3	-0.01 (-0.02, 0.03)	1.3

Values are regression coefficients (95% confidence interval) or percentages of the mean and reflect the difference in placental and fetal flow characteristic for maternal smoking in third trimester of pregnancy. PI, Pulsatility Index; PSV, Peak Systolic Velocity; CI, Confidence interval. Models adjusted for gestational age and gender.

Figure 2 presents the associations of continued third trimester smoking with estimated fetal weight and birth weight within tertiles of fetal umbilical artery PI. Smoking ≥5 cigarettes/day in third trimester of pregnancy and having an umbilical artery PI in the highest tertile resulted in a lower estimated fetal weight and birth weight (p-value <0.01) compared to the reference group (non-smoking mothers who are in the lowest tertile of umbilical artery PI). Within each smoking category, the largest effect on birth weight was found in the highest umbilical artery tertile.

Figure 2. Associations of fetal umbilical artery PI with estimated third trimester fetal weight and birth weight in each smoking category



** Tests for trend (P_{trend}) for the associations of umbilical artery PI with third trimester estimated fetal weight and birth weight within in each smoking category; p-value <0.01.*

Table 4 shows that placental and fetal arterial resistance characteristics, which were associated with maternal smoking, were not associated with postnatal systolic and diastolic blood pressure or with left ventricular mass in the offspring at the age of two years. An increased third trimester umbilical artery PI was associated with a reduced aortic root diameter. An increased middle cerebral artery PI, a decreased ascending aorta diameter and a decreased left cardiac output resulted in a decreased left atrial diameter at the age of two years (Table 4).

Table 4. Associations of placental and fetal cerebral and cardiac flow characteristics and postnatal blood pressure and left cardiac structures at the age of two years

	Blood pressure at two years of age (95% CI)		**Left cardiac size and function at two years of age (95% CI)**		
	Systolic blood pressure (mmHg)	**Diastolic blood pressure (mmHg)**	**Aortic root diameter (mm)**	**Left atrial diameter (mm)**	**Left ventricular mass (grams)**
Placental flow characteristic					
Uterine artery PI (SD = 0.21)	0.11 (-0.78, 0.10)	-0.20 (-1.11, 0.73)	-0.09 (-0.21, 003)	-0.12 (-0.33, 0.08)	-0.12 (-0.57, 0.33)
Umbilical artery PI (SD = 0.17)	0.56 (-0.33, 1.45)	-0.47 (-1.38, 0.45)	-0.17 (-0.30, -0.05)	-0.03 (-0.24, 0.18)	-0.04 (-0.49, 0.42)
Cerebral flow characteristic					
Middle cerebral artery PI (SD = 0.33)	-0.75 (-1.63, 0.14)	-0.80 (-1.71, 0.11)	-0.02 (-0.13, 0.10)	-0.18 (-0.38, -0.03)	-0.06 (-0.50, 0.38)
Middle cerebral artery PSV, cm/s (SD = 8.3)	0.23 (-0.70, 1.15)	-0.52 (-1.47, 0.42)	-0.04 (-0.16, 0.07)	0.12 (-0.10, 0.33)	0.14 (-0.32, 0.59)
Cardiac flow characteristic					
Ascending aorta diameter, mm (SD = 0.7)	0.33 (-0.53, 1.19)	0.35 (-0.55, 1.24)	0.06 (-0.06, 0.18)	0.52 (0.32, 0.72)	0.28 (-0.17, 0.73)
Ascending aorta PSV, cm/s (SD = 12.4)	-0.15 (-1.02, 0.72)	-0.69 (-1.58, 0.20)	-0.02 (-0.14, 0.10)	-0.06 (-0.27, 0.15)	-0.26 (-0.71, 0.20)
Left cardiac output, mL/min (SD = 174)	0.48 (-0.40, 1.35)	0.14 (-0.75, 1.03)	0.03 (-0.09, 0.15)	0.39 (0.18, 0.60)	0.10 (-0.36, 0.55)

Values are regression coefficients (95% confidence interval) and reflect the difference in blood pressure and left cardiac structure at the age of two years per standard deviation increase in placental and fetal cerebral and cardiac flow characteristics in third trimester of pregnancy. SD, standard deviation; PI, Pulsatility Index; PSV, Peak Systolic Velocity; CI, Confidence interval. Models are adjusted for age at visit, gender and current weight and height.

Discussion

In a large prospective cohort study, we found that continued maternal smoking during pregnancy is associated with third trimester fetal arterial resistance adaptations. Furthermore, the effect of maternal smoking during pregnancy on third trimester fetal growth is stronger in children with these fetal circulatory adaptations. The hemodynamic adaptations seem to have consequences for cardiovascular development in early postnatal life.

The major strengths of our study are its prospective design from early fetal life and the size of the cohort. Fetal resistance and flow characteristics could be measured in 99% of the mothers participating in this study in third trimester of pregnancy. Postnatal cardiac ultrasound measurements had been performed in 85% of the children participating in the visit at two years of age. A limitation is that the current study was performed in a healthy, population-based cohort study. As a consequence, generalizability is limited to children born preterm or with low birth weight. Another limitation is that we were not able to assess the effect of maternal smoking as dose-response on continuously measured number of cigarettes. This may have led to loss of power and an underestimation of the associations.

Previous studies showed inverse associations between birth weight and risk factors for cardiovascular disease. The specific adverse fetal exposures and mechanisms underlying these associations are not known. To unravel these associations, we used maternal smoking and fetal arterial resistance indices as specific exposure and underlying mechanism, respectively. We found that continued smoking during pregnancy (≥5 cigarettes/day) was associated with both an increased umbilical and uterine artery PI, indicating a higher resistance in the fetal-placental vascular bed compared to non-smokers. We did not find associations of first trimester smoking and continued smoking <5 cigarettes/day during pregnancy with placental and fetal hemodynamic adaptations. Our results suggest a dose-response effect. Smoking creates a state of chronic hypoxia resulting from both carbon dioxide in the maternal bloodstream that crosses the placenta and the vasoactive effect of nicotine.[25] A more chronic effect of smoking could be a reduction in the ability of placental vessels to dilate, due to reduced levels of prostacyclin and nitric oxide synthesis.[26] Furthermore, studies showed that smoking may reduce the dimensions of fetal capillaries in the placenta.[8, 27] Inadequate placental perfusion and function may explain the association between maternal smoking and fetal growth retardation.[16] We also found an association of continued smoking during pregnancy and an increased middle cerebral artery PI, indicating a higher resistance in the cerebral vascular bed compared to fetuses of non-smokers. This could be an indication of the brain sparing effect in these fetuses, which is usually seen in growth retarded fetuses. The increased middle cerebral artery PI might be a direct effect of nicotine, causing vasoconstriction in the vessels of the brain. Continued smoking during pregnancy was associated with a smaller diameter and lower flow of the ascending aorta, which might indicate an increased resistance in the main arterial vascular bed. This is supported by a tendency towards a lower left

cardiac output in the present study. An explanation for the decreased flow in the aorta might be smaller blood vessels as a result of suboptimal development of these vessels. Several mechanisms may lead from maternal smoking during pregnancy to increased fetal arterial resistance indices. First, impairment in elastin synthesis due to these hemodynamic changes may result in a reduced compliance of the aorta and large arteries, leading to higher pulse and mean blood pressures.[28] The process of aging causes a gradual loss of elastin and replacement by collagen, which amplifies the postnatal increase in blood pressure. Another mechanism could be that increased placental impedance will increase ventricular afterload and reduce fetal cardiac flow, which lead to a suboptimal stimulus for development of the fetal cardiovascular system.[29]

Maternal smoking during pregnancy is associated with smaller fetal growth characteristics.[7] In addition, fetal growth retardation was correlated with fetal cardiovascular adaptations.[10] We found that in mothers who continued smoking during pregnancy, estimated fetal weight and birth weight were mostly affected in children with the highest umbilical artery resistance, which implies that the effect of maternal smoking during pregnancy on fetal growth is at least partly mediated by fetal circulatory adaptations.

Eventually, persistence of the increased arterial resistance during life may predispose a person to the development of essential hypertension, left ventricular hypertrophy and cardiovascular disease in adulthood.[30] This hypothesis is supported by the finding that increased third trimester umbilical artery resistance was associated with a reduced aortic root diameter at two years of age. An increased middle cerebral artery resistance, a decreased ascending aorta diameter and left cardiac output during fetal life were also associated with a decreased left atrial diameter at the age of two years. No associations were found with blood pressure and left ventricular mass. These findings may suggest that increased arterial resistance first affects left atrium and aortic root development. Since previous studies did not show associations between maternal smoking in pregnancy and blood pressure before the age of five years, adaptations in left ventricular mass and blood pressure may still follow in a later stage.

In conclusion, we found that maternal smoking during pregnancy is associated with fetal hemodynamic adaptations consistent with increased arterial resistance. These adaptations lead both to fetal growth retardation and postnatal cardiovascular developmental changes. Future studies are needed to identify whether these fetal hemodynamic adaptations have consequences for the development of cardiovascular disease in later life.

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Chapter 4.4

Maternal anthropometrics in pregnancy and left ventricular mass in infancy

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Abstract

Background: Pregnancy and early life factors may permanently affect left ventricular growth and development in the offspring. The aim of this study was to examine the associations of maternal anthropometrics during pregnancy with left ventricular mass in infancy.

Methods: This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards. Maternal anthropometrics were obtained in early, mid- and late pregnancy. Echocardiographic follow-up measurements were performed in 791 infants aged 6 weeks and 6 months.

Results: We found no associations of maternal height, weight or body mass index with longitudinally measured left ventricular mass from 6 weeks to 6 months. Maternal weight gain until late pregnancy was associated with an increased growth of left ventricular mass from 6 weeks to 6 months (difference 0.46 grams per week for the highest tertile of weight gain compared to the lowest tertile (p-value <0.05)).

Conclusions: Maternal weight gain until late pregnancy is associated with larger left ventricular mass at the age of 6 months, suggesting that maternal health status during pregnancy may have permanent consequences for left ventricular mass in their children. Further studies are needed to identify the underlying causal mechanisms and the long-term consequences.

Introduction

Left ventricular hypertrophy is a strong and independent risk factor of cardiovascular morbidity and mortality.[1] Left ventricular mass tracks from childhood to adulthood, meaning that relative to body size, the size of the heart of a subject remains in a given rank order over time compared to other subjects.[2] The human heart has its highest growth rates in fetal and early postnatal life.[3] After this period, increase in cardiac muscle mass is mainly due to enlargement of pre-existing cells.[4] This rapid growth in fetal and early postnatal life suggests that early life factors may permanently affect cardiac growth and development.[3]

The "developmental origins of health and disease hypothesis" postulates that adverse environmental exposures in fetal and early postnatal life lead to adaptations that permanently program the fetus' structure, physiology and metabolism.[5,6] This hypothesis is supported by both animal studies and epidemiological studies, which have consistently shown associations of fetal and early postnatal growth characteristics with development of cardiovascular disease and its risk factors.[7,8] However, fetal cardiovascular adaptations may be present without overt changes in fetal and early postnatal growth characteristics.[7] Maternal anthropometrics during pregnancy are related to their nutritional and health status and thereby to fetal environmental exposures.[9,10] Several studies have shown that maternal anthropometric factors such as body mass index, weight gain during pregnancy, and insulin resistance are associated with fetal growth characteristics and adverse pregnancy outcomes.[11-15] Recently, it has also been suggested that both maternal gestational diabetes and macrosomia in children of mothers without overt gestational diabetes were associated with newborn left ventricular mass.[16] Thus, maternal nutritional and health status during pregnancy may lead to an adverse fetal environment and might affect cardiac growth and development.

We hypothesized that maternal anthropometrics during pregnancy affect cardiac growth and development in early postnatal life. Therefore, we examined in a population-based, prospective cohort study whether maternal height, weight and body mass index in various periods of pregnancy and maternal weight gain during pregnancy are associated with infant left ventricular mass growth between the ages of 6 weeks and 6 months.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. This study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail.[17,18] In total, the cohort includes 9,778 mothers and their children living in Rotterdam, the Netherlands. A vast majority of mothers were enrolled in the first

trimester of pregnancy.[17, 18] Assessments in pregnancy included physical examinations, fetal ultrasounds, biological samples and questionnaires and were planned in early (gestational age <18 weeks), mid- (gestational age 18-25 weeks) and late pregnancy (gestational age ≥25 weeks) to collect information about fetal growth and its main determinants. Their partners are assessed once during this period. Additionally, more detailed assessments of fetal and postnatal growth and development are conducted in a subgroup of 1,232 Dutch mothers and their children, referred to as the Generation R Focus cohort. This subgroup is ethnic homogeneous to exclude possible confounding or effect modification by ethnicity. Dutch ethnicity was defined as having two parents and four grandparents born in the Netherlands.[18] Between February 2003 and April 2005 all women pregnant of children who met this criterion were approached. No other exclusion criteria were used. Of all approached women, 80% agreed to participate in this subgroup study in late pregnancy (gestational age of 30 weeks). In this subgroup postnatal echocardiograms were performed in infants at the ages of 6 weeks and 6 months. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants.

Population for analysis

In total, 1,232 women were enrolled in the Generation R Focus Study at a gestational age of 30 weeks. The present analysis was limited to singleton live births (n = 1,215). Twin pregnancies (n = 15) and pregnancies leading to intra-uterine or perinatal death (n = 2) were excluded from the analysis. None of the children in this analysis had congenital heart disease. Of the singleton live births, 74% (n = 900) participated in the postnatal assessment at 6 weeks of age and 74% (n = 901) participated in the postnatal assessments at 6 months of age. Echocardiograms were performed in 88% (n = 791) of these infants at 6 weeks and in 87% (n = 785) of these infants at 6 months of age. Missing echocardiograms were mainly due to crying behavior or unavailability of equipment or echocardiographer.

Maternal anthropometrics

Maternal anthropometrics were measured in one of the research centers at the visits in early (gestational age <18 weeks), mid- (gestational age 18-25 weeks) and late pregnancy (gestational age ≥25 weeks). We measured weight (kg) and height (cm) and we calculated maternal body mass index (kg/m^2) for each pregnancy period. The gestational age medians (95% range) for these assessments were 12.6 (9.6 - 16.9) weeks, 20.4 (18.6 - 22.5) weeks and 30.2 (28.5 - 32.5) weeks, respectively. Information about maternal weight just before pregnancy was obtained from questionnaires. Self-reported weight just before pregnancy was highly correlated to measured weight at intake (r = 0.97, p-value <0.01). Weight gain was defined as the difference between weight

before pregnancy and in late pregnancy (gestational age 30 weeks). This is a measure of two trimester weight gain.

Left cardiac structure

Two-dimensional M-mode were performed on Kretz Voluson 530D equipment in the children at 6 weeks and 6 months of age. The examination was carried out in a quiet room with the baby quietly awake in a supine position. One echocardiographer performed the vast majority (86%) of these measurements. Two other echocardiographers performed the other measurements. In a parasternal long-axis view, left ventricular end diastolic diameter (LVEDD), interventricular septal thickness (IVST) and left ventricular posterior wall thickness (LVPWT) were measured using methods recommended by the American Society of Echocardiography.[19] Left ventricular mass was computed by use of the formula derived by Devereux *et al.*:

Left ventricular mass = $0.80 * 1.04 ((IVST + LVEDD + LVPWT)^3 – (LVEDD)^3) + 0.6$.[20]

The intraobserver intraclass correlation coefficients for the echocardiographic measurements in the present study were similar as previously demonstrated in large scale multicenter studies in young children.[21, 22]

Covariates

All anthropometrics in the infants were measured without clothes at the same visits as the echocardiograms at 6 weeks and 6 months of age. Weight was measured to the nearest gram using electronic scales. Length was measured in supine position to the nearest millimeter using a neonatometer. Birth weight, date of birth and gender were obtained from midwife and hospital registries.

Statistical analysis

Associations of maternal anthropometrics with repeatedly measured left ventricular mass at 6 weeks and 6 months of age were first assessed using repeated measures regression analysis using the Proc Mixed module of SAS.[23] This is a regression technique which takes the correlation of multiple measurements within one subject into account and is used to examine response trends over time.[23] Based on tertiles of weight gain until late pregnancy within our study population, we constructed three categories for increase in weight (<6.5 kg, 6.5 - 9.0 kg and ≥9.0 kg). Weight gain during pregnancy was included in the model as an interaction term with age (p-value <0.05). This model enables us to asses both the time independent and time dependent effects of weight gain on left ventricular mass. The model can be written as:

Left ventricular mass = $\beta_0 + \beta_1$*increase in weight + β_2*age + β_3*increase in weight*age.

In this model, the term including '$\beta_0 + \beta_1$' reflects the intercept and the term including 'β_2' reflects the slope of growth in left ventricular mass per week. The term including 'β_3' reflects the differences in growth of left ventricular mass between the different categories of increase in weight. First we examined the association of maternal weight, body mass index and weight gain in pregnancy with left ventricular mass in the offspring adjusted for gender (Model A). All models were additionally adjusted for current weight and current length of the children (Model B). Models with maternal weight gain as independent variable were additionally adjusted for maternal weight and height just before pregnancy (Model B).

Subsequently, we performed multiple linear regression models to study the associations of maternal anthropometrics with left ventricular mass at the ages of 6 weeks and 6 months separately. These models were first adjusted for gender and age at the visit (Model A) and additionally for current weight and current length of the children (Model B). All measures of association are presented with their 95% confidence intervals (CI). Statistical analyses were performed using the Statistical Analysis System version 8.2 (SAS, Stata corporation, College station, TX, USA), including the Proc Mixed module for unbalanced repeated measurements and the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Characteristics of infants who participated in the postnatal echocardiogram studies and their mothers are presented in Table 1. The percentage of boys was 52%. Birth weight was larger in boys than in girls. The overall median age in infants at their 6 weeks visit was 6.4 (95% range: 4.4 – 12.5) weeks and at their 6 months visit 27.3 (95% range: 23.7 – 35.6) weeks. Weight, length and left ventricular mass were somewhat smaller in girls than in boys. Infants who had a postnatal echocardiogram (n = 791) did not differ from the postnatal non-responders (n = 424) in maternal weight (difference 0.6 (95% CI: -1.1, 2.3) kg) and maternal body mass index (difference 0.2 (95% CI: -0.4, 0.7) kg/m^2) just before pregnancy. Figure 1 shows the correlations between infant characteristics and left ventricular mass.

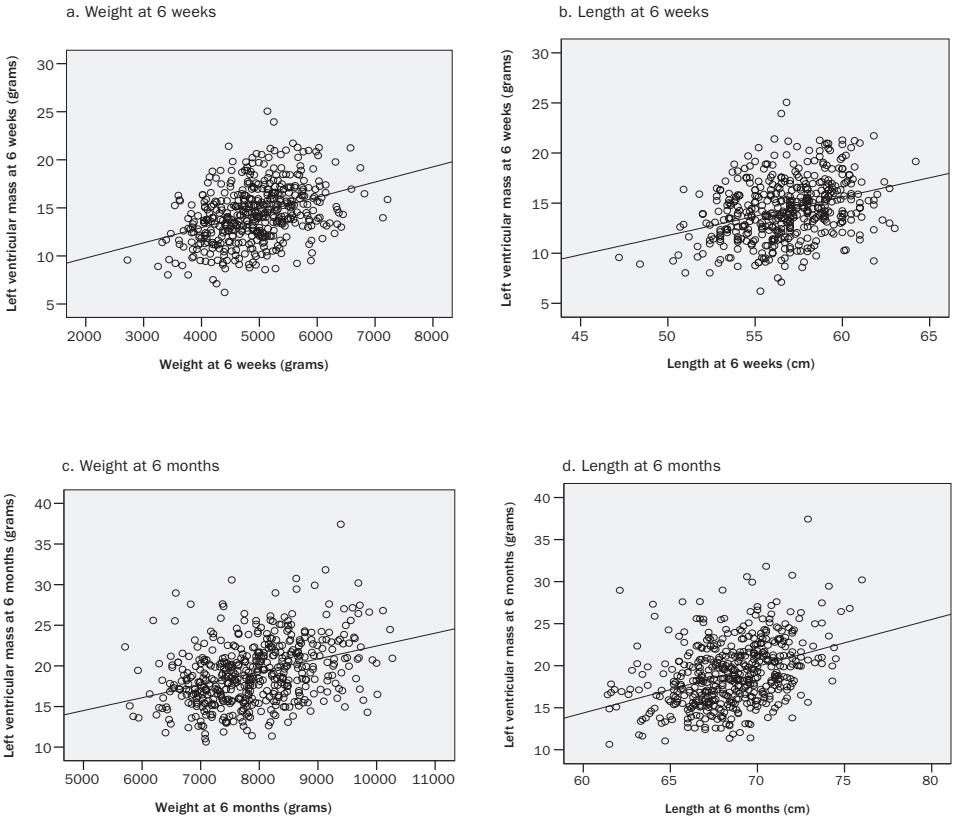
Table 1. Subject characteristics

	Boys (n = 413)	**Girls** (n = 378)	**P-value**
Pregnancy and birth characteristics			
Maternal age (years)	31.8 (4.0)	32.3 (3.8)	0.09
Maternal height (cm)	170.9 (6.0)	170.9 (6.8)	0.66
Maternal weight before pregnancy (kg)	71.0 (13.2)	71.9 (12.5)	0.20
Maternal BMI before pregnancy (kg/m^2)	24.3 (4.3)	24.6 (4.2)	0.24
Maternal weight in late pregnancy (kg/m^2)	78.9 (12.8)	79.7 (12.6)	0.38
Birth weight (grams)	3559 (535)	3463 (542)	0.05
Gestational age (weeks)	39.9 (35.3-42.1)	40.0 (35.7-42.1)	0.79
Postnatal characteristics visit 6 weeks			
Age at visit (weeks)	6.4 (4.5-12.9)	6.4 (4.4-11.9)	0.38
Weight (grams)	5088 (749)	4758 (627)	<0.01
Length (cm)	58 (3)	56 (3)	<0.01
Left ventricular mass (grams)	13.8 (2.7)	12.7 (2.5)	<0.01
Postnatal characteristics visit 6 months			
Age at visit (weeks)	27.4 (23.9-35.0)	27.3 (23.6-36.1)	0.67
Weight (grams)	8191 (840)	7628 (812)	<0.01
Length (cm)	70 (3)	68 (3)	<0.01
Left ventricular mass (grams)	18.0 (3.7)	16.4 (3.5)	<0.01

Values are means (SD) or medians (95% range) for variables with skewed distribution.

Of the total group, data were missing on maternal weight before pregnancy (n = 140), maternal body mass index before pregnancy (n = 140), weight 6 weeks (n = 14), length 6 weeks (n = 18), weight 6 months (n = 26), length 6 months (n = 26), left ventricular mass 6 weeks (n = 102) and left ventricular mass 6 months (n = 120). P-values were based on unpaired sample t-test examining the differences between boys and girls.

Figure 1. Correlations of individual measurements of infant weight and height with left ventricular mass at the ages of 6 weeks and 6 months



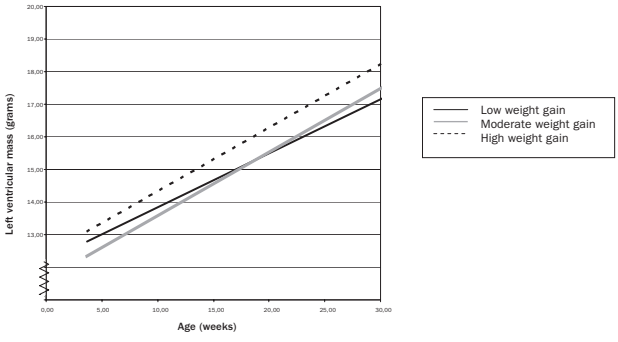
The associations between maternal anthropometrics during pregnancy and longitudinal measured left ventricular mass in infancy are presented in Table 2. No associations were found of maternal weight and body mass index in early, mid- and late pregnancy with growth of left ventricular mass. Compared to mothers with the lowest increase in weight during pregnancy, growth of left ventricular mass was 0.28 (95% CI:-0.17, 0.72) grams per 10 weeks lower in mothers with moderate increase in weight during pregnancy and 0.46 (95% CI: 0.02, 0.90) grams per 10 weeks higher in mothers with the highest increase in weight during pregnancy. Figure 2 presents the adjusted trend lines of left ventricular growth between the ages of 6 weeks and 6 months for the different categories of weight gain during pregnancy.

Table 2. Maternal anthropometrics and tertiles of weight gain in pregnancy and longitudinal measured growth in left ventricular mass

	Change in left ventricular mass (grams/10 weeks)	
	Model A	**Model B**
Early pregnancy		
Height (cm)	0.06 (0.01, 0.09)	0.01 (-0.03, 0.06)
Weight (kg)	0.01 (-0.01, 0.03)	0.01 (-0.01, 0.02)
Body mass index (kg/m^2)	0.03 (-0.02, 0.07)	0.02 (-0.02, 0.06)
Mid-pregnancy		
Weight (kg)	0.02 (0.01, 0.03)	0.01 (-0.01, 0.02)
Body mass index (kg/m^2)	0.04 (-0.01, 0.08)	0.03 (-0.01, 0.07)
Late pregnancy		
Weight (kg)	0.02 (0.00, 0.05)	0.01 (-0.01, 0.03)
Body mass index (kg/m^2)	0.04 (-0.01, 0.09)	0.03 (-0.01, 0.08)
Weight gain (kg)		
<6.5	Reference	Reference
6.5 – 9.0	-0.22 (-0.68, 0.24)	-0.28 (-0.72, 0.17)
≥9.0	0.62 (0.17, 1.08)	0.46 (0.02, 0.90)

Values are regression coefficients (95% confidence interval) and reflect the difference in growth of left ventricular mass for the different categories of maternal anthropometrics and weight gain in pregnancy. Model A: adjusted for gender; Model B: additionally adjusted for current weight and current length. Models with weight gain as independent variable are additionally adjusted for maternal weight and height just before pregnancy.

Figure 2. Weight gain in pregnancy and left ventricular mass in early infancy



Values present the adjusted trend lines of left ventricular growth between the ages of 6 weeks and 6 months for the different categories of weight gain during pregnancy. The dotted line represents the category of the highest weight gain in pregnancy and shows the steepest increase of left ventricular mass in early infancy. These data are adjusted for gender and current weight and length.

Table 3 gives the associations between maternal weight gain during pregnancy with left ventricular mass at the ages of 6 weeks and 6 months separately. We did not find an association of maternal weight gain during pregnancy with left ventricular mass at 6 weeks of age (Table 3). However, weight gain during pregnancy was positively associated with postnatal left ventricular mass at the age of 6 months. For each kg increase in weight during pregnancy, left ventricular mass at the age of 6 months increased by 0.08 (95% CI: 0.02, 0.15) grams. This means an increase of 0.47%. The associations did not materially change after adjustment for maternal weight and height just before pregnancy, and gender, age, weight and length of the child (Table 3).

Table 3. Maternal weight gain during pregnancy and left ventricular mass at 6 weeks and 6 months

	Difference in left ventricular mass 6 weeks (grams)			
	Model A		**Model B**	
Weight gain (kg)	0.06 (-0.01, 0.12)	0.45%	0.03 (-0.03, 0.09)	0.23%
	Difference in left ventricular mass 6 months (grams)			
	Model A		**Model B**	
Weight gain (kg)	0.11 (0.04, 0.18)	0.64%	0.08 (0.02, 0.15)	0.47%

Values are regression coefficients (95% confidence interval) and reflect the difference in left ventricular mass per unit weight gain in maternal weight. Model A: adjusted for maternal weight and height just before pregnancy, age and gender; Model B: additionally adjusted for current weight and current length.

Discussion

Our study demonstrates for the first time that higher maternal weight gain during pregnancy is associated with a higher increase in growth of left ventricular mass during the first 6 months of life. These findings suggest that maternal anthropometrics during pregnancy may have consequences for left ventricular mass in the offspring.

Postnatal follow-up echocardiograms were successfully obtained in 65% of all singleton live births of the mothers participating in the Generation R Focus Study. Maternal anthropometrics in pregnancy were similar between infants who had a postnatal echocardiogram and the postnatal non-responders. The observed effect estimates would be biased if the associations of maternal anthropometrics with left ventricular mass differ between those included and not included in the present analyses. This seems unlikely. The missing values of left ventricular mass among infants

who did participate in postnatal visits were mainly due to crying behavior or unavailability of equipment or echocardiographer and are unlikely to lead to biased results.

Weight gain was partly based on self-reported weights, which is known to introduce measurements error. Based on an assessment of validity of self-reported weight in the US Third National Health System, women in this age group may systematically underestimate their weights.[24] Since self-reported weight just before pregnancy was highly correlated (r = 0.97) to measured weight at intake, we do not think that this would lead to biased results.

In the present analysis, we defined maternal weight gain as the difference between weight in late pregnancy and weight just before pregnancy. In-fact this is the increase in weight in the first two trimesters of pregnancy. Ideally weight gain during pregnancy should be defined as the difference between the highest weight in pregnancy and the weight just before pregnancy. Since the largest differences in maternal weight gain during pregnancy are expected to appear in late pregnancy, our associations between weight gain and left ventricular mass may be underestimated.

No associations were found of maternal weight and body mass index in early, mid- and late pregnancy with left ventricular mass in infancy. We found that mothers who increase more in weight during pregnancy have children with an increased growth of the left ventricular mass in the first 6 months, resulting in larger left ventricular mass at the age of 6 months. These associations were independent of maternal weight just before pregnancy.

Anthropometrics during pregnancy reflect maternal nutritional and health status and may be measures of fetal environmental exposures.[9] Increased maternal weight gain during pregnancy may be caused by several factors including nutrition, obesity, fluid retention and lower physical activity levels.[25] Furthermore, increased maternal weight gain during pregnancy is associated with various pregnancy complications including insulin resistance, gestational diabetes and pregnancy-induced hypertension. Therefore, several underlying biological pathways may explain the associations between maternal anthropometrics in pregnancy and left ventricular mass in infancy. The usual increase in insulin resistance seen in late pregnancy is higher in mothers who show a marked increase in weight during pregnancy.[26] Insulin resistance causes higher glucose levels in the mother. Since glucose passes the placenta to the child, fetal insulin production will be increased. Insulin is the main fetal growth factor and may stimulate fetal body and heart growth.[27] Offspring of mothers with gestational diabetes have increased cardiac sizes in the neonatal phase.[16] However, this larger cardiac size is usually not present anymore at the age of 6 months.[28] In our study, data on sub-clinical insulin resistance or increased glucose levels during pregnancy were not available. The number of pregnant women with doctor-diagnosed gestational diabetes in our study was too small (n = 45) to assess the effect on left ventricular mass. Another biological pathway may directly be related to the nutrition intake of the mother during pregnancy.[29, 30] Excessive fetal exposure to energy sources may lead to rapid growth and cardiac enlargement. Further studies, in which measures of insulin resistance such as insulin, glucose or HbA1c levels and maternal nutrition in pregnancy have been measured, are needed to identify the underlying causal pathways.

The associations could also be explained by postnatal factors related to postnatal growth, nutrition, blood pressure development and genetic susceptibility.[31-33] We showed that the effect of weight gain on left ventricular mass was still present after adjustment for current weight. Thus we do not think that postnatal anthropometrics totally explain the associations. However, previous studies showed that in adolescents, increase in body mass index from early childhood, rather than current body mass index is associated with left ventricular mass.[31, 32] Thus, postnatal growth patterns or determinants of these growth patters, which are also associated with maternal weight gain in pregnancy may explain part of these associations. Further follow up studies in our and other cohorts may reveal these underlying mechanisms.

The effect estimates shown in our study are unlikely to be of any clinical significance in infancy. However, our results suggest an etiological role of maternal anthropometrics during pregnancy in left ventricular mass development. Increased left ventricular mass may precede the development of left ventricular hypertrophy and increased blood pressure. Left ventricular hypertrophy and hypertension are risk factors for cardiovascular morbidity and mortality.[1] Whether and to what extend the effects of maternal anthropometrics on left ventricular mass persists in childhood and in adulthood needs to be further studied.

Study implications

Our findings demonstrated that maternal anthropometrics, as measure of maternal and fetal environment, are associated with left ventricular mass in infancy. The effect sizes seem to be too small for clinical implications. However, these results are of interest from a developmental perspective and add new support for “the developmental origins of health and disease hypothesis”. Further studies examining the underlying biological pathways and the long-term consequences are needed.

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Chapter 4.5

Glucocorticoid receptor-9β polymorphism and blood pressure and heart growth in early childhood

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Abstract

Background: Glucocorticoid receptor-9β polymorphism (GR-9β, rs6198) is associated with the susceptibility for cardiovascular disease. Our aim was to examine whether the GR-9β variant is also associated with blood pressure and heart growth in early childhood.

Methods: This study was embedded in a prospective cohort study from fetal life onwards. Left cardiac structures (aortic root diameter, left atrial diameter and left ventricular mass), shortening fraction and heart rate were measured postnatally at the ages of 1.5, 6 and 24 months. Blood pressure was measured at 24 months of age. Analyses were based on 857 children.

Results: At the age of 24 months, homozygous variants showed a higher systolic blood pressure of 2.65 mmHg (95% CI: 0.16, 5.14), a higher heart rate of 9.14 beats per minute (95% CI: 0.22, 18.1) and a higher left ventricular mass of 5.01 grams (95% CI: 1.32, 8.71) compared to homozygous references. GR-9β polymorphism was significantly associated with left ventricular mass growth during the first 2 years.

Conclusions: Our findings suggest that genetically determined differences in cortisol exposure affect cardiovascular development in early life. Future studies are needed to replicate these findings and should assess whether these relations are associated with development of cardiovascular disease.

Introduction

Glucocorticoids are important regulators of cardiovascular function and metabolism. High levels of glucocorticoids results in unfavorable cardiovascular risk factors, such as visceral obesity, steroid-induced diabetes and hypercholesterolemia.[1] The effects of these hormones are mediated by glucocorticoid receptors, which show a large interindividual variation in their sensitivity.[2] Genetic variants in the glucocorticoid receptor gene (GR or NR3C1) seem to contribute to this difference in sensitivity.[3]

In humans, two isoforms of the glucocorticoid receptor exists: hGRα and hGRβ.4 hGRα resides in the cytoplasm, can bind glucocorticoids, and can alter gene transcription by binding to glucocorticoid respons elements.[5] hGRβ does not bind hormone and is transcriptionally inactive.[6] In vitro studies have shown that it can act as a dominant negative inhibitor of hGRα's transactivation properties.[7] A polymorphism in the glucocorticoid receptor gene, the GR-9β variant (rs6198), has been identified that is associated with cortisol sensitivity. A few studies demonstrated associations of the GR-9β variant with a reduced risk of bacterial colonization with Staphylococcus aureus in the nose[8] and an increased susceptibility to rheumatoid arthritis and a more aggressive disease course in multiple sclerosis.[9,10] Recently, it was demonstrated in a middle-aged population-based cohort in The Netherlands that this polymorphism of the glucocorticoid receptor gene is associated with the risk of myocardial infarction and coronary heart disease.[11] Variation in cortisol sensitivity may not only harm glucocorticoid treatment of immune related diseases, but may also directly affect cardiovascular growth and development in the fetus and subsequently increase the risk of cardiovascular disease in adulthood.[12,13] It was shown in sheep fetus that cortisol treatment resulted in an increase in cardiac mass without myocyte enlargement, indicating that cortisol stimulates hyperplastic growth of cardiomyocytes but not hypertrophic growth.[14] It is unknown what the effect is of variation in cortisol levels on fetal and early postnatal cardiovascular growth and development. The relative effect of variants of the glucocorticoid receptor gene on blood pressure and cardiac structures might be larger in childhood, when the effect of various environmental factors, such as life style habits, is limited.

We hypothesize that the GR-9β variant of the glucocorticoid receptor gene affects blood pressure and cardiac growth in early life. These early cardiovascular changes may predispose the individual to cardiovascular disease in adulthood. Therefore, we studied in a prospective cohort study from fetal life onwards the effects of the GR-9β polymorphism of the glucocorticoid receptor gene on blood pressure and left cardiac structures and function during the first two years of life.

Methods

Design

This study was embedded in the Generation R Study, a prospective cohort study from fetal life until young adulthood in Rotterdam, the Netherlands.[15, 16] Assessments in pregnancy included physical examinations, fetal ultrasounds, biological samples and questionnaires. More detailed assessments of fetal and postnatal growth and development are conducted in a subgroup, the Focus cohort.[15,16] The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants.

Population for analysis

A total of 1,232 women were enrolled in the Focus cohort. The present analysis was limited to singleton live births (n = 1,215). Twin pregnancies (n = 15) and pregnancies leading to perinatal death (n = 2) were excluded from the analysis. None of the remaining 1,198 children had congenital heart disease. DNA was collected from cord blood and available for 884 subjects. Reasons for nonavailability of DNA were mainly logistic constraints for blood collection at birth. Genotyping was successfully performed in 857 subjects. Of these children, a total of 88% (n = 752) participated in the postnatal assessments at 1.5 and 6 months of age, and 84% (n = 719) participated in the postnatal assessments at 24 months of age. Echocardiograms were successfully performed in 88%, 89% and 84% at 1.5, 6 and 24 months of age. Missing echocardiograms were mainly due to crying or unavailability of equipment or echocardiographer.

Genotyping

All participants were genotyped for the GR-9β polymorphism.[3, 17, 18] Genotyping of the GR-9β polymorphisms was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C (15 minutes), then 40 cycles of 94° C (15 seconds) and 60° C (1 minute)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 97% of the samples for this genotype. To confirm the accuracy of the genotyping results, 276 randomly selected samples were genotyped for a second time with the same method. The error rate was less than 1%. Overall, the distribution of the GR-9β genotypes in our study sample was 75.1% homozygous reference (AA), 23.5% heterozygous (AG) and 1.4% homozygous variant (GG) for the polymorphism. In our study sample, the minor allele frequency of the glucocorticoid receptor-9β allele was 13.1%.

Genotype and allele frequencies were according to the Hardy Weinberg equilibrium (p-value = 0.32).

Blood pressure and cardiac measurements

At 24 months of age, systolic and diastolic blood pressure were measured twice to the nearest mmHg at the left upper arm by using an automatic sphygmomanometer (Vital Signs Monitor CAS 740, CAS Medical Systems, Inc., Branford, Connecticut, USA).[19] The mean of the two readings was used for data analysis. Blood pressure was successfully measured in 577 subjects. In 122 children, it was not possible to measure blood pressure twice due to crying and oppositional behaviors. Thus, their blood pressure was based on one measurement.

Two-dimensional M-mode and Doppler echocardiograms were performed using an ATL-Philips Model HDI 5000 (Seattle, Washington, USA) equipment when the children were aged 1.5, 6 and 24 months. The vast majority (86%) of the measurements was performed by a single echocardiographer; the other measurements were performed by two other echocardiographers. Left ventricular end diastolic diameter (LVEDD), interventricular septal thickness (IVST), left ventricular posterior wall thickness (LVPWT) and shortening fraction were measured using methods recommended by the American Society of Echocardiography.[20] Left ventricular mass was computed using the formula derived by Devereux *et al.*[21] Other measurements of left cardiac structures were aortic root diameter and left atrial diameter. We used shortening fraction and heart rate as a quantification of cardiac function.

To assess reproducibility of ultrasound measurements, the intraclass correlation coefficient between and among observers were calculated in 30 subjects and varied between 0.85 and 0.99 for the main outcome measures. This is similar as previously demonstrated in large-scale multicenter studies in young children.[22, 23]

Covariates

The infants' anthropometrics, including weight and length, were all measured at the ages of 1.5, 6 and 24 months. Date of birth, birth weight and gender were obtained from midwife and hospital registries.

Statistical analysis

Differences in subject characteristics were quantified between the different genotypes. Associations of the GR-9β genotype with blood pressure measured at 24 months and cardiac structures (aortic root diameter, left atrial diameter, left ventricular mass) and function (heart rate, shortening fraction) repeatedly measured at the ages of 1.5, 6 and 24 months were assessed using regression

models. These models were adjusted for gender and age. Height and weight at two years of age were correlated with left ventricular mass at the same age ($r = 0.39$ and $r = 0.39$, $p < 0.001$). We found no significant correlations between the genetic variant and height and weight until the age of two years. Adjustment for anthropometrics measured at the visit of the cardiac ultrasound did not materially change the effect estimates in the associations of the glucocorticoid receptor-9β polymorphism with left cardiac structures. Therefore, weight and length were not included in the regression models. Furthermore, since population genotype distribution is assumed to be unrelated to covariates such as breastfeeding, they were not included in the models.[24]

Second, for the repeatedly measured cardiac structures and function, we performed repeated measures regression analysis using the Proc Mixed module of SAS, which takes the correlation of multiple measurements within one subject into account, allows for incomplete outcome data and assesses both the time independent and time dependent effects of the GR-9β genotype on left cardiac structures and function.[25] The GR-9β polymorphism was included in the model as intercept and as interaction term with age (p-value = 0.005). Also, these models were adjusted for age at visit and gender. Other covariates including weight, length and gestational age at birth did not change the effect estimates and were therefore not included in the model. The final model can be written as:

$$\text{Cardiac measurement} = \beta_0 + \beta_1 * \text{GR-9}\beta \text{ polymorphism} + \beta_2 * \text{age} + \beta_3 * \text{GR-9}\beta \text{ polymorphism} * \text{age} + \beta_4 * (1/\sqrt{\text{age}}).$$

In this model, the term including '$\beta_0 + \beta_1$' reflects the intercept and the term including 'β_2' reflects the slope of growth in left cardiac structure per month. The term including 'β_3' reflects the differences in growth of cardiac measurement between the different genotypes of the GR-9β polymorphism. Again, we compared the homozygous variants and the heterozygous to the homozygous references. In addition, to study the dominant effect of the GR-9β polymorphism, we merged the group heterozygous and homozygous variant subjects and performed the same analyses.

Results

Table 1 presents the baseline characteristics of infants who participated in the postnatal echocardiogram studies. The percentage of boys was 52%. The overall median ages (95% range) in infants at their visits were 1.5 months (1.0 – 2.9), 6.3 months (5.5 – 8.3) and 25.1 months (23.6 – 28.3). At all ages, aortic root diameter, left ventricular diameter and left ventricular mass were larger in boys than in girls.

Table 1. Subject characteristics (n = 857)

Subject characteristics	Homozygous reference (n = 644)	Heterozygous (n = 201)	Homozygous variant (n = 12)	P-value
Characteristics at birth				
Gestational age at birth (weeks)	40.0 (36.4-42.3)	40.3 (36.7-42.3)	40.1 (36.7-42.6)	
Birth weight (grams)	3529 (515)	3578 (463)	3225 (711)	0.05
Characteristics at 24 months				
Age at visit (months)	25.1 (23.6-28.6)	25.0 (23.5-27.8)	25.7 (24.2-29.0)	0.10
Weight at visit (grams)	12,669 (1,428)	12,590 (1,278)	13,500 (845)	0.19
Length at visit (cm)	89.1 (3.3)	88.7 (3.2)	90.2 (3.0)	0.31
Cardiovascular measurements				
Systolic blood pressure (mmHg)	101.0 (9.5)	100.1 (10.1)	103.7 (6.9)	0.04
Diastolic blood pressure (mmHg)	62.3 (9.6)	62.3 (10.7)	62.6 (4.9)	0.99
Heart beat (beats/min)	105.4 (10.7)	106.0 (11.1)	114.5 (27.0)	0.07
Aortic root diameter (mm)	16.3 (1.5)	16.5 (1.4)	16.6 (0.7)	0.43
Left atrial diameter (mm)	20.5 (2.5)	20.8 (2.4)	21.0 (2.5)	0.46
Left ventricular diameter ED (mm)	31.3 (2.5)	31.7 (2.4)	31.7 (2.3)	0.34
Interventricular septal thickness ED (mm)	4.9 (0.8)	4.8 (0.7)	5.5 (0.9)	0.04
Left ventricular posterior wall thickness ED (mm)	4.6 (0.8)	4.8 (0.9)	5.0 (0.9)	0.01
Left ventricular mass (grams)	31.1 (5.8)	32.4 (5.7)	36.1 (3.9)	<0.01
Shortening fraction (%)	35.4 (4.8)	35.3 (4.6)	38.1 (5.3)	0.18

Values are means (standard deviation) or medians (95% range). Differences between boys and girls were compared using ANOVA tests.

Table 2 shows that systolic blood pressure at the age of 24 months was higher in children with the homozygous variant of the GR-9β genotype with 2.65 mmHg (95% CI: 0.16, 5.14) compared to homozygous references, indicating an increase of 2.6%. No association was found with diastolic blood pressure.

Table 2. Associations of the GR-9β polymorphism of the glucocorticoid receptor gene with cross-sectionally measured systolic and diastolic blood pressure at the age of 24 months

GR-9β genotype	**% of persons**	**Systolic blood pressure (mmHg) (Difference (95% CI))** (n = 486)	**Diastolic blood pressure (mmHg) (Difference (95% CI))** (n = 486)
Homozygous reference	76.4	Reference	Reference
Heterozygous	22.2	-0.91 (-9.30, 7.45)	-0.01 (-2.08, 2.06)
Homozygous variant	1.4	2.65 (0.16, 5.14)	0.28 (-7.09, 7.64)

Values mean differences in systolic and diastolic blood pressure compared to the reference group. Models are adjusted for age and gender; 95% CI, 95 % confidence interval.

Table 3 shows that GR-9β genotype was not associated with left cardiac structures and function at the ages of 1.5 and 6 months. At the age of 24 months, GR-9β homozygous variants showed a raised heart rate (9.14 (95% CI: 0.22, 18.1) beats per minute, indicating an increase of 8.6%). Left ventricular mass was not only higher in homozygous variants (difference 5.01 (95% CI: 1.32, 8.71) grams compared to homozygous references), but also in heterozygous subjects (difference 1.32 (95% CI: 0.27, 2.37) grams compared to homozygous references). This means an increase of 16% and 4.2%, respectively. Subsequently, we examined the effect of the combined group (heterozygous and homozygous variant subjects) on the main findings of our study and compared these dominant to the recessive models. In the dominant models, only the association between the GR-9β genotype with left ventricular mass was still significant (p-value = 0.01). These results suggest a recessive effect of the GR-9β polymorphism. When we applied Bonferroni approach our presented associations were still significant.

Table 3. *Associations of the GR-9β polymorphism of the glucocorticoid receptor gene with cross-sectionally measured left cardiac structures and function at the age of 1.5, 6 and 24 months*

GR-9β genotype	% of persons	Heart beat (beats/min) (Difference (95% CI))	Aortic root diameter (mm) (Difference (95% CI))	Left atrial diameter (mm) (Difference (95% CI))	Left ventricular mass (grams) (Difference (95% CI))	Shortening fraction (%) (Difference (95% CI))
		Left cardiac structures and function at 1.5 months of age (n = 752)				
Homozygous reference	76.2	Reference	Reference	Reference	Reference	Reference
Heterozygous	22.4	-1.08 (-4.79, 2.63)	0.10 (-0.12, 0.32)	-0.13 (-0.50, 0.23)	0.31 (-0.98, 0.37)	-0.26 (-1.24, 0.72)
Homozygous variant	1.4	1.11 (-12.4, 14.6)	0.26 (-0.62, 1.13)	1.25 (-0.19, 2.68)	1.21 (-1.38, 3.79)	-1.53 (-5.28, 2.21)
		Left cardiac structures and function at 6 months of age (n = 752)				
Homozygous reference	74.6	Reference	Reference	Reference	Reference	Reference
Heterozygous	23.9	0.41 (-9.63, 10.4)	0.16 (-0.63, 0.95)	0.09 (-0.25, 0.44)	0.07 (-0.70, 0.83)	-0.32 (-1.29, 0.62)
Homozygous variant	1.5	-2.44 (-5.43, 0.55)	0.21 (-0.01, 0.43)	0.38 (-0.86, 1.62)	0.68 (-2.33, 3.68)	2.35 (-1.34, 6.21)
		Left cardiac structures and function at 24 months of age (n = 719)				
Homozygous reference	76.4	Reference	Reference	Reference	Reference	Reference
Heterozygous	22.2	0.62 (-1.87, 3.12)	0.15 (-0.13, 0.42)	0.27 (-0.21, 0.76)	1.32 (0.27, 2.37)	-0.07 (-1.37, 1.24)
Homozygous variant	1.4	9.14 (0.22, 18.1)	0.25 (-0.72, 1.23)	0.52 (-1.21, 2.25)	5.01 (1.32, 8.71)	2.68 (-1.87, 7.23)

Values mean differences in left cardiac structures and function compared to the reference group. Models are adjusted for age and gender; 95% CI, 95 % confidence interval.

Figure 1 presents the adjusted values of left ventricular mass between the ages of 1.5 months and 24 months for the different genotype groups of the GR-9β polymorphism of the glucocorticoid receptor gene. Values are based on repeated measurements regression analysis. Homozygous variants showed a significantly higher increase in left ventricular mass compared to homozygous references (difference 2.62 (95% CI: 0.46, 4.79) grams per month). No associations were found with the other cardiovascular outcomes.

Table 4 shows associations of the GR-9β variant with the left ventricular dimensions at the age of 24 months. A significant trend in larger interventricular septal thickness and left ventricular posterior wall thickness was seen in children homozygous for the GR-9β genotype.

Figure 1. GR-9β polymorphism and left ventricular mass until the age of 24 months

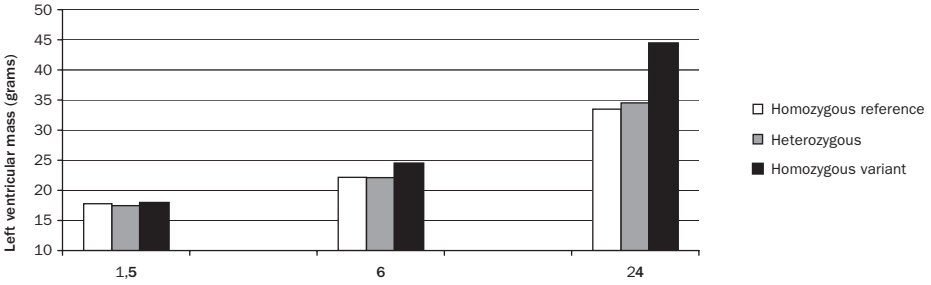

Figure 1 presents the adjusted values of left ventricular mass for the different genotypes of the GR-9β polymorphism. Values are based on repeated measurements regression analysis.

Table 4. Associations of the GR-9β polymorphism of the glucocorticoid receptor gene with cross-sectionally measured left cardiac dimensions at the age of 24 months

		Left cardiac structures at 24 months of age (n = 719)		
GR-9β genotype	**% of persons**	**Left ventricular diameter ED (mm) (Difference (95% CI))**	**Interventricular septal thickness ED (mm) (Difference (95% CI))**	**Left ventricular posterior wall thickness ED (mm) (Difference (95% CI))**
Homozygous reference	76.4	Reference	Reference	Reference
Heterozygous	22.2	0.44 (-0.01, 0.88)	-0.06 (-0.22, 0.09)	0.19 (0.02, 0.36)
Homozygous variant	1.4	0.44 (-1.12, 2.01)	0.58 (0.04, 1.11)	0.43 (-0.17, 1.02)
		P_{trend} = 0.34	P_{trend} = 0.04	P_{trend} = 0.01

Values mean differences in left cardiac dimensions compared to the reference group.
Models are adjusted for age and gender; 95% CI, 95 % confidence interval.

Discussion

In our prospective cohort study we found that the GR-9β polymorphism (rs6198), which has previously been shown to be related to myocardial infarction and coronary heart disease, is associated with higher systolic blood pressure and higher left ventricular mass at the age of 24 months. No associations were found for the other cardiac measurements.

The major strengths of our study are its prospective design from early fetal life and the size of the cohort. Furthermore, the relative effect of variants of the glucocorticoid receptor gene on blood pressure and cardiac structures might be larger in childhood, when the effect of various environmental factors, such as life style habits, is limited. A limitation could be that not in all children participating in the Generation R measurements cardiac characteristics could not be measured. The effect estimates would be biased if the associations of GR-9β polymorphism with blood pressure and left cardiac structures differed between those included and not included in the present analyses. This seems unlikely. We had 12 homozygous variant subjects for the GR-9β polymorphism in the study. Although we recognize this limitation, to our knowledge our study has the largest study group examining these associations in this age group so far. Furthermore, the distribution of genotype frequencies did not significantly differ between children who could and who could not be phenotyped. A third possible limitation is that the current study was designed to assess heart development in a healthy, prospective cohort study. Thus generalizability is limited to children born preterm or with low birth weight.

This is the first study that showed a higher systolic blood pressure, heart rate and left ventricular mass in carriers of the GR-9β genotype at such a young age. This is in line with a previous study in older-aged persons that found an association between the GR-9β polymorphism and an increased risk of cardiovascular disease.[11] They concluded that the GR-9β polymorphism, which is related to a diminished suppressive effect of cortisol on the pro-inflammatory system, is associated to the risk of myocardial infarction and coronary heart disease.[11] This finding was supported by higher levels of hs-CRP and Interleukin-6, suggesting an inflammatory pathogenesis of cardiovascular disease. Recently, Kumsta *et al.* showed that in the glucocorticoid receptor gene the rs10482605 Single Nucleotide Polymorphism (SNP) is in high linkage disequilibrium (r^2 = 0.915) with the A/G SNP in exon 9β (rs6198).[26] They suggest that these two functional SNPs have additive effects, leading to a relative glucocorticoid resistance on systemic level. The rs10482605 SNP may also be involved in the associations we found and will be responsible for at least part of the effect. Further research is needed to study the functional characteristics of this particular SNP.

We found strong effects of the GR-9β genotype on blood pressure and left ventricular mass at 2 years of age. These associations were not yet significant at the ages of 1.5 and 6 months. Results of our repeated measurements regression analyses suggest that the GR-9β genotyping affects cardiac growth during the first 2 years, which leads to a significantly higher left

ventricular mass at 2 years of age. We hypothesized a priori that the glucocorticoid receptor-9β polymorphism affected cardiac growth and thus the effect increased over time. Entering the interaction term of age with genotype in our repeated measurements models was prespecified. The significant interaction term of genotype and age ($p = 0.005$) confirmed this increasing effect. The GR-9β variant is described to be associated with a decreased sensitivity to glucocoticoids. Recently is shown that cortisol stimulates cell cycle activity in the cardiomyocyte of the sheep fetus.[14] Thus higher exposure to glucocorticoids in fetal and early postnatal life might lead to higher blood pressure and left ventricular mass in early childhood. Furthermore, a study in 4-wk old rats concluded that reduced ventricular weight and systolic dysfunction found in 4-weeks-old rats after neonatal dexamethasone treatment is transient, presumably due to compensatory cellular hypertrophy. However, they found that depressed systolic function became manifest in elderly animals, showing evidence of compensatory dilatation to maintain normal cardiac output.[27] It is unknown whether these results are applicable to humans. A study in three American ethnic groups reported a decreased systolic blood pressure in homozygous variants of the GR-9β polymorphism (GG) compared to homozygous references (AA) (difference of 16 mmHg; p-value = 0.006).[28] To our knowledge, this association has not been replicated in other studies. We found an association between the GR-9β genotype and a higher blood pressure and left ventricular mass. We studied a relatively healthy group of young European children, in which the GR-9β polymorphism may have different cardiovascular effects. Furthermore, environmental factors during pregnancy or during early life could also interact with genetically determined glucocorticoid sensitivity and affect the development of blood pressure. Since results seem inconsistent and underlying pathways are not known, future research is needed. The cardiovascular adaptations we found in this study might have consequences in later life. Blood pressure and heart beat are both related to the GR-9β genotype and to left cardiac structures. However, adjustment for cardiovascular function (i.e. blood pressure and heart beat) did not change the effect estimates in the associations of the GR-9β genotype and left cardiac structures and dimensions. Changes in systolic blood pressure and heart rate may be a first sign of the clinical consequences of this cardiac remodelling. Further studies are needed to identify these pathways.

In conclusion, we showed in a large prospective cohort that the GR-9β polymorphism of the glucocorticoid receptor gene influences systolic blood pressure and left cardiac dimensions at young age. Furthermore, we found a trend of an increasing effect during the first years of life. These results support our a priori hypothesis but should be replicated in other cohorts. Furthermore, follow-up studies are needed to assess whether these relations persist during later life and whether this genetic variant is indeed related to an increased risk of cardiovascular disease in adulthood.

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Chapter 4.6

Hypertensive disorders of pregnancy and offspring blood pressure

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Abstract

Background: Offspring of women who develop hypertensive disorders of pregnancy are at increased risk of cardiovascular complications later in life, but the mechanisms underlying these associations are unclear. The aim of this study was to examine the role of maternal and offspring adiposity as explanations for the association of hypertensive disorders of pregnancy with variation in blood pressure in the offspring.

Methods: Using data from 6,668 nine-year-old participants of the Avon Longitudinal Study of Parents and Children, we examined the association between hypertensive disorders of pregnancy (preeclampsia and gestational hypertension) and offspring blood pressure and adiposity measures.

Results: Maternal body mass index (BMI) was positively associated with both preeclampsia and gestational hypertension. Preeclampsia was associated with lower birth weight and increased risk of preterm delivery, whereas gestational hypertension was not associated with either of these outcomes. Gestational hypertension was associated with greater offspring adiposity at age 9 (assessed by BMI, waist circumference and fat mass) and with greater risk of obesity, but these associations all attenuated towards the null with adjustment for maternal and paternal BMI. Preeclampsia was inversely associated with offspring lean mass at age 9 in unadjusted and all multivariable models, it was also inversely associated with BMI, waist circumference, fat mass and obesity after adjustment for maternal BMI.

Preeclampsia and gestational hypertension were both associated with systolic and diastolic blood pressure in the nine-year-old offspring, with associations being of similar magnitude in the confounder adjusted models (including adjustment for maternal and paternal BMI and offspring adiposity at age 9): mean difference in systolic blood pressure of 2.05 mmHg (95% CI: 0.72, 3.38) and 2.04 mmHg (95% CI: 1.42, 2.67), respectively for preeclampsia and gestational hypertension, compared to those with no hypertensive disorders of pregnancy, with equivalent results for diastolic blood pressure being 1.00 mmHg (95% CI: -0.01, 2.10) and 1.07 mmHg (0.60, 1.54). The association of preeclampsia with offspring systolic and diastolic blood pressure attenuated towards the null with further adjustment for birth weight and gestational age, whereas these adjustments did not attenuate the association of gestational hypertension with offspring blood pressure.

Conclusions: The associations of hypertensive disorders of pregnancy with higher offspring blood pressure in later life do not appear to be explained by familial adiposity. Our findings suggest that different mechanisms underlie the association of preeclampsia and gestational hypertension with offspring blood pressure, with some evidence that the former is in part mediated by the effect of preeclampsia on intrauterine growth restriction and preterm delivery.

Introduction

Hypertensive disorders of pregnancy, consisting of preeclampsia and gestational hypertension, are the most common complications of pregnancy and associated with adverse health outcome for mother and her offspring. Preeclampsia, diagnosed by elevated blood pressure and poteinuria after 20 weeks of gestation in women with no previous history of hypertension, occurs in about 2-7% of otherwise healthy nulliparous women.[1-3] It is characterized by abnormal vascular response to placentation resulting in increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation system, and endothelial cell dysfunction.[4] It is a major cause of perinatal deaths, preterm birth, and intrauterine growth restriction.[2, 5, 6] Gestational hypertension, is defined as elevated blood pressure after 20 weeks of gestation in women with no previous history of hypertension, with no proteinuria. It has been found to occur in 6-17% of otherwise healthy pregnancies and is associated with preterm delivery and small for gestational age infants.[1, 3] Preeclampsia and gestational hypertension are sometimes considered to be different manifestations of the same underlying condition, differing only by severity and they do share several key risk factors, including maternal obesity.[7, 8] However, there are also important differences in their etiology.[1] For example, primiparity is described to be independently associatiated with preeclampsia, but not with gestational hypertension.[9]

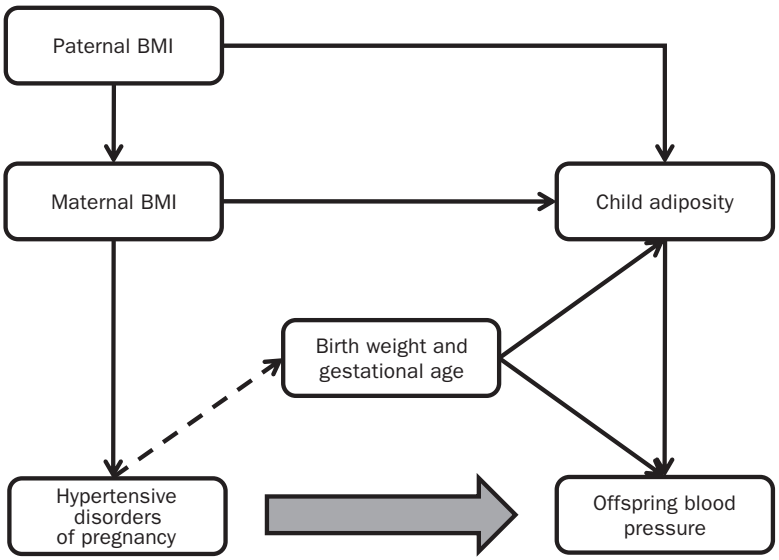
Maternal preeclampsia and gestational hypertension have been associated with higher blood pressure in the offspring during childhood and adolescence. Palti and Rothschild reported that 6-year-old boys, but not girls, born to mothers with preeclampsia had higher diastolic blood pressures than their control children born to mothers without preeclampsia.[10] By contrast Seidman *et al.* concluded that 17-year-old girls from mothers with preeclampsia had higher systolic and diastolic blood pressures than their control females, whereas only the mean systolic blood pressure differed between the males at the same age.[11] Reports of gender differences such as these are often chance findings and two further studies that have reported positive associations of hypertensive disorders of pregnancy with offspring blood pressure in childhood reported no gender differences.[12-14] In the second of these studies there were positive associations with both random blood pressure and 24 hour blood pressure in the offspring.[13, 14]

The underlying mechanisms explaining the associations of gestational hypertension and preeclampsia with offspring blood pressure remain unclear and to our knowledge previous studies have not examined whether associations differ between preeclampsia and gestational hypertension. The familial clustering of preeclampsia is well-known[15, 16] and evidence suggests that both maternal and fetal (from paternal genes) genes contribute to the risk of preeclampsia.[17-19] In one of the largest family studies to examine this, Cnattingius *et al.* concluded that genetic factors account for more than half of the liability of preeclampsia, and that maternal genes contribute more than fetal genes.[18] Furthermore, the similar association of maternal (non-pregnant) and paternal blood pressure with offspring blood pressure, suggest shared familial genetic or lifestyle characteristics

underlie familial clustering of high blood pressure risk in general, rather than a strong intrauterine mechanism.[19]

Given the strong association of maternal obesity with both preeclampsia and gestational hypertension risk[7, 8], as well as with blood pressure in general (including in children)[20], together with familial clustering of adiposity[21, 22], family adiposity might be important in the mechanism linking maternal hypertensive disorders of pregnancy to offspring blood pressure. Furthermore, the epidemiological and pathophysiological links between preeclampsia and intrauterine growth restriction and the positive associations of birth weight with later fat mass and body mass index could provide a further link between preeclampsia and offspring blood pressure. The aim of this study is to examine the associations of gestational hypertension and preeclampsia with offspring blood pressure at the age of 9 years, and to study whether these associations are explained by parental and offspring anthropometry. Figure 1 shows the pathways that we have examined in this study.

Figure 1. Pathways concerning the association of hypertensive disorders of pregnancy and offspring blood pressure that are explored in this paper.



The straight lines represent positive associations and the dashed line represents an inverse association. The pathways in this figure are not intended to represent all pathways that might link maternal hypertensive disorders of pregnancy but focus on the role that shared familial adiposity and birth weight and gestational age might have in explaining any association of hypertensive disorders of pregnancy with offspring blood pressure.

Methods

Design

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a prospective population-based birth cohort study that recruited 14,541 pregnant women resident in Avon, UK with expected dates of delivery 1st April 1991 to 31st December 1992 (http://www.alspac.bris.ac.uk.).[23] There were 13,678 mother-offspring pairs from singleton live births who survived to at least one year of age; only singleton pregnancies are considered in this paper. Of these women 94% gave consent for abstraction of data from their obstetric records. 7,722 children attended the 9 year follow-up clinic, which is approximately 65% of those invited (participants who were still alive and still agreeing to be involved with the study).

Study population

In total 6,668 mother-offspring pairs had abstracted maternal antenatal data and an offspring who attended the 9 year follow-up clinic. Of the 6,668 mother-offspring eligible pairs, complete data on hypertensive disorders of pregnancy, including preeclampsia and gestational hypertension, offspring anthropometry, blood pressure and potential confounders were available for 6,343 (95%) and these form the analysis cohort for this study.

Maternal characteristics

Six trained research midwives abstracted data from obstetric medical records. There were no between-midwife variation in mean values of abstracted data and repeated data entry checks demonstrated error rates consistently <1%. Obstetric data abstractions included every measurement of systolic and diastolic blood pressure entered into the medical records and the corresponding gestational age and date at the time of the blood pressure measurement. According to the definitions of the International Society for the Study of Hypertension in Pregnancy, a hypertensive disorder during pregnancy was defined as a systolic blood pressure greater than 139 mmHg or a diastolic blood pressure greater than 89 mmHg, measured on at least two occasions after 20 weeks of gestation in a woman without known hypertension prior to pregnancy.[24] Preeclampsia was defined as the development of hypertension as stated above and proteinuria, diagnosed if the protein reading on dipstick testing (Albustix; Ames Company, Elkhart, Indiana) was at least 2+ on at least two occasions, occurring at the same time as the episodes of raised blood pressure. Women with hypertensive disorder of pregnancy who did not fulfil the criteria of preeclampsia were classified as having gestational hypertension. Thus, all women were categorised into one of three mutually exclusive categories of no hypertensive disorder of pregnancy, gestational hypertension and preeclampsia.

Offspring measurements at the age of nine years

Our outcomes in the current study are systolic and diastolic blood pressure (SBP and DBP). As illustrated in Figure 1 we were concerned with the way in which maternal and offspring anthropometry might explain the primary association between hypertensive disorders of pregnancy and offspring blood pressure. Offspring blood pressure, body mass index (BMI), waist circumference, total fat and lean mass were obtained around the age of 9 years, when all ALSPAC children were invited to attend a clinic session.

Blood pressure was measured using a Dinamap 9301 Vital Signs Monitor. Two readings of systolic and diastolic blood pressure (SBP and DBP) were recorded, with the child at rest, and the mean of each was used.

Weight and height were measured in light clothing and without shoes. Weight was measured to the nearest 0.1 kg using Tania scales. Height was measured to the nearest 0.1 cm using a Harpenden stadiometer. Waist circumference was measured to the nearest 1 mm at the mid-point between the lower ribs and the pelvic bone with a flexible tape. Fat and lean mass was assessed using dual energy X-ray densitometry (DXA). A Lunar prodigy narrow fan beam densitometer was used to perform a whole body DXA scan where bone content, lean and fat mass were measured. For our main analyses we examined BMI, waist circumference, and total body lean and fat mass as continuously measured variables. We also examined binary outcomes of overweight/obese (BMI) and centrally obese (waist circumference). General overweight and obesity were defined using the International Obesity Taskforce (IOTF) age- and sex-specific thresholds for childhood BMI.[25] Central obesity was defined as an age- and sex-specific waist circumference $\geq 90^{th}$ percentile[26], based on waist circumference percentile curves derived for British children.[27]

Covariates

Maternal age, parity, mode of delivery (caesarean section / vaginal delivery) and the child's sex and birth weight were obtained from the obstetric records. Current age of the child was recorded in months as they arrived at the assessment clinic. At the time of recruitment, mothers were asked to report their pre-pregnancy weight and height, which were used to calculate maternal pre-pregnancy BMI. Maternal self-report of pre-pregnancy weight and her measured weight at the first antenatal clinic were highly correlated (Pearson's correlation coefficient = 0.95; $p < 0.0001$). At the time of recruitment, mothers were also ask to pass a questionnaire to the father of the child; in this questionnaire the father was asked to record his height and weight, from which BMI (at the start of his partner's pregnancy) was calculated, and also his date of birth, so that his age could be derived. Based on questionnaire responses, the highest parental occupation was used to allocate the children to family social class groups (classes I (professional / managerial) to V (unskilled manual workers), using the 1991 British Office of Population and Census Statistics

(OPCS) classification). Mothers were repeatedly asked about their smoking throughout pregnancy and these data were used to generate a categorical variable: never smoked; smoked before pregnancy or in the first trimester and then stopped; smoked throughout pregnancy. Any note of diabetes during the pregnancy in the medical records was recorded and women were categorised as existing diabetes (already known to have diabetes before the start of pregnancy), gestational diabetes (a new diagnosis of diabetes during the pregnancy noted in the medical records) or no evidence of diabetes. Universal screening (for example with a fasting or random blood sample) for gestational diabetes was not undertaken in this population.

Statistical analysis

Maternal, paternal and offspring characteristics (means (standard deviation) or medians (mid-95% range) for continuous variables and N (%) for categorical variables) are presented across the three categories of no hypertensive disorder of pregnancy, gestational hypertension and preeclampsia; f-tests and chi-squared tests were used to test for statistical evidence of differences across these three categories.

We created age- and sex-specific z-scores of the adiposity measures. This enabled us to investigate whether the magnitude of the relationship between hypertensive disorders of pregnancy and adiposity differs between the four adiposity measures, since all results will be on the standard deviation scale.

We used multivariable regression models (linear regression for continuous outcomes and logistic regression for binary outcomes) to explore all associations outlined in Figure 1. These associations include the associations of maternal pre-pregnancy BMI with hypertensive disorders of pregnancy and with offspring adiposity at the age of nine years; the associations of hypertensive disorders of pregnancy with birth size, gestational age and offspring adiposity at the age of nine years; the associations of birth size with markers of adiposity at the age of nine years and the associations of hypertensive disorders of pregnancy with offspring blood pressure at the age of nine years. In all analyses gestational hypertension and preeclampsia were examined as separate exposures with the comparison group always being those women with no evidence of either condition. In all models we adjusted first for offspring gender and age at examination (Model I). Subsequently, we adjusted for potential confounding factors (maternal age, parity and smoking during pregnancy, parental BMI, family socioeconomic position (parental education and occupational social class), and in models with fat mass or lean mass for offspring height and height-squared) (Model II). Since there were only 26 women (0.4%) with diagnosed gestational diabetes and 39 women (0.6%) with pre-existent diabetes, we did not consider gestational diabetes as a confounding variable in our models, as these numbers would be too small to have any important effect on the associations examined.

Finally, we adjusted for potential mediating factors (Model III). Mediators were considered as characteristics that occur after the main exposure variables (here hypertensive disorder of pregnancy) and are believed to be caused by them and in turn are believed to cause the main outcome of interest (here offspring blood pressure). Hence we considered gestational age at birth, birth weight, and mode of delivery to be potential mediators of the main association since hypertensive disorder of pregnancy can cause preterm birth (by induction of labour) and low birth weight (by shared pathophysiology)28 and sibling studies suggest that these may be causally (via intrauterine mechanisms) related to later offspring blood pressure.19, 29 By contrast offspring adiposity might be primarily a confounding factor since the most likely pathway linking it to both hypertensive disorders of pregnancy and offspring blood pressure would be via maternal adiposity. Maternal and offspring adiposity together would thus represent potential confounding by shared familial factors that are related to adiposity in family members and hence greater blood pressure in family members.

We consider the confounder-adjusted model (Model II) to be the main estimate of a potential causal effect of hypertensive disorders of pregnancy on our outcome. Results are presented jointly for mothers of female and male offspring as there was no strong and consistent evidence of interactions with gender in any of the associations examined (all p-values >0.1). All statistical analyses were performed using the Statistical Package for the Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Table 1 presents the characteristics of mother and offspring. Of the 6,668 women included in this study, 205 women (3.1%) were diagnosed with preeclampsia and 1,118 women (17.3%) were diagnosed with gestational hypertension. Mothers included in this study did not differ in the amount of hypertensive disorders of pregnancy compared to the mothers not included because of missing outcome or covariable data (202 (3.6%) of the 5,609 excluded women had preeclampsia (p-value for difference 0.10) and 920 (16.4%) of the 5,609 excluded women had gestational hypertension (p-value for difference 0.59)).

Table 1. Subject characteristics (n = 6,668)

	No hypertensive disorder of pregnancy (n = 5,345)	Gestational hypertension (n = 1,118)	Preeclampsia (n = 205)	P-value*
Mothers				
Age at delivery (years)	29.1 (4.5)	28.8 (4.7)	28.8 (5.4)	0.26
Body mass index (kg/m^2)	22.5 (3.3)	24.6 (4.8)	25.3 (5.7)	<0.01
Height (cm)	164.0 (6.6)	164.9 (6.8)	163.4 (7.3)	<0.01
C-section (%)	502 (9.5)	172 (15.4)	55 (26.8)	<0.01
Length of gestation (weeks)	40.0 (35.0 - 42.0)	40.0 (36.0 - 42.0)	39.0 (29.0 - 42.0)	<0.01
Preterm birth (<37 weeks) (%)	236 (4.4)	53 (4.7)	47 (22.9)	<0.01
No previous pregnancies (%)	2254 (43.7)	631 (58.4)	138 (69.7)	<0.01
Smoked throughout pregnancy (%)	1013 (19.4)	162 (14.8)	27 (13.5)	<0.01
Manual social class (%)	711 (14.5)	152 (14.9)	25 (13.6)	0.25
Fathers				
Age (years)	31.3 (5.4)	31.1 (5.4)	31.1 (6.7)	0.53
Body mass index (kg/m^2)	25.0 (3.2)	25.6 (3.6)	25.3 (3.7)	<0.01
Offspring				
Male (%)	2613 (48.9)	584 (52.2)	109 (53.2)	0.07
Birth weight (grams)	3446 (509)	3435 (565)	3103 (859)	<0.01
Age at visit (months)	118.0 (114.0 - 128.0)	118.5 (114.0 - 129.0)	118.5 (114.0 - 127.0)	0.76
Systolic blood pressure (mmHg)	102.2 (9.1)	105.2 (10.1)	104.5 (8.8)	<0.01
Diastolic blood pressure (mmHg)	57.2 (6.4)	58.2 (6.0)	58.6 (6.6)	<0.01
Weight at visit (kg)	34.5 (7.3)	36.2 (8.3)	34.4 (7.6)	<0.01
Height at visit (cm)	139.4 (6.3)	140.4 (6.2)	138.8 (6.2)	<0.01
BMI at visit (kg/m^2)	17.6 (2.8)	18.2 (3.2)	17.7 (3.0)	<0.01
Obese based on BMI (%)	219 (4.1)	90 (8.1)	12 (5.9)	<0.01
Overweight or obese based on BMI (%)	1161 (22.0)	315 (28.5)	51 (25.0)	<0.01
Waist circumference (cm)	62.6 (7.6)	64.2 (8.7)	63.0 (8.3)	<0.01
Total body fat mass (grams)	8404 (5016)	9416 (5636)	8439 (5197)	<0.01
Total body lean mass (grams)	24493 (3212)	25108 (3340)	24208 (3271)	<0.01
Centrally obese (%)	2066 (38.8)	519 (46.5)	83 (40.5)	<0.01

Values are means (standard deviation), medians (95% range) or number of children (%).

**p-value chi-square test or f-test for the null hypothesis of no differences across the 3 categories (i.e. 2 degrees of freedom).*

Table 2 shows the associations of maternal characteristics and paternal BMI with preeclampsia and gestational hypertension. Both preeclampsia and gestational hypertension were more common in women in their first pregnancy and were less common in women who smoked in pregnancy. Maternal BMI was positively associated with both preeclampsia and gestational hypertension The associations of nulliparity, smoking and maternal BMI all appeared stronger for preeclampsia than they did for gestational hypertension, but there was no strong statistical evidence that any of these associations differed from each other (p-value for heterogeneity between the two estimates all >0.3). Paternal BMI was associated with an increased risk of gestational hypertension, but this association attenuated to the null after adjustment for maternal BMI (maternal BMI adjusted association of paternal BMI with gestational hypertension: 1.03 (95% CI: 1.01, 1.06)). Social class was not associated with either hypertensive disorder of pregnancy.

Table 2. Associations of parental characteristics with hypertensive disorders of pregnancy

	No hypertensive disorder of pregnancy (n = 5,345)	**Gestational hypertension** (n = 1,118)	**Preeclampsia** (n = 205)
No previous pregnancies, OR	Reference	1.78 (1.56, 2.02)	2.83 (2.10, 3.80)
Smoked throughout pregnancy, OR	Reference	0.72 (0.60, 0.86)	0.65 (0.43, 0.98)
Manual social class, OR	Reference	1.03 (0.86, 1.25)	0.93 (0.61, 1.43)
Maternal body mass index (per 1SD z-score), OR	Reference	1.67 (1.56, 1.78)	1.79 (1.59, 2.02)
Paternal body mass index (per 1SD z-score), OR	Reference	1.20 (1.12, 1.29)	1.09 (0.92, 1.28)

Values are odds ratios (95% confidence interval).

Table 3 presents the unadjusted associations of hypertensive disorders of pregnancy with offspring characteristics. Preeclampsia was associated with both lower birth weight (mean difference comparing offspring of mothers with preeclampsia to those with no hypertensive disorder of pregnancy: -341.9 grams (95% CI: -416.0, -267.9)) and preterm birth (OR comparing offspring of mothers with preeclampsia to those with no hypertensive disorder of pregnancy: 6.39 (95% CI: 4.50, 9.08)). These effect estimates attenuated after adjustment for mode of delivery, and gestational age at birth (-109.2 grams (95% CI: -172.3, -46.1)) or birth weight (OR 1.91 (95% CI: 0.99, 3.69)), respectively for birth weight and preterm delivery. Gestational hypertension was not associated with birth weight or preterm delivery either before or after adjustment for mode of delivery

and birth weight or gestational age. In these unadjusted analyses gestational hypertension was associated with greater offspring adiposity at the age of nine years, whereas preeclampsia did not show strong evidence of an association with offspring adiposity. Preeclampsia was inversely associated with lean mass, suggesting that infants of women who experienced preeclampsia having less lean mass than those who did not experience any hypertensive disorder of pregnancy. Finally, in these unadjusted models, preeclampsia and gestational hypertension were both positively associated with offspring systolic and diastolic blood pressure.

Table 3. Associations of hypertensive disorders of pregnancy with offspring characteristics at birth and at the age of nine years

	No hypertensive disorder of pregnancy (n = 5,345)	**Gestational hypertension** (n = 1,118)	**Preeclampsia** (n = 205)
Offspring characteristic			
Birth weight, mean difference (grams)	Reference	-10.3 (-43.9, 23.3)	-341.9 (-416.0, -267.9)
Preterm birth, OR	Reference	1.07 (0.79, 1.45)	6.39 (4.50, 9.08)
Adiposity measure			
BMI, mean difference (z-score)	Reference	0.22 (0.15, 0.28)	0.04 (-0.10, 0.17)
Waist circumference, mean difference (z-score)	Reference	0.20 (0.14, 0.27)	0.03 (-0.11, 0.16)
Fat mass, mean difference (z-score)	Reference	0.22 (0.16, 0.29)	0.03 (-0.11, 0.17)
Lean mass, mean difference (z-score)	Reference	0.18 (0.11, 0.24)	-0.14 (-0.29, -0.01)
Obese, OR	Reference	2.05 (1.59, 2.64)	1.45 (0.80, 2.64)
Overweight or obese, OR	Reference	1.43 (1.23, 1.65)	1.19 (0.86, 1.65)
Central obesity, OR	Reference	1.39 (1.22, 1.58)	1.09 (0.82, 1.45)
Blood pressure			
SBP, mean difference (mmHg)	Reference	3.06 (2.46, 3.66)	2.36 (1.09, 3.64)
DBP, mean difference (mmHg)	Reference	1.44 (1.03, 1.86)	0.99 (0.10, 1.89)

Values are means of differences or odds ratios (OR) (95% confidence interval) and reflect the difference in offspring characteristic compared to women without any hypertensive disorder of pregnancy (reference group).

Multivariable associations of hypertensive disorders of pregnancy and offspring adiposity are presented in Table 4. The inverse association of preeclampsia with offspring lean mass at age 9 was essentially unaltered by adjustment for potential confounding factors and mediation by birth size and gestational age. With adjustment for potential confounding factors the previously null associations of preeclampsia with all measures of offspring adiposity became inverse. The main covariable that resulted in this unmasking of an inverse association was maternal pre-pregnancy BMI. Thus, offspring of women with preeclampsia are less adipose and less likely to be obese once the positive association of maternal BMI with preeclampsia risk and with offspring adiposity has been controlled for.

Table 4. Associations of hypertensive disorders of pregnancy with offspring adiposity measures

	No hypertensive disorder of pregnancy (n = 5,345)	**Gestational hypertension** (n = 1,118)			**Preeclampsia** (n = 205)		
		Model I	**Model II**	**Model III**	**Model I**	**Model II**	**Model III**
BMI, z-score	Reference	0.23 (0.16, 0.30)	0.05 (-0.02, 0.12)	0.07 (-0.01, 0.14)	0.03 (-0.12, 0.18)	-0.19 (-0.34, -0.05)	-0.17 (-0.32, -0.03)
Waist circumference, z-score	Reference	0.21 (0.14, 0.28)	0.04 (-0.03, 0.10)	0.05 (-0.02, 0.12)	0.01 (-0.15, 0.16)	-0.20 (-0.34, -0.05)	-0.19 (-0.33, -0.04)
Fat mass, z-score	Reference	0.24 (0.17, 0.31)	0.04 (-0.03, 0.10)	0.04 (-0.03, 0.10)	0.03 (-0.12, 0.19)	-0.09 (-0.23, 0.05)	-0.09 (-0.23, 0.05)
Lean mass, z-score	Reference	0.19 (0.12, 0.26)	0.01 (-0.04, 0.05)	0.02 (-0.03, 0.06)	-0.20 (-0.36, -0.04)	-0.19 (-0.28, -0.09)	-0.15 (-0.25, -0.05)
Obese, OR	Reference	2.29 (1.71, 3.06)	1.37 (0.99, 1.89)	1.41 (1.02, 1.95)	1.21 (0.56, 2.63)	0.50 (0.21, 1.20)	0.50 (0.21, 1.22)
Overweight or obese, OR	Reference	1.54 (1.31, 1.82)	1.08 (0.90, 1.30)	1.10 (0.92, 1.32)	1.10 (0.75, 1.62)	0.60 (0.39, 0.93)	0.61 (0.39, 0.95)
Central obesity, OR	Reference	1.40 (1.21, 1.61)	1.04 (0.89, 1.21)	1.08 (0.92, 1.26)	1.19 (0.86, 1.65)	0.81 (0.57, 1.15)	0.84 (0.59, 1.20)

Values are regression coefficients or odds ratios (95% confidence interval) and reflect the difference in offspring characteristic for maternal hypertensive disorder of pregnancy. CI, confidence interval; OR, Odds ratio; BMI, Body mass index.

Model I: adjusted for offspring sex and age at the 9-y-old visit.

Model II: additionally adjusted for maternal age at delivery, parental pre-pregnancy body mass index, parity, social class, maternal smoking during pregnancy, and offspring weight, height and height squared at the 9-y-old visit (the confounder adjusted model).

Model III: additionally adjusted for mode of delivery, gestational age at birth and birth weight (to examine mediation by these determinants).

With adjustment for potential confounders the previous positive association of gestational hypertension and offspring adiposity was considerably attenuated to the null, with adjustment for parental BMI being the key covariables responsible for this attenuation. After adjustment for mode of delivery, gestational age at birth and birth weight, gestational hypertension had some association with obesity (OR 1.41 (95% CI: 1.02, 1.95)). This increase in magnitude of the association was largely due to adjustment for birth weight.

Associations of hypertensive disorders of pregnancy with offspring blood pressure are presented in Table 5. Both preeclampsia and gestational hypertension were associated with greater systolic and diastolic blood pressure in the nine-year-old offspring, with the magnitudes of these associations being similar for each hypertensive disorder of pregnancy in the confounder adjusted model (Model II): on average both preeclampsia and gestational hypertension were associated with a 2 mmHg greater systolic blood pressure and a 1 mmHg diastolic blood pressure in the 9-year-old offspring. With further adjustment for mode of delivery, birth weight and gestational age the association of preeclampsia with offspring blood pressure attenuated towards the null, whereas that of gestational hypertension was not attenuated.

Table 5. Associations of hypertensive disorders of pregnancy with offspring blood pressure

	No hypertensive disorder of pregnancy (n = 5,345)	Gestational hypertension (n = 1,118)			Preeclampsia (n = 205)		
		Model I	Model II	Model III	Model I	Model II	Model III
SBP (mmHg)	Reference	3.17 (2.50, 3.85)	2.04 (1.42, 2.67)	2.50 (1.80, 3.20)	2.23 (0.78, 3.67)	2.05 (0.72, 3.38)	1.13 (-0.35, 2.60)
DBP (mmHg)	Reference	1.47 (1.00, 1.94)	1.07 (0.60, 1.54)	1.25 (0.76, 1.73)	0.96 (-0.06, 1.97)	1.00 (-0.01, 2.01)	0.65 (-0.38, 1.69)

Values are regression coefficients or odds ratios (95% confidence interval) and reflect the difference in offspring characteristic for maternal hypertensive disorder of pregnancy. CI, confidence interval; SBP, Systolic blood pressure; DBP, Diastolic blood pressure.

Model I: adjusted for offspring sex and age at the 9-y-old visit.

Model II: additionally adjusted for maternal age at delivery, parental pre-pregnancy body mass index, parity, social class, maternal smoking during pregnancy, and offspring weight, height and height squared at the 9-y-old visit (the confounder adjusted model).

Model III: additionally adjusted for mode of delivery, gestational age at birth and birth weight (to examine mediation by these determinants).

Discussion

In our prospective population-based cohort study, offspring of women with a history of preeclampsia or gestational hypertension had increased systolic and diastolic blood pressure at the age of nine years compared to women without hypertensive disorders of pregnancy. These associations did not appear to be explained by shared familial characteristics (genetic or behavioral) related to family adiposity, since they remained with adjustment for maternal and paternal pre-pregnancy BMI and offspring adiposity. The association of preeclampsia with offspring blood pressure appeared to be, at least in part, mediated by the association of preeclampsia with lower birth weight and preterm delivery, as adjustment for these markedly attenuated the confounder adjusted association; adjustment for these infant characteristics did not attenuate the association of gestational hypertension with offspring blood pressure. An additional interesting finding of our study was that of an inverse association of preeclampsia with offspring lean mass in all multivariable models and also an inverse association with measurements of adiposity and obesity once maternal BMI had been taken into account. These associations did not markedly attenuate with adjustment for birth size and preterm birth and suggest that preeclampsia might have long-term effects on reduced offspring lean and fat mass.

The major strengths of our study are its prospective design, the large sample size and the ability to unpick associations between hypertensive disorders of pregnancy, offspring blood pressure and familial adiposity. To our knowledge this is the first study examining the associations of hypertensive disorders of pregnancy with measures of adiposity in detail, including highlighting the differences between preeclampsia and gestational hypertension. We were able to define preeclampsia and gestational hypertension by applying standard definitions to detailed clinical data (repeated assessments throughout pregnancy of blood pressure and proteinuria) abstracted from the antenatal medical records rather than having to rely on retrospective maternal report as some previous studies have. Routine clinical data may be less accurate than data collected for research purposes and examining the antenatal blood pressure used in this study shows digit preference for values ending in 5 or 0 (e.g. systolic blood pressures of 105, 110, 115, 120, 125 mmHg, etc.). Since the clinical staff collecting these data could not have had any knowledge of future offspring blood pressure this measurement error (rounding) would be non-differential by the main outcome of offspring blood pressure and would have the expectation of biasing any association towards the null. The findings of expected associations of preeclampsia and gestational hypertension with established risk factors (maternal age, parity, smoking and BMI) in our study suggest that measurement error has not resulted in marked bias. Whilst the majority (95%) of participants attending the nine year follow-up clinic had adequate data on blood pressure, fat mass, other anthropometric measurements and all confounding factors, there has been lost to follow-up with 65% of those eligible to attend the nine year follow-up clinic attending. Participants who attend follow-up clinics are more likely to be from higher socioeconomic position families, less

likely to have had teenage mothers and mothers who smoked during pregnancy.[30] However, those attending the clinic had mothers with similar proportions of preeclampsia and gestational hypertension compared to those who were not included in the study and we can think of no reasons why the associations we have examined here should be markedly different in those lost to follow-up.

We found positive associations of both preeclampsia and gestational hypertension with offspring systolic and diastolic blood pressure at the age of nine years, with associations of each being somewhat weaker for diastolic blood pressure compared to systolic blood pressure. Given smaller numbers of mothers with preeclampsia the association with diastolic blood pressure is imprecisely estimated and includes the null value. In the basic model adjusting only for gender and age appears that gestational hypertension might have somewhat stronger associations with offspring blood pressure than preeclampsia, but this is largely driven by the stronger association of maternal (and paternal) BMI with gestational hypertension than with preeclampsia. When parental BMI, offspring adiposity and other potential confounders are taken into account positive associations of both preeclampsia and gestational hypertension remain with systolic and diastolic blood pressure with the point estimates for associations being very similar for preeclampsia and gestational hypertension. With further adjustment for birth weight and gestational age the point estimates for the associations of preeclampsia with both systolic and diastolic blood pressure are reduced by about 40%, whereas these adjustments make very little difference to the positive associations of gestational hypertension with systolic and diastolic blood pressure. Though the confidence intervals for Model III overlap with those for Model II, suggesting that the two estimates are consistent with each other.

Our findings are in line with several other studies that have reported positive associations between hypertensive disorders of pregnancy and offspring blood pressure.[10, 11, 13, 14] Our study extends this previous work by suggesting that family adiposity does not explain these associations. It also suggests that intrauterine growth restriction might in part mediate the association of preeclampsia with offspring blood pressure. By contrast since gestational hypertension was not associated with lower birth weight it does not mediate the similar magnitude of association of gestational hypertension with offspring blood pressure.

We are not aware of previous studies showing a reduced lean and fat mass (after adjustment for maternal BMI) in offspring of mothers who experienced preeclampsia in their pregnancy. Several mechanisms could explain this association. Preeclampsia is often accompanied by fetal growth restriction and preterm delivery, due to placental hypoperfusion[28], and we confirmed a specific association of preeclampsia with lower birth weight and increased risk of preterm delivery, which was not seen for gestational hypertension, in our study. Since lower birth weight is associated with reduced lean and fat mass in later life, a finding previously demonstrated in ALSPAC, the association of preeclampsia with later offspring reduced fat and lean mass may be explained by its effect on IUGR. Another explanation for the association of preeclampsia and decreased measures of adiposity could be maternal smoking during pregnancy. Smoking is

associated with lower BMI and is protective against preeclampsia.[31] However, several studies have shown a positive association between maternal smoking in pregnancy and greater offspring BMI in later life.[32] Thus, when we control for maternal BMI we may unmask a positive association of maternal smoking with offspring BMI and because of the protective effect of smoking on preeclampsia an inverse association between it and offspring BMI. Whilst these explanations are plausible the inverse association was present even with adjustment for maternal smoking and was unaffected by adjustment for birth weight and gestational age. Thus, further research is required to examine whether this finding is replicated in other independent cohorts and if so to explore likely underlying mechanisms.

In conclusion, our findings suggest that women who experience hypertensive disorders of pregnancy have children with higher blood pressure at the age of nine years. These associations are not explained by the associations of maternal BMI with hypertensive disorders of pregnancy and with offspring BMI and whilst interventions to reduce obesity might reduce the occurrence of hypertensive disorders of pregnancy these are unlikely to reduce its link to offspring higher blood pressure. The mechanisms underlying the associations of preeclampsia and gestational hypertension with offspring blood pressure may differ from each other, with evidence that the relationship between preeclampsia and IUGR plays a role in its association but does not have any role in the association of gestational hypertension with offspring blood pressure.

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General discussion

5

Introduction

Many epidemiological studies from different geographical regions demonstrated a strong relationship between low birth weight and the risk of cardiovascular disease.[1-3] These associations are consistent and cannot be explained by preterm birth.[4, 5] Also, they seem to be independent of influences in adult life including social class, obesity and smoking habits.[6, 7] The mechanisms underlying these associations are not well known. It has been suggested that developmental adaptations due to suboptimal fetal nutrition permanently program the fetus and lead to an increased risk of coronary heart disease many decades later.[8] It has also been argued that the associations between size at birth and later disease could primarily be the result of common genetic influences. Based on more recent studies, a more general developmental plasticity hypothesis has been proposed.[9] Developmental plasticity is defined as the ability of an organism to develop in various ways, depending on the particular environment or setting.[10] In this process, early environmental influences induce anatomical, physiological and biochemical adaptations in later life. These adaptations may be beneficial for short-term survival but could have adverse long-term consequences. This latter conceptual basis is extended with the Predictive Adaptive Response (PAR) theory, stating that long-term consequences of these early environmental influences may be especially harmful if the actual postnatal, mature environment differs from the environment predicted during the plastic, developmental phase.[11] The greater the degree of mismatch, the greater the disturbance in physiology and the greater the risk of disease.

Studies in humans generally use birth weight as a measure of adverse fetal exposure. Birth weight might indeed be an indicator of the quality of the intra-uterine environment.[12] However, the same birth weight might be the result of different growth patterns and exposures in fetal life. Furthermore, a period of compensatory growth will follow after nutritional deficit in utero. The most rapid growth acceleration in low birth weight children occurs in the first weeks after birth.[13] This accelerated growth rate might also have important consequences later in life.[14] These findings suggest that children with both restricted fetal and infant growth and accelerated childhood weight gain are at particular risk of development of cardiovascular disease in adult life and its risk factors.[15, 16]

In this chapter, we discuss epidemiological studies designed to identify mechanisms underlying the associations of fetal growth retardation and low birth weight with the development of cardiovascular disease in adulthood. We will focus on specific adverse fetal exposures, cardiovascular adaptations and perspectives for future studies.

Specific fetal exposures

Fetal undernutrition

Maternal anthropometrics, maternal diet and placenta function

The fetal nutrition supply line includes maternal anthropometrics, diet and placenta function. Maternal anthropometrics during pregnancy are related to their nutritional and health status.[17, 18] Several studies have shown that maternal anthropometric factors such as prepregnancy body mass index and weight gain during pregnancy are associated with fetal growth characteristics and adverse pregnancy outcomes.[19-21] Thus, the maternal nutritional and health status during pregnancy may lead to an adverse fetal environment and might affect fetal growth and development (Table 1).

Table 1. Maternal anthropometrics and diet in association with cardiovascular disease later in life

Maternal anthropometric characteristic during pregnancy	Cardiovascular outcome
Maternal anthropometric	
Short stature	Increased death rates from coronary heart disease
Low triceps skin fold thickness	Increased blood pressure
High weight gain during pregnancy	Increased left ventricular mass
Maternal diet	
Total energy intake	Increased risk and earlier onset coronary artery disease
Low protein intake	Increased blood pressure
Low calcium intake	Increased blood pressure
Low folate intake	Endothelial dysfunction

Studies examining the direct effect of maternal anthropometrics on cardiovascular outcomes in the offspring demonstrated conflicting results (Table 1). Most of these studies were based on retrospective cohorts. We found in a prospective cohort study that maternal weight gain during pregnancy is associated with larger left ventricular mass at the age of 6 months, independent of maternal weight just before pregnancy.[22] Additionally, a recent study showed that greater maternal gestational weight gain is associated with greater offspring body mass index into early adulthood and that this may translate into higher systolic blood pressure in the offspring.[23] Several underlying biological pathways may explain the associations between maternal anthropometrics during pregnancy and vascular development and cardiac growth and development postnatally. One explanation could be that the usual increase in insulin resistance seen in late pregnancy is higher

in mothers who show a marked increase in weight during pregnancy, resulting in increased fetal body and cardiac growth.[24, 25] Further studies, in which measures of insulin resistance such as insulin, glucose or HbA1c levels in pregnancy have been measured, are needed to identify the potential role of maternal insulin resistance for fetal cardiovascular development. The major limitation of weight gain is that the total amount depends on both an increase in fluid and fat mass. Also, it is not clear whether maternal pre-pregnancy weight or weight gain during pregnancy affects cardiovascular development in the offspring.

The fetal nutrition supply line may also be directly affected by the dietary intake of the mother during pregnancy. A follow-up study of persons who were conceived during the Dutch famine during the second World War demonstrated a doubled risk and an earlier onset of coronary artery disease among subjects who were exposed to famine during fetal life.[16] These results were independent of size at birth and current smoking and social economic status and suggest that severe maternal malnutrition during early gestation contributes to the occurrence of coronary artery disease in the offspring. However, these results could not be replicated in a cohort of adults who were exposed as fetuses to severe famine during the siege of Leningrad.[26] The associations of less extreme variations in maternal intake of macronutrients and micronutrients with the development of risk factors for cardiovascular disease in the offspring have also been studied. Prospective cohort studies showed that variation in maternal total protein intake during pregnancy does not program offspring blood pressure already in infancy or in adolescence.[27-29]

Stronger relations have been reported between micronutrients and risk factors for cardiovascular disease in the offspring. Maternal calcium supplementation during pregnancy has been described as being associated with lower systolic blood pressure in the offspring.[30-32] In some follow-up studies the effect disappeared later in childhood and no associations were reported in twins.[33, 34] Therefore, it is still unclear whether ensuring adequate calcium intake among pregnant women could be a way to prevent hypertension in the next generation. Low folate and high homocystein levels, in combination or independently, have been shown to be risk factors for endothelial dysfunction and cardiovascular disease.[35, 36] In healthy infants, a relationship between maternal folate levels during pregnancy and vascular endothelial function was demonstrated.[37] More recently developed statistical approaches, such as dietary pattern analysis may give more detailed information about the effect of maternal nutrition intake during pregnancy on development of risk factors for cardiovascular disease later in life.

Placental function, reflected by placental weight and hemodynamic function is one of the most important determinants of the fetal supply line. While the size of the placenta reflects only an indirect measure of its capacity to transfer nutrients to the fetus, it is strongly associated with fetal size at birth.[38] A review focused on the associations of placental weight with the risk of cardiovascular disease in the offspring reported no consistent associations.[39] Preeclampsia is considered as the most extreme form of hemodynamic placental dysfunction. According to recent studies, women with prior preeclamptic pregnancies are at increased risk of cardiovascular dis-

ease.[40, 41] Furthermore, preeclampsia has been associated with elevated blood pressure in offspring during childhood and adolescence.[42] Fetal growth restriction due to placental dysfunction or common genetic variants may at least partly explain these associations. A milder form of placental hemodynamic dysfunction is reflected by resistance in the umbilical artery. An increased umbilical artery resistance is associated with fetal growth restriction and low birth weight.[43]

Maternal smoking during pregnancy

Maternal smoking during pregnancy is strongly associated with fetal growth retardation.[44-46] The effects of smoking on placental vessels could be due to nicotine or hypoxia. This association is partly mediated by restricted blood flow in the vascular beds of the placenta due to increased resistance of the umbilical-placental circulation.[47, 48] Fetal exposure to maternal smoking might also have adverse and persistent consequences for cardiovascular growth and development. Recent studies showed associations between intra-uterine exposure to maternal smoking and high blood pressure in childhood and adulthood.[49, 50] However, the association of maternal smoking and offspring blood pressure might be confounded by a comprehensive range of indicators of social economic position. Confounding by social and familial factors is further supported by the similarity of maternal and paternal smoking effects, suggesting that modest differences in childhood blood pressure associated with maternal smoking could be the result of not only biological effects on the intrauterine environment, but also common adverse familial factors.[51] Studies focused on fetal cardiovascular development may unravel these associations. We found in a prospective cohort study that maternal smoking during pregnancy is associated with placental and fetal hemodynamic adaptations indicating increased arterial resistance.[52] These fetal hemodynamic adaptations were subsequently associated with fetal growth retardation and changes in postnatal cardiac structures. Similarly, maternal smoking in pregnancy has also been suggested to be directly associated with changes in fetal cardiac dimensions and volumes.[53, 54]

Genetic susceptibility

The association of fetal growth restriction and low birth weight with the increased risk of cardiovascular disease may also be explained by common genetic variants related to insulin sensitivity or angiogenesis (Table 2).[55] Insulin and insulin-like growth factors are important fetal growth factors.[56] Genetic factors related to insulin or insulin-like growth factors production and sensitivity may lead to both impaired fetal growth and to type 2 diabetes and cardiovascular disease in later life.[55] It has been suggested previously that Single Nucleotide Polymorphisms (SNPs) in the *IGF1* gene and the *INS* VNTR gene are associated with fetal and postnatal growth. However, these effects seem to be inconsistent.[57, 58]

Table 2. Common genetic variants (PPARγ2 and GR gene) studied to explain the associations between low birth weight with type 2 diabetes and cardiovascular disease later in life

First author (year)	Main finding: effect on pre- and postnatal growth	Main finding: effect on risk factors type 2 diabetes	Main finding: effect on risk factors cardiovascular disease
PPARγ2 (rs 1801282)			
Masud et al (2003) [171]		Pro12Ala genotype is associated with higher BMI and obesity.	
Bennett et al (2008) [59]		Pro12Ala genotype is not associated with birth weight.	
Pfab et al (2006) [60]	Pro12Ala genotype is not associated with intra-uterine growth, size at birth and insulin resistance.	Pro12Ala genotype is not associated with intra-uterine growth, size at birth and insulin resistance.	
Mook-Kanamori et al (2009) [61]	Ala12 allele is associated with an increased growth rate in early life. This effect may be influenced by the duration of breastfeeding.	Ala12 allele is associated with an increased growth rate in early life. This effect may be influenced by the duration of breastfeeding.	
Eriksson et al (2003) [63]		Ala12 allele and a lower birth weight is associated with risk of increased lipid levels.	Ala12 allele and a lower birth weight is associated with risk of increased lipid levels.
Yliharsila et al (2004) [64]		Pro12Pro genotype modifies the association between low birth weight and hypertension.	Pro12Pro genotype modifies the association between low birth weight and hypertension.
GR gene (NR3C1)			
Van Rossum et al (2002) [172]		ER22/23EK polymorphism is associated with decreased sensitivity to glucocorticoids and low insulin levels.	ER22/23EK polymorphism is associated with decreased sensitivity to glucocorticoids and low cholesterol levels.
Finken et al (2007) [173]	ER22/23EK polymorphism is associated with a protecting effect against postnatal growth failure and insulin resistance after preterm birth.	ER22/23EK polymorphism is associated with a protecting effect against postnatal growth failure and insulin resistance after preterm birth.	
Van Rossum et al (2003) [174]		G-allele of the *Bcl*I polymorphism is associated with increased glucocorticoid sensitivity and lower BMI.	
Rosmond et al (2000) [175]		G-allele of the *Bcl*I polymorphism is associated with increased abdominal obesity and higher cortisol levels in GG-carriers compared to CC-carriers.	
Bueman et al (1997) [176]		G-allele of the *Bcl*I polymorphism is associated with increased abdominal visceral fat in lean GG-carriers, but not in overweight GG-carriers.	
Huizenga et al (1998) [177]		N363S polymorphism is associated with increased glucocorticoid sensitivity, increased insulin response to Dexamethasone and increased BMI.	

Table 2. continued

First author (year)	Main finding: effect on pre- and postnatal growth	Main finding: effect on risk factors type 2 diabetes	Main finding: effect on risk factors cardiovascular disease
Watt et al (1992) [178]			Homozygosity for the G-allele of the *BclI* polymorphism was more frequent in the group with personal and parental hypertension.
Di Blasio et al (2003) [179]			Carrying both the N363S and the *BclI* polymorphism is associated with higher systolic and diastolic blood pressure and serum cholesterol levels.
Rosmond et al (2001) [180]		N363S polymorphism is not associated with BMI or sensitivity to glucocorticoids.	
Lin et al (2003) [181]		N363S polymorphism is associated with obesity and overweight, but not with type 2 diabetes.	N363S polymorphism is not associated with hypertension.
Rosmond et al (2000) [182]		*TthIII* polymorphism is associated with diurnal cortisol levels, but not with any anthropometric or glucose related phenotype.	
Van den Akker et al (2008) [69]			GR-9β polymorphism is associated with an increased risk of cardiovascular disease.
Geelhoed et al (2009)	GR gene polymorphisms are not associated with growth in fetal and early postnatal life, neither to size at birth or catch-up growth until the age of 2 years.	GR gene polymorphisms are not associated with growth in fetal and early postnatal life, neither to size at birth or catch-up growth until the age of 2 years.	
Geelhoed et al (2009)			GR-9β polymorphism is associated with increased systolic blood pressure and increased left ventricular mass at the age of 2 years.

Common polymorphisms of type 2 diabetes gene PPARγ2 may also explain previously suggested associations of growth in early life with the risk of cardiovascular disease in later life. Two large birth cohort studies found no association between the PPARγ2 polymorphism and birth weight.[59, 60] We recently showed that the PPARγ2 Ala12 allele is associated with an increased growth rate in early life. This effect was modified by the duration of breastfeeding.[61] In addition to the PPARγ2 polymorphism, other common type 2 diabetes genetic susceptibility variants seem to affect size at birth directly through the fetal genotype. Risk alleles at CDKAL1 and HHEX-IDE were both associated with reduced birth weight. These findings suggest the associations between low birth weight and type 2 diabetes might at least be explained in part by common genetic variants.[62] Furthermore, it has been demonstrated that the PPARγ2 polymorphism modifies the associations of low birth weight with lipid levels and hypertension.[63, 64]

Glucocorticoids are important regulators of cardiovascular function and metabolism. Studies in rats showed that activity of placental 11β-hydroxysteroid dehydrogenase type 2, which converts physiological glucocorticoids to inactive products, correlates positively with birth weight and negatively with placental weight.[65] In addition, administration of low-dose dexamethasone to pregnant rats not only reduces birth weight but also leads to high blood pressure in young adult offspring.[65] In human studies, it has been demonstrated that fetuses with the greatest exposure to maternal glucocorticoids have low birth weight and high placental weight and might be at a higher risk of subsequent hypertension.[66] Increased exposure to cortisol in adults leads to increased risks of cardiovascular disease, type 2 diabetes and obesity.[67, 68] Thus higher exposure to glucocorticoids in fetal and early postnatal life might affect cardiovascular development in fetal life and early childhood. The effects of these hormones, including cortisol, are mediated by glucocorticoid receptors. Glucocorticoid receptor gene (NR3C1) SNPs may explain part of the associations between growth characteristics in early life and disease in adulthood by increasing glucocorticoid sensitivity in the fetus for maternal glucocorticoids. A previous study in adults found an association between the GR-9β polymorphism and an increased risk of cardiovascular disease.[69] We found an association of this GR-9β polymorphism with increased left ventricular mass and systolic blood pressure in children aged two years.[70] No associations were observed between this GR-9β polymorphism and fetal and early postnatal growth.[71] Other recent identified glucocorticoid related polymorphisms, such as the brain-derived neurotrophic factor (BDNF) and the mineral corticoid gene, may affect fetal and postnatal growth by influencing the glucocorticoid metabolism.

Thus far, results of studies focused on the associations of common genetic variants related to both early growth and the risk of cardiovascular disease later in life are inconsistent. Further systematic searches for common genetic variants by means of genome-wide association studies will enable us to obtain a more complete understanding of what genes and polymorphisms are involved in both growth in fetal life and infancy and development of cardiovascular disease in adulthood.

Cardiovascular adaptations

Cardiac development

Stimuli for cardiac development

It has been demonstrated that fetuses have stiffer fetal ventricles than neonates and the diastolic filling patterns in normally grown fetuses mimic those of the diseased adult heart.[72] Left ventricular elastic compliance increases with gestational age and left ventricular stiffness significantly decreases. In growth restricted fetuses, this process may be affected.[72] Furthermore, reduced end-diastolic flow velocities in fetal umbilical and maternal uteroplacental arteries have been associated with increased peripheral vascular resistance.[73] This increase in peripheral arterial resistance and subsequently in fetal cardiac afterload may lead to an increase in fetal cardiac performance and to persistent structural left ventricular changes.[74] Decreased fetal growth was also associated with fetal adaptations in cardiac function in the whole range of fetal growth.[75] Furthermore, birth weight and measures of placental vascular resistance and fetal cardiac output were associated with left cardiac structures until the age of two.[76] This is in line with a previous prospective cohort study in children which demonstrated positive associations of birth weight with total coronary artery diameter, aortic root diameter and left ventricular outflow tract diameter in children aged nine.[77] However, inverse associations between birth weight and left ventricular mass in adolescents and adults have been demonstrated.[78, 79] It should be investigated whether these relations persist during later life and are related to the development of cardiovascular disease in adulthood.

Adverse cardiac adaptations

Studies in children showed that left ventricular mass tracks from childhood to adulthood.[80, 81] This implies that children with smaller left cardiac structures in early postnatal life tend to keep their relatively smaller left cardiac structures in childhood.[76, 80, 81] However, a relatively smaller left ventricle and aortic root may lead to insufficient cardiac functioning for increasing metabolic demands in postnatal life. Subsequently, the heart may respond by growth and adverse remodeling. Since the number of heart cells is established largely in fetal life, adaptation and growth of existing cells may eventually lead to left ventricular dysfunction and hypertrophy. Left ventricular hypertrophy is a strong and independent risk factor of cardiovascular morbidity and mortality in adulthood.[82, 83] Thus, fetal exposures affecting cardiac development may have life-long consequences.

Renal development

Stimuli for renal development

Nephrons start to form from day 30 of gestation.[84] Numerous factors, including the renin-angiotensin

system (RAS), various growth factors, apoptosis and an adequate supply of nutrients are required for nephrogenesis.[85-87] Nephrogenesis continues until 36 weeks of gestation and the induction of nephron number ceases thereafter.[88, 89] On average about 750,000 nephrons per kidney are present, with a wide interindividual range (250,000 – 2,000,000).[90-93]

Animal studies have shown that various determinants of fetal nutrition including low protein intake, relative vitamin A deficiency, reduced placenta perfusion and administration of steroids in late pregnancy cause fetal growth restriction, smaller kidneys and a permanently reduced nephron number.[94-97] Human studies demonstrated that low birth weight infants have lower kidney weight with a reduced number of nephrons.[98, 99] It was also demonstrated that hypertensive subjects have lower nephron numbers.[100] The best surrogate measure for assessing nephron number in epidemiological studies appears to be kidney weight or size measured by ultrasound.[89] Few studies investigated renal length or volume in early life. Being small for gestational age (SGA) is associated with small kidneys at birth and impaired kidney growth in early childhood.[101] Kidney length in preterm SGA infants follows closely the other anthropometric parameters during the first year of life.[102] SGA term infants had shorter kidney length at birth compared to what is appropriate for gestational age infants, but a similar length from the 3rd to 24th month of life.[103] This may represent either an accelerated renal maturation or early compensatory kidney hypertrophy in the SGA infants. Maternal anthropometrics, fetal abdominal circumference, fetal blood flow redistribution, and raised placental resistance are associated with both third trimester fetal kidney volume and kidney volume at the age of two years.[104, 105] Since kidney size tracks from the third trimester of pregnancy to early childhood, these adaptations may have persistent consequences.[104]

Adverse renal adaptations

The kidneys respond to this reduced number of nephrons by hyperperfusion and remodelling.[106] According to the hyperfiltration theory, this will lead to more sodium reabsorption, increased systolic blood pressure and albuminuria.[107] This process may be in favor of short-term renal function but may eventually lead to glomerular hypertrophy and damage.[106] Finally, this may predispose the individual to renal failure and hypertension. It has been shown that low birth weight is associated with early onset chronic renal failure. In subjects aged less than 50 years, those who weighed less than 2.5 kg at birth had a higher risk for end-stage renal disease than people who weighted 3-3.5 kg at birth.[108] This association was shown in all groups of primary causes of end-stage renal failure in adults (hypertension, diabetes and other causes). Studies in younger subjects have focused on urine albumin excretion, a predictor of cardiovascular and renal disease in diabetic and non-diabetic subjects.[109] Low birth weight is associated with microalbuminuria in children and adults independent of blood pressure and measures of insulin resistance.[110, 111] The pathway leading from small kidneys to hypertension may include the renin-angiotensin system, which has been demonstrated to be altered in the early phase of primary hypertension.[112] An increased activity of the renin-angiotensin system could be a compensatory mechanism in a decreased

number of nephrons in order to maintain normal renal filtration. It has been demonstrated that renin activity in umbilical cord blood is inversely related with the size of the kidney at birth.[113]

Vascular development

Hemodynamic stimuli for fetal vascular development

Fetal growth retardation leads to a preferential blood flow to the brain and heart, which deprives other organs of adequate oxygen and nutrients supply. This brain-sparing suggests organ specific vasodilatation and vasoconstriction and has been demonstrated in growth restricted fetuses.[114] In addition, we recently demonstrated that decreased fetal growth is associated with adaptive fetal cardiovascular changes.[75] These changes already occured before the onset of clinically apparent fetal growth restriction. Future studies are needed to identify whether these adaptations in fetal hemodynamics have consequences for the development of cardiovascular disease in later life.

Endothelial function

The endothelium controls vascular tone, coagulation and inflammatory responses.[115] Endothelial dysfunction is an early event of atherosclerosis, preceding structural changes in the vascular wall.[116] Atherosclerosis is thought to begin in childhood and to develop silently before clinical events such as myocardial infarction or stroke occur. Many studies demonstrated atherosclerotic wall thickening in the arteries of children with cardiovascular risk factors using ultrasound imaging.[117] Furthermore, studies have shown that risk factors for cardiovascular disease measured in childhood track into adulthood.[118, 119] There is limited direct evidence that risk factors measured in childhood are predictive of atherosclerosis in adulthood.[120, 121] Only a few studies examined the associations of fetal and maternal factors during pregnancy and vascular changes in childhood. Low maternal folate levels during pregnancy and low birth weight are demonstrated to be associated with vascular endothelial dysfunction in newborn infants.[37, 122, 123]

Arterial stiffness

Flow may determine vascular growth in the fetal cardiovascular system and thereby a reduction in flow may alter later vascular behavior.[124] One mechanism that could underlie the association of low birth weight with raised blood pressure may be a suboptimal development of the fetal cardiovascular system due to these circulatory changes, thus increasing the stiffness of the vessel wall and comprising the cardiovascular function.[125] Several studies have attempted to test this hypothesis by investigating the relationship between birth weight and indicators of arterial stiffness such as pulse wave velocity and pulse pressure, but with conflicting results.[126-128] Another mechanism could be that these hemodynamic adaptive changes in the fetal circulation, which occur in a critical period of blood vessel development, may influence rates of elastin synthesis.[129] This may result in a reduced compliance of the large arteries, leading to higher pulse and mean blood pressures.

Furthermore, the process of ageing causes a gradual loss of elastin and replacement by collagen, which amplifies the increase in blood pressure.[130] Persistent alteration in conduit artery function and arterial stiffness may predispose a person to hypertension and cardiovascular disease in adulthood.

Methodological considerations

The studies performed in this thesis have mainly been conducted within the Generation R Study, a population-based prospective cohort study. The prospective design enables detailed data collection and provides the opportunity to assess temporal relationships. Types of bias that might have affected our results include selection bias, information bias, and confounding.[137]

Selection bias

When the relation between determinant and outcome is different in those who participate and those who were eligible, but do not participate, selection bias may occur. Of all eligible children at birth, 61% participated in the Generation R Study.[131] Non-response due to non-participation is not likely to be random. The percentages of mothers from ethnic minorities and lower socioeconomic status, and of mothers and children with medical complications are lower among the participants than expected from the population figures in Rotterdam.[138] Especially in the Generation R Focus Study, including the Dutch women participating in the Generation R Study and with a delivery date between February 2003 and August 2005, this is the case. We do not think that this selection towards a more affluent and healthy study population substantially affected our etiological association studies, since these selection mechanisms are not related to both the determinant and the outcome and we do not expect that these associations differ between the study population and the total eligible population. However, this selection will probably affect frequency rates and, as a consequence, the statistical power and the generalizibility of our studies. The group of children born with low birth weight or small size for gestational age was too small for specific analyses focused on these groups and our results can therefore not easily be extrapolated to preterm or low birth weight children.

Selection bias may not only be introduced by selective non-response, but also by selective loss to follow-up. Most of the studies presented in this thesis are performed in the Generation R Focus Study. Of all mothers who were enrolled in the Focus cohort, 80% participated in the postnatal detailed examinations. These examinations included the ultrasound examinations of the heart and the kidneys, which were the main outcomes in chapters 3 and 4 of this thesis. Loss to follow-up would lead to bias if the associations of various determinants with these ultrasound measures differ between those who participate and those who were loss to follow-up. Since the association between exposure and disease in non-participants is unknown, the effect of selective

loss to follow-up can only be inferred. A previous analysis showed that biased estimates in large cohort studies primarily arise from loss to follow-up rather than from non-response at baseline.[139]

Information bias

Information on the determinants and outcomes in the studies described in this thesis was mainly obtained by physical examinations, ultrasound examinations and parental questionnaires. Random misclassification would lead to bias towards the null. When misclassification of the determinant is related to the outcome, bias may occur.[140] Exposure data in our studies, including fetal growth characteristics measured by ultrasound and other cardiovascular risk factors, were collected before assessment of the outcome, which makes differential misclassification of the exposure unlikely. Information about maternal smoking in pregnancy was obtained prospectively by questionnaires sent to the mothers. Although the mothers were not aware of the specific research questions addressed in this thesis, assessment of adverse life style habits by questionnaires may lead to underreporting. If underreporting would be selectively present among heavy smoking mothers who report low- to moderate smoking, the estimated differences in renal and cardiovascular structures between the offspring of non-smoking and low smoking mothers would be overestimated. To overcome the limitation of underreporting of smoking, other studies have used biomarkers of tobacco exposure including cotinine in maternal urine, saliva or blood samples or nicotine in indoor air.[141, 142] However, so far these studies have demonstrated that these biomarkers are not superior to self-report when studying the effect of maternal smoking on several outcomes of their offspring.[143, 144] In most of our studies, the outcome was assessed using ultrasound examination. Validation studies of these ultrasound measurements showed good repeatability and reproducibility between and among observers. Furthermore, the observers were blinded to the exposure status, which makes differential misclassification of the outcome less likely.

Confounding

The Generation R Study data collection provides information on many variables related to growth, development and health of the children. Therefore many potential confounders were available for the analyses. Confounding may be considered as biased effects, in which the apparent effect of the exposure of interest is distorted because the effect of an extraneous factor is mistaken for or mixed with the actual exposure effect.[1] A confounding factor should be associated with both the exposure and the outcome, and cannot be an intermediate in the causal chain from exposure to outcome. Adjustment for an intermediate in the causal pathway from exposure to outcome, or adjustment for a variable that is causally related to the exposure but only correlated to the outcome, is inappropriate. Although we had information on many variables of interest, we may have missed potential confounders. Residual confounding due to unmeasured variables such as ma-

ternal nutrition, medication use, and physical activity during pregnancy, might still be possible. According to the developmental plasticity hypothesis, adverse environmental exposures in fetal and early postnatal life lead to adaptations that permanently program the fetus' structure, physiology and metabolism, leading to both low birth weight and cardiovascular disease in adulthood. Thus, missing information on specific adverse exposures in fetal and early postnatal life may have introduced residual confounding in the studies presented in this thesis.

Most of the studies presented in this thesis were performed in the Generation R Focus cohort. Confounding might be less since the studied determinants were not or only moderately related to sociodemographic variables, due to the homogeneity (all children had Dutch ethnicity, defined as two parents and four grandparents born in The Netherlands, and 63% finished high education) of our sample. This underlines the benefits of studying etiological associations within a restricted and homogenous sample.

The study presented in chapter 4.6 was performed in the Avon Longitudinal Study of Parents and Children (ALSPAC). ALSPAC is a similar population-based prospective cohort study as the Generation R Study and investigates the health and development in children (www.alspac.bris.ac.uk).[132] Detailed information about the children has been collected from questionnaires administered through childhood, and clinical measurements have been performed on the entire cohort annually since the age of 7 years. In total, 6,668 mother-offspring pairs, approximately 65% of those invited, attended the 9 year follow-up clinic. Data on hypertensive disorders during pregnancy, offspring anthropometry, blood pressure and potential confounders were available for 6,343 (95%) of these children. Blood pressure and adiposity could be measured in 99% of the children participating in this study at the 9-year-old visit. Those attending the clinic did not differ in the amount of hypertensive disorders during pregnancy from the ones who were not included in the study. There was a minimal missing data amongst clinic participants for most variables. As results are based on complete case analysis, we do not think that these missing data has resulted in major bias.

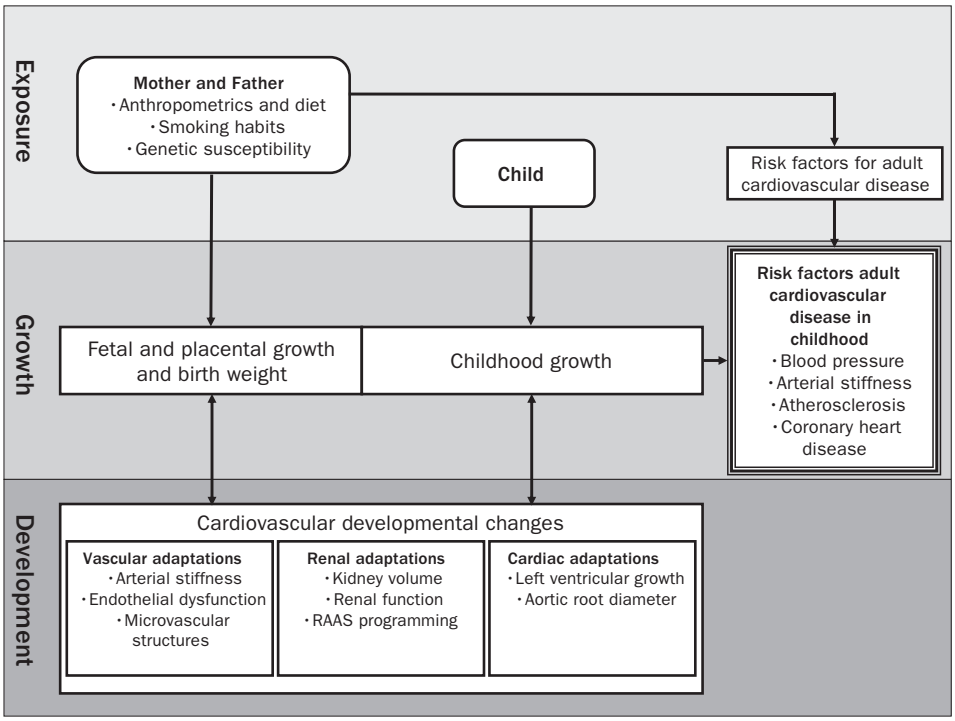
Perspectives

Study designs

The associations of fetal growth retardation and low birth weight with cardiovascular disease are not yet fully understood. Methodological issues, such as the role of potential confounders in the associations, should be further examined. Well-designed epidemiological studies are necessary to overcome current methodological issues. Population-based prospective cohort studies starting in the preconceptional period or in early fetal life, in which the offspring is followed from early fetal life until young adulthood, seem to be the most suitable epidemiological design (Figure 1). This epidemiological design is the best design for assuring quality of the data, taking account of

potential confounders and identifying growth patterns at risk. Recently, various population-based prospective cohort studies have been started worldwide.[131-134] However, a limitation of this design is the long period needed for the studied adult disease to develop. The design of a retrospective cohort study may lead to an earlier availability of data according to cardiovascular disease. However, this design will not take account of all potential confounders and will probably not be able to study the effects of fetal or early postnatal influences.

Figure 1. A developmental origins study model, studying the fetal origins of cardiovascular disease in epidemiological studies



This model presents core associations that have to be studied to unravel the underlying mechanisms of the associations of fetal growth restriction and low birth weight and cardiovascular disease later in life. The upper part shows associations in mother and father, identifying both determinants of fetal and postnatal growth patterns and environmental and genetic mechanisms. The lower part demonstrates associations in the offspring that have to identify growth patterns and developmental changes in fetal and early postnatal life, resulting in an increased risk of cardiovascular disease in adulthood.

RAAS, Renin-angiotensin-aldosterone system

The effects of many adverse fetal exposures due to maternal life style habits can only be studied in observational designs. However, for assessing the effects of nutritional exposures on fetal growth restriction and the risk of cardiovascular disease, randomised controlled trials would be the design of choice. For example, the impact of calcium, folate and other micronutrients on offspring birth weight and blood pressure is examined in randomised controlled trials.[30, 135, 136] Randomised controlled trials should be able to overcome current methodological issues and identify mechanisms in the causal pathway that underlie the associations.

Detailed exposure studies

Nutrition

In Western countries, maternal variation in dietary patterns and intake of micronutrients seems more relevant than a restricted total energy and macronutrient intake for having an effect on fetal growth retardation.[145] Future studies should focus on the intake of particular macronutrients and micronutrients, including calcium, folic acid and homocystein, in specific periods of pregnancy. Identifying critical periods in pregnancy will result in detailed dietary advices aimed at these specific time points in pregnancy. Additionally, dietary patterns during the preconception period have been associated with biomarker concentrations and related to complex diseases, such as cardiovascular disease.[146, 147] Overall dietary patterns may be easier for the public to interpret or translate into diets.

Genome-wide association studies

Common genetic variants may explain the associations between growth in early life and diseases in adulthood. Large scale genome-wide association studies (GWAS) have revealed links between DNA sequence variation and a growing range of diseases and continuous traits, such as cardiovascular disease and type 2 diabetes.[148-150] The effect of these common genetic variants on growth characteristics and growth patterns may already be present in fetal and early postnatal life. Relating these genetic loci to early growth patterns might identify genes related to both fetal growth retardation and cardiovascular disease.

Epigenetics

Epigenetics is understood as the heritable changes in gene expression potential that are not caused by changes in DNA sequence.[151, 152] DNA methylation is one of the best understood type of epigenetic modification and is a key epigenetic contributor to maintenance of gene silencing. CpG islands are mostly located in the control regions of genes, for example the promoter region of actively transcribed gene, and are generally unmethylated. Methylation of this promoter region leads to decreased binding of transcription factors and thereby to a reduced gene expression.[153] Maternal diet has been shown to dynamically affect DNA methylation status.[154] Methyl-supple-

mented diets include the methyl donors such as folic acid, vitamin B12, choline, L-methionine and zinc. Studies in sheep showed associations between reductions in the availability of Vitamin B12 and folate during the periconceptual period and alterations in methylation status of CpG islands in the offspring.[155] This may lead to widespread epigenetic modifications to the genome of the offspring. In humans, lower DNA methylation of the IGF-2 gene was reported in adults who were exposed to the Dutch Famine in the periconceptional period.[156] These results suggest that early-life environmental conditions can cause epigenetic changes that persist throughout life and could lead to the development of risk factors for hypertension and cardiovascular disease in adult offspring. However, future research is needed to better understand the role of epigenetic dysregulation in the associations between adverse fetal nutritional exposures and diseases in adult life.[157] Since the periconceptional period is a crucial period for establishing and maintaining epigenetic marks, cohort studies starting in the preconceptional period would give important knowledge.[158]

Paternal influences

We discussed substantial affects on fetal programming by maternal prenatal behaviors, specifically by maternal smoking, maternal diet and maternal genetic susceptibility. However, paternal influences, for example paternal genetic susceptibility, could also underlie the pathways of fetal growth retardation and low birth weight to the development of cardiovascular disease in adulthood. Also, comparing maternal and paternal effects provides a method of separating intra-uterine effects from associations related to familial or environmental factors. Similar associations of maternal and paternal exposures with offspring health outcomes suggest that common factors related to the family or environment may drive the associations, rather than intrauterine effects only. Future research is required to compare associations of both maternal and paternal exposures during pregnancy with components of offspring health.

Detailed cardiovascular development studies

To disentangle the mechanisms underlying the associations of fetal growth restriction and low birth weight with the development of cardiovascular disease, future research should focus on early markers of cardiovascular adaptation. Good repeatability and reproducibility of most left cardiac structures in children measured by 2D ultrasound has been previously demonstrated in large-scale multicenter studies in young children.[159,160] However, newer imaging techniques, such as three-dimensional echocardiography and magnetic resonance imaging, can more precisely calculate physiologic variables of interest.[161] In the future, these new imaging techniques will offer great opportunities for detailed cardiovascular measurements in epidemiological research. Thereby, subtle developmental cardiovascular changes can be identified already in fetal or early postnatal life. Furthermore, focusing on other biomarkers of cardiac development, including

detailed repeated measurements of right cardiac structures, may give newer inside in the pathway leading to the development of cardiovascular disease.

Although there is limited direct evidence that risk factors measured in childhood are predictive of atherosclerosis in adulthood[162, 163], less is known about the associations of fetal and maternal factors during pregnancy and vascular changes in childhood. Endothelial function and structural arterial changes, including arterial resistance and arterial stiffness, can be measured noninvasively in early childhood with high resolution ultrasound to measure brachial artery flow-mediated dilatation and carotid artery intima-media thickness, respectively.[164, 165] Furthermore, future studies should focus on additional markers of vascular structures and function, including retinal arteriolar narrowing. This may lead to peripheral vascular resistance and thereby predispose a person to the development of hypertension and cardiovascular disease.[166]

Clinical implications and future research

The associations between low birth weight and cardiovascular disease in later life seem to be one of the most intriguing and controversial epidemiological findings of the last decades and might have clinical and public health implications. However, the effect size of low birth weight on cardiovascular disease and blood pressure in adulthood presented in current studies seems to be small.[1, 167] Furthermore, the specific adverse exposures and underlying mechanisms are not known. To explore the underlying mechanisms further and to assess potentials for clinical implications, research has to move onwards from birth weight association studies to adverse fetal exposures and detailed early cardiovascular development studies. Since plasticity operates across the entire range of the environment and leads to multigenerational cycles of disease, any rational approach to health care should start early in life and take a cross-generational perspective.[168] Data from prospective cohort studies confirm the existence of a window of opportunity for intervention in early childhood.[169, 170] The growing awareness of the importance of the preconceptual period, when nutrition can have long-lasting effects without causing any change in birth weight, underscores the importance of healthy nutrition during the prepregnancy period. Research should also focus on identifying individuals at greater risk of later poor health and develop strategies aimed at these specific individuals.

Conclusion

The associations between low birth weigh and cardiovascular disease are present within the normal and physiological ranges, and suggest that specific exposures in different periods of fetal and early postnatal life have permanent consequences for cardiovascular growth and function. The mechanisms underlying these associations are not known, but may include environmental and genetic determinants. Future studies should be focused on these mechanisms and epigenetic

modifications of specific genes related to cardiovascular development. Furthermore, studies should focus on detailed cardiovascular adaptive responses in fetal life and early childhood by use of new imaging and functional techniques. Eventually results from these studies may lead to improved health in childhood and adulthood by promoting a better fetal environment.

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6

Summary

In **chapter 1** the background and scope of the studies presented in this thesis are given. Many lines of evidence indicate an important role of early life events in influencing later susceptibility to cardiovascular disease. These observations resulted in the "developmental plasticity hypothesis", which suggest that an organism may develop in various ways, depending on the particular environment or setting. In this process, adverse environmental exposures in fetal and early postnatal life lead to adaptations that permanently program the fetus' structure, physiology and metabolism. Birth weight has been the most widely studied measure of fetal growth in studies examining the associations of birth-related measures with risk factors of disease in later life. For further exploring the underlying mechanisms and to assess whether these associations are causal, research has to move onwards from birth weight association studies to adverse fetal exposure and detailed early cardiovascular development studies.

In **chapter 2** the associations between genetic determinants with fetal and postnatal growth are described. In **chapter 2.1** we hypothesized that variants of the insulin-like growth factor-1 (*IGF1*) gene are associated with growth patterns from fetal life until infancy. Eight alleles of the *IGF1* promoter region were identified. In total, 43% of the subjects were homozygous for the most common 192-bp allele (wild-type), 45% were heterozygous, and 12% were non-carrier of the 192-bp allele. No differences were found in birth weight between the three groups. However, non-carriers had a lower estimated fetal weight in mid-pregnancy, which may be an indication of growth restriction in early pregnancy, followed by an increased growth rate until 6 months in comparison to 192-bp homozygotes. A similar difference in growth rate was found for length. An explanation for our findings could be that the absence of the wild-type 192-bp allele might alter the transcription rate of *IGF1*. Alternatively, the *IGF1* promoter region may be in linkage disequilibrium with other regulatory elements, which in turn influence IGF-1 levels.

Chapter 2.2 shows the associations between the insulin gene variable number of tandem repeats (*INS* VNTR) and growth patterns in fetal life and infancy. The genotype distribution was I/I 50.8%; I/III 40.0%; and III/III 9.2%. Our data suggest that the III/III genotype may be associated with asymmetrical growth in mid-pregnancy, but not in late pregnancy. III/III individuals had a shorter gestational age and a lower birth weight. There were no differences in birth weight after adjusting for gestational age. Class III homozygotes had a smaller abdominal circumference/head circumference ratio in mid-pregnancy, but not in late pregnancy. Also, III/III subjects had a relative decrease in head circumference (SDS) from mid-pregnancy to the age of 14 months. No other differences in pre- and postnatal growth characteristics and patterns were found. Alterations in insulin or IGF2 levels may explain part of the associations we found.

Glucocorticoids have an important role in early growth and development. Glucocorticoid receptor gene polymorphisms have been identified that contribute to the variability in glucocorticoid

sensitivity. In **chapter 2.3** we hypothesized that these glucocorticoid receptor gene polymorphisms are associated with growth in fetal and early postnatal life. The studied glucocorticoid receptor gene polymorphisms included *Bcl*I (rs41423247), *Tth*III*I* (rs10052957), GR-9β (rs6198), N363S (rs6195) and R23K (rs6189 and 6190). We showed that glucocorticoid receptor gene polymorphisms were not associated with fetal weight, birth weight and early postnatal weight. Also, no associations were found with length and head circumference. Neither were these polymorphisms associated with the risks of low birth weight or catch-up growth from birth to 24 months of age. Therefore, we concluded that these polymorphisms do not affect early growth. Further systematic searches for common genetic variants by means of genome-wide association studies will enable us to obtain a more complete understanding of what genes and polymorphisms are involved in growth in fetal life and infancy.

Chapter 3 of this thesis describes early determinants of kidney growth and development from third trimester of pregnancy until the age of two years. In **chapter 3.1** we demonstrated good reproducibility and agreement of most renal dimensions in children measured by ultrasound. Intraobserver intraclass correlation coefficients ranged from 0.93 (left and right renal width and right renal thickness) to 0.99 (left renal length) and interobserver intraclass correlation coefficients ranged from 0.64 (right renal thickness) to 0.90 (right renal length). These results confirm that ultrasound is an appropriate measure to assess renal dimensions in both clinical and epidemiological studies. Reference growth curves were constructed for kidney growth from third trimester of pregnancy until the age of two years are demonstrated in **chapter 3.2**.

Studies focused on kidney growth determinants are described in **chapters 3.3, 3.4 and 3.5**. In **chapter 3.3** we showed that maternal height and pre-pregnancy weight were associated with fetal kidney volume. This association may be explained by both environmental (maternal nutritional status) and common genetic factors. After adjustment for the same characteristics in late pregnancy, fetal growth and blood flow parameters in mid-pregnancy were not associated with kidney volume in late pregnancy, suggesting that the main influence of fetal growth on kidney volume in late pregnancy exerts after mid-pregnancy. In late pregnancy, all fetal growth characteristics were positively associated with kidney volume. Signs of fetal blood flow redistribution and raised placental resistance were associated with reduced kidney volume in late pregnancy. Furthermore, kidney volume was positively associated with amniotic fluid quantity, which may be a measure of fetal kidney function. Our results are in line with studies that found that the period of maximum kidney growth to occur between 26-34 weeks of gestation Growth restriction in this period most likely affects kidney size and volume considerably.

Chapter 3.4 showed that maternal smoking during pregnancy affects kidney development in fetal life. Compared to non-smoking, first trimester only and continued smoking were not associated with any fetal kidney characteristics. We observed a curved-shaped association between the number of cigarettes smoked in third trimester of pregnancy with fetal kidney size. Compared

to non-smoking, smoking less than 5 cigarettes per day was associated with larger combined kidney volume (difference 2.10 (95% confidence interval (CI): 0.87, 3.32) cm^3), while smoking more than 10 cigarettes per day was associated with smaller combined kidney volume (difference -1.90 (95% CI: -3.73, -0.06) cm^3). These results suggest a differential effect of maternal smoking during pregnancy, depending on the specific period and number of cigarettes smoked. The vasoactive mechanisms and effect of nicotine in different tissues could explain at least part of these associations.

In **chapter 3.5** we demonstrated that small kidney size in fetal life tends to persist in early childhood. Maternal height and pre-pregnancy weight were positively associated with kidney volume at the age of 2 years. This association may be explained by both genetic and environmental determinants. Third trimester fetal head circumference, abdominal circumference and estimated weight and postnatal length were positively associated with kidney volume at the age of 2 years, suggesting that adverse fetal growth patterns lead to smaller kidneys with a lower amount of nephrons postnatally. Preferential fetal blood flow to the brain was also associated with smaller kidneys, predisposing an individual to an increased risk of renal disease and hypertension in later life. Further research should be aimed at disentangling the causal mechanisms underlying the associations of various determinants with kidney growth and the long-term consequences.

Early determinants of cardiac growth and development until the age of two years are presented in **chapter 4**. We found good reproducibility and agreement of left cardiac structures in children measured by ultrasound in **chapter 4.1**. High intraobserver and interobserver intraclass correlation coefficients were found, ranging from 0.91 for interventricular septal thickness (95% CI: 0.78, 0.96) to 0.99 for aortic root diameter (95% CI: 0.97, 1.00), confirming that left cardiac structures can be measured in both clinical and epidemiological research projects.

Chapter 4.2 demonstrated moderate tracking of left cardiac structures during the first two years of life. Furthermore, small size at birth and hemodynamic variations in fetal life, including resistance indices of the umbilical and uterine arteries and cardiac output, have consequences for postnatal cardiac size and function. These results suggest that undernutrition in third trimester of pregnancy leads to persisting increase of left ventricular structures, mediated through cardiovascular adaptive changes in utero.

Chapter 4.3 examined whether maternal smoking during pregnancy leads to fetal arterial resistance adaptations and subsequently to fetal growth retardation and changes in postnatal blood pressure and cardiac development. First trimester smoking was not associated with third trimester placental and fetal blood flow adaptations. Continued smoking of ≥5 cigarettes/day was associated with increased resistance in the uterine, umbilical, and middle cerebral arteries and a decreased flow and diameter of the ascending aorta. Among mothers who continued smoking, estimated fetal weight at 30 weeks and birth weight were most affected in children with the highest umbilical artery resistance. Fetal arterial resistance indices were also associated

with aortic root diameter and left atrial diameter. Our findings suggest that fetal arterial resistance adaptations may be involved in the pathways leading from maternal smoking during pregnancy to both low birth weight and cardiovascular developmental changes in childhood in the offspring.

In **chapter 4.4** we showed that maternal weight gain until late pregnancy was associated with an increased growth of left ventricular mass from 6 weeks to 6 months (difference 0.46 grams per week for the highest tertile of weight gain compared to the lowest tertile (p-value <0.05)), suggesting that maternal health status during pregnancy may have permanent consequences for left ventricular mass in their children. We found no associations of maternal height, weight or body mass index with longitudinally measured left ventricular mass from 6 weeks to 6 months. The increased heart growth could be attributed to either the indirect growth stimulating effect of insulin or the direct growth stimulating effect of increased maternal energy intake during pregnancy.

In **chapter 4.5** we showed an association of the Glucocorticoid receptor-9β polymorphism (GR-9β, rs6198) with blood pressure and heart growth in early childhood. At the age of 24 months, homozygous variants showed an increased systolic blood pressure of 2.65 mmHg (95% CI: 0.16, 5.14), an increased heart rate of 9.14 beats per minute (95% CI: 0.22, 18.1) and an increased left ventricular mass of 5.01 grams (95% CI: 1.32, 8.71) compared to homozygous references. GR-9β polymorphism was significantly associated with left ventricular mass growth during the first 2 years. Our findings suggest that genetically determined differences in cortisol exposure affect cardiovascular development in early life. Future studies are needed to replicate these findings and should assess whether these relations are associated with development of cardiovascular disease.

Chapter 4.6 examined the associations of hypertensive disorders of pregnancy with blood pressure in the nine-year-old offspring. Offspring of women with a history of preeclampsia or gestational hypertension had increased systolic and diastolic blood pressure at the age of nine years (increase in systolic blood pressure of 2.05 mmHg (95% CI: 0.72, 3.38) and 2.04 mmHg (95% CI: 1.42, 2.67), respectively, compared to those with no hypertensive disorders of pregnancy). These associations do not appear to be explained by shared familial characteristics (genetic or behavioral) related to familial adiposity. Our findings suggest that different mechanisms underlie the associations of preeclampsia and gestational hypertension with offspring blood pressure, with some evidence that the former is in part mediated by the effect of preeclampsia on intrauterine growth restriction and preterm delivery.

In **chapter 5** the combined results of the aforementioned chapters were discussed in a broader perspective. In conclusion, the associations between fetal undernutrition and genetic susceptibility with cardiovascular development are present within the normal and physiologic ranges, and suggest that specific exposures in different periods of fetal and early postnatal life have permanent consequences for cardiovascular growth and function. The mechanisms underlying these associations are not known, but may include environmental and genetic determinants. Further studies are needed to unravel these mechanisms and to identify whether these cardiovascular adaptations have consequences for the development of cardiovascular disease in later life.

Samenvatting

In **hoofdstuk 1** worden de achtergrond en het doel van de studies in dit proefschrift besproken. Verschillende onderzoekslijnen hebben de afgelopen jaren verbanden aangetoond tussen een ongunstige foetale omgeving en het risico op hart- en vaatziekten op latere leeftijd. Deze observaties hebben geresulteerd in de *"developmental plasticity"* hypothese, die veronderstelt dat een organisme zich op verschillende manieren kan ontwikkelen, afhankelijk van een bepaalde omgeving of plaats. In dit proces leidt een ongunstige foetale omgeving tot aanpassingen in de ontwikkeling die permanent de structuur, fysiologie en het metabolisme van de foetus beïnvloedt. Op korte termijn leidt dit tot foetale groeivertraging en een laag geboortegewicht, maar op lange termijn kan het het risico op hart- en vaatziekten vergroten. Het geboortegewicht is de meest gebruikte maat als weerspiegeling van de foetale groei in studies die kijken naar verbanden tussen een ongunstige foetale omgeving en risicofactoren voor verschillende aandoeningen op latere leeftijd. Om na te gaan of deze relaties een oorzakelijk verband hebben en om nog onbekende onderliggende mechanismen te identificeren, is vervolgonderzoek nodig dat gericht is op foetale blootstellingen en groeipatronen in plaats van op het geboortegewicht. Tevens zal de focus meer moeten liggen op het nog gedetailleerder in beeld brengen van de vroege ontwikkeling van het hart- en vaatstelsel.

Voor mijn onderzoek werd gebruik gemaakt van gegevens verzameld in het Generation R onderzoek, een populatiegebaseerd prospectief cohort onderzoek vanaf het vroege foetale leven tot de jongvolwassenheid. Het doel van dit onderzoek is om de vroege omgevingsfactoren en genetische oorzaken van de normale en abnormale groei, ontwikkeling en gezondheid vanaf het foetale leven tot aan de jongvolwassenheid te identificeren. In totaal zijn 9.778 moeders opgenomen met een bevallingsdatum tussen april 2002 en januari 2006. In de vroege zwangerschap (zwangerschapsduur <18 weken), halverwege de zwangerschap (zwangerschapsduur 18-25 weken) en laat in de zwangerschap (zwangerschapsduur ≥25 weken) werden gegevens verzameld van zowel de ouders als de foetus met behulp van lichamelijk onderzoek, vragenlijsten, foetaal echo-onderzoek en bepalingen in bloed en urine. De meeste studies in mijn proefschrift zijn verricht in een subgroep van dit cohort, het Generation R Focus cohort. Dit is een groep van 1.232 moeders en hun kinderen met een Nederlandse nationaliteit. Deze groep werd nauwkeurig na de geboorte gevolgd, waarbij gedetailleerde metingen van onder andere de lengte, het gewicht, de lichaamssamenstelling, de groei en ontwikkeling van het hart en de nieren en de bloeddruk tot de leeftijd van twee jaar werden verricht.

In **hoofdstuk 2** wordt de rol van genetische factoren op de foetale en postnatale groei beschreven. In **hoofdstuk 2.1** testen we de hypothese dat variaties in het Insulin-like growth factor-1 (IGF-1) gen gerelateerd zijn aan groeipatronen vanaf het foetale leven tot aan de kinderleeftijd. We hebben acht allelen in de promotor regio van het IGF-1 gen geïdentificeerd. In totaal is 43% van

de onderzoekspopulatie homozygoot voor het meest voorkomende 192-bp allel (wild-type), 45% is heterozygoot en 12% is geen drager van het 192-bp allel. Er is geen verschil in het geboortegewicht tussen deze drie groepen. Daarentegen is het geschatte gewicht in het tweede trimester van de zwangerschap lager bij de groep die geen drager is van het 192-bp allel, wat een aanduiding kan zijn van groeivertraging in het eerste trimester. Tevens toont deze groep een versnelde gewichtstoename in de eerste zes maanden na de geboorte. Een vergelijkbaar verschil in groeisnelheid vinden wij voor de lengte van het kind. Toekomstig onderzoek is nodig om een vollediger beeld te krijgen van de functionaliteit van het IGF-1 gen en de relatie met groei en ziekte op de kinderleeftijd en later in het leven.

Hoofdstuk 2.2 toont de verbanden tussen een aantal zich herhalende frequenties in het insuline gen (*INS* VNTR) en groeipatronen vanaf het foetale leven tot aan de kinderleeftijd. Op basis van de hoeveelheid zich herhalende frequenties in het insuline gen is een indeling gemaakt in klasse I (30-44 herhalende frequenties) en klasse III (rond de 150 herhalende frequenties) dragers; klasse II is heel zeldzaam in de blanke populatie. De genotype verdeling is I/I 50.8%; I/III 40.0% en III/III 9.2%. Kinderen met het III/III genotype hebben een kortere zwangerschapsduur en een lager geboortegewicht, hoewel het verschil in geboortegewicht wegvalt na correctie voor zwangerschapsduur. Onze resultaten wijzen er op dat het III/III genotype gerelateerd is aan asymmetrische groei (een kleinere buikomvang/hoofdomvang ratio) in het tweede trimester van de zwangerschap, maar niet in het derde trimester van de zwangerschap. Vervolgonderzoek is nodig om een beter inzicht te krijgen in de functionaliteit van het *INS* VNTR gen en de relatie tussen de groei en het risico op ziekte op de kinderleeftijd en later in het leven.

Glucocorticoïden spelen een belangrijke rol bij de groei en ontwikkeling al vroeg in het leven. De afgelopen jaren zijn er variaties in het gen dat codeert voor de glucocorticoïd receptor geïdentificeerd. Deze genetische variaties spelen mogelijk een rol in de mate van gevoeligheid voor glucocorticoïden. In **hoofdstuk 2.3** onderzoeken we of deze variaties in het glucocorticoïd receptor gen gerelateerd zijn aan de groei in het foetale en vroeg postnatale leven. De volgende variaties in het glucocorticoïd receptor gen zijn bestudeerd: *Bcl*I, *TthIII*, GR-9β, N363S en R23K. Onze resultaten laten zien dat variaties in het glucocorticoïd receptor gen niet gerelateerd zijn aan het foetaal gewicht, het geboortegewicht en het gewicht op de kinderleeftijd. Evenmin vinden wij een verband met de lengte en hoofdomvang van de kinderen. Ook vinden wij geen verband met een groeiversnelling in de eerste twee levensjaren. Daarom concluderen wij dat deze genetische variaties geen invloed hebben op de groei vroeg in het leven. Toekomstige studies die gebruik maken van een genoom brede screen, zullen waarschijnlijk meer inzicht brengen in genen en genetische variaties die van invloed zijn op de groei in het foetale leven en op de kinderleeftijd.

Hoofdstuk 3 van dit proefschrift beschrijft verschillende factoren die vroeg in het leven van invloed zijn op de groei en ontwikkeling van de nieren vanaf het derde trimester van de zwangerschap tot de leeftijd van twee jaar. In **hoofdstuk 3.1** laten we zien dat echografie een betrouwbare methode

is om verschillende structuren van de nier te meten. We vinden intraobserver intraklasse correlatiecoefficiënten variërend van 0.93 (linker en rechter nierbreedte en nierdikte) tot 0.99 (linker nierlengte) en interobserver intraklasse correlatiecoefficiënten variërend van 0.64 (rechter nierdikte) tot 0.90 (rechter nierlengte). Deze resultaten laten zien dat het herhaald meten van dezelfde kinderen door één onderzoeker of door twee verschillende onderzoekers tot vergelijkbare resultaten leidt. Het echo-onderzoek is dus een geschikte methode om nierstructuren in zowel klinisch- als epidemiologisch onderzoek in beeld te brengen en als uitkomstmaat te gebruiken. Vervolgens hebben we referentiecurven gecreëerd voor de groei van de nieren vanaf het derde trimester van de zwangerschap tot de leeftijd van twee jaar. Deze curven worden gepresenteerd in **hoofdstuk 3.2**.

De **hoofdstukken 3.3, 3.4 en 3.5** beschrijven onderzoek gericht op factoren die de niergroei beïnvloeden. In **hoofdstuk 3.3** laten we zien dat lengte en gewicht van de moeder voorafgaand aan de zwangerschap gerelateerd zijn aan het niervolume tijdens het foetale leven. Deze verbanden kunnen verklaard worden door zowel omgevingsfactoren (zoals voedingsstatus van de moeder) als door genetische factoren. Foetale groei karakteristieken en de doorbloeding van de foetus in het tweede trimester van de zwangerschap zijn niet gerelateerd aan het niervolume in het derde trimester van de zwangerschap. In het derde trimester van de zwangerschap zijn alle foetale groei karakteristieken positief gerelateerd aan het foetaal niervolume. Tevens zijn tekenen van een herverdeling van de foetale doorbloeding en een verhoogde weerstand in het vaatbed van de placenta gerelateerd aan een kleiner niervolume in het derde trimester van de zwangerschap. Het niervolume is ook positief gerelateerd aan de hoeveelheid amnionvocht, wat een maat zou kunnen zijn voor de nierfunctie. Onze resultaten zijn in lijn met andere studies die hebben aangetoond dat tussen 26 en 34 weken zwangerschap de nieren hun maximale groeisnelheid hebben. Groeivertraging in deze periode van de zwangerschap zal dus het grootste effect hebben op de niergrootte en het niervolume.

In **hoofdstuk 3.4** laten we zien dat roken tijdens de zwangerschap invloed heeft op de groei en ontwikkeling van de foetale nieren. Roken in het eerste trimester van de zwangerschap heeft geen invloed op het niervolume in de foetale periode. We vinden wel effecten op de foetale niergroei bij vrouwen die bleven roken in de zwangerschap. Het roken van minder dan vijf sigaretten per dag is gerelateerd aan een groter gecombineerd niervolume (linker en rechter niervolume samen), terwijl het roken van meer dan tien sigaretten gerelateerd is aan een kleiner gecombineerd niervolume. Deze resultaten wijzen er op dat het effect van roken tijdens de zwangerschap op de nierontwikkeling afhangt van zowel het aantal sigaretten dat gerookt wordt als de periode van de zwangerschap waarin gerookt wordt. Het effect van roken op niervolume kan voor een deel verklaard worden door het zowel vernauwende als verwijdende effect van nicotine op de vaten in verschillende weefsels.

In **hoofdstuk 3.5** tonen we aan dat kinderen met kleine nieren in het foetale leven een grotere kans hebben op kleine nieren op de leeftijd van twee jaar. Dit verschijnsel wordt "*tracking*"

genoemd. De lengte en het gewicht van de moeder voorafgaand aan de zwangerschap zijn positief gerelateerd aan het niervolume op de leeftijd van twee jaar. Ook de hoofdomvang, de buikomvang en het geschat gewicht in het derde trimester van de zwangerschap en de lengte na de geboorte zijn positief gerelateerd aan het niervolume op de leeftijd van twee jaar. Dit wijst er op dat een ongunstige foetale omgeving en groei leiden tot kleinere nieren postnataal met mogelijk een verminderd aantal nefronen. Dit kan betekenen dat een individu een grotere kans heeft om op latere leeftijd een nieraandoening of een hoge bloeddruk te ontwikkelen. Toekomstig onderzoek zal zich voornamelijk moeten richten op de oorzakelijke mechanismen die de verbanden tussen foetale groei en de ontwikkeling van de nieren kunnen verklaren. Tevens zal de focus moeten liggen op de consequenties van onze bevindingen op de lange termijn.

Vroege invloeden op de groei en ontwikkeling van het hart in de eerste twee levensjaren staan beschreven in **hoofdstuk 4**. In **hoofdstuk 4.1** laten we zien dat verschillende structuren van de linkerhelft van het hart op deze leeftijd betrouwbaar te meten zijn met behulp van echografie. Hoge intraobserver intraklasse correlatiecoefficiënten, variërend van 0.91 voor de interventriculaire septumdikte (95% BI: 0.78, 0.96) tot 0.99 voor de diameter van de aortabasis (95% BI: 0.97-1.00), bevestigen dat structuren van het linkerhart betrouwbaar gemeten kunnen worden in zowel klinisch- als epidemiologisch onderzoek.

In **hoofdstuk 4.2** laten we zien dat kinderen met een kleiner linkerhart op de leeftijd van zes weken na de geboorte een grotere kans hebben om ook een kleiner linkerhart te hebben op de leeftijd van twee jaar. Tevens constateren we dat een laag geboortegewicht en foetale aanpassingen in de doorbloeding, zoals een hogere vaatweerstand in de arteria uterina en de arteria umbilicalis, evenals een veranderde output van het hart, consequenties hebben voor de grootte en de functie van het hart op de kinderleeftijd. Dit betekent dat factoren in het derde trimester van de zwangerschap mogelijk leiden tot permanente aanpassingen in structuren van het linkerhart.

Hoofdstuk 4.3 behandelt het effect van roken tijdens de zwangerschap op de groei en de ontwikkeling van het hart en de bloedvaten van de foetus en het kind na de geboorte in de eerste twee levensjaren. Roken in het eerste trimester van de zwangerschap is niet gerelateerd aan bloedstroom profielen van de foetus en de placenta in het derde trimester van de zwangerschap. Het roken van meer dan vijf sigaretten per dag gedurende de hele zwangerschap is gerelateerd aan een verhoogde weerstand in de arteria uterina, de arteria umbilicalis en de arteria cerebri media. Tevens is dit gerelateerd aan een verminderde bloedstroom en een kleinere diameter van de foetale aorta ascendens. Daarnaast constateren we dat de combinatie van roken gedurende de hele zwangerschap en een hoge weerstand in de arteria umbilicalis leidt tot de grootste aanpassing in het geschatte gewicht van de foetus bij dertig weken zwangerschap en het grootste verschil in het geboortegewicht ten opzichte van de referentiegroep. De weerstand in het arteriële vaatbed van de foetus is ook gerelateerd aan de diameter van de aortabasis en de diameter van

het linker atrium. Onze bevindingen wijzen er op dat aanpassingen in de foetale circulatie mogelijk betrokken zijn bij het proces waarbij roken tijdens de zwangerschap tot een laag geboortegewicht leidt, en uiteindelijk tot de ontwikkeling van hart- en vaatziekten later in het leven.

In **hoofdstuk 4.4** laten we zien dat de gewichtstoename van de moeder tijdens de zwangerschap leidt tot een versnelde groei van de linker ventrikel in de periode van zes weken tot zes maanden na de geboorte. Dit wijst er op dat de voedingsstatus van de moeder tijdens de zwangerschap permanente gevolgen kan hebben voor de ontwikkeling van de massa van de linkerventrikel bij haar kind. We vinden geen verband tussen de lengte, het gewicht en de body mass index van de moeder voorafgaand aan de zwangerschap en de massa van de linkerventrikel bij haar kind op de leeftijd van zes weken tot zes maanden na de geboorte. De versnelde hartgroei bij deze kinderen kan zowel het gevolg zijn van een indirect groeistimulerend effect van insuline als een direct groeistimulerend effect van de energie-inname van de moeder tijdens de zwangerschap.

In **hoofdstuk 4.5** tonen we aan dat er een verband bestaat tussen een variatie in het glucocorticoïd receptor-9β gen (GR-9β) en de bloeddruk en de groei van het hart op de kinderleeftijd. Homozygote dragers hebben een hogere bloeddruk en hartslag en een grotere massa van de linkerventrikel in vergelijking met kinderen die geen drager zijn van deze genetische variant. Onze bevindingen wijzen er op dat genetisch bepaalde verschillen in cortisol receptor gevoeligheid, invloed hebben op de ontwikkeling van het hart- en vaatstelsel op jonge leeftijd. Toekomstige studies zullen moeten uitwijzen of onze resultaten ook in andere populaties bevestigd kunnen worden en of deze kinderen later in het leven ook daadwerkelijk een groter risico hebben om hart- en vaatziekten te ontwikkelen.

Voor de laatste studie in dit proefschrift wordt gebruik gemaakt van gegevens uit de Avon Longitudinal Study of Parents and Children (ALSPAC), een populatiegebaseerd prospectief cohort onderzoek vanaf de geboorte tot de jongvolwassenheid. Het doel van dit onderzoek is de vroege omgevings- en genetische oorzaken van de normale en abnormale groei, ontwikkeling en gezondheid te identificeren. In totaal zijn 14.541 moeders, wonend in drie verschillende districten van Bristol (Groot-Brittannië) en met een bevallingsdatum tussen april 1991 en januari 1993, opgenomen in de studie. Gedetailleerde gegevens over de groei en ontwikkeling van de kinderen werden verkregen met behulp van vragenlijsten. Tevens werd het hele cohort tot de leeftijd van zeven jaar jaarlijks gezien in het onderzoekscentrum, waarbij diverse metingen werden verricht, zoals lengte, gewicht, lichaamssamenstelling, bloed- en urinebepalingen en de bloeddruk.

Gebruik makend van deze gegevens, tonen we in **hoofdstuk 4.6** aan dat een hoge bloeddruk van de moeder tijdens de zwangerschap, zoals pre-eclampsie en zwangerschapshypertensie, leidt tot een hogere bloeddruk bij het kind op de leeftijd van negen jaar in vergelijking met moeders zonder een hoge bloeddruk tijdens de zwangerschap. Dit verband blijkt onafhankelijk te zijn van een genetische aanleg om dik te worden. Onze bevindingen wijzen er op dat verschillende mechanismen ten grondslag liggen aan het verband tussen pre-eclampsie en zwangerschapshypertensie enerzijds en een hoge bloeddruk bij het kind op de leeftijd van negen jaar anderzijds. De relatie

met pre-eclampsie lijkt, tenminste voor een deel, verklaard te kunnen worden door het verband tussen pre-eclampsie en zowel foetale groeivertraging als vroeggeboorte.

In **hoofdstuk 5** worden de resultaten van dit proefschrift beschreven en in een breder perspectief geplaatst. In het algemeen kunnen we concluderen dat er ook in een relatief gezonde onderzoekspopulatie verbanden bestaan tussen factoren die wijzen op een ongunstige foetale omgeving en genetische factoren enerzijds en de ontwikkeling van het hart- en vaatstelsel anderzijds. Deze bevindingen wijzen er op dat invloeden in verschillende perioden tijdens het foetale en vroeg postnatale leven permanente gevolgen hebben voor de groei en functie van het hart en de bloedvaten. De mechanismen die hieraan ten grondslag liggen zijn voor een groot deel nog onbekend, maar zowel omgevingsfactoren als genetische invloeden lijken een belangrijke rol te spelen. Toekomstig onderzoek is van groot belang om deze mechanismen te ontrafelen en om te onderzoeken of de aanpassingen op jonge leeftijd ook consequenties hebben voor de ontwikkeling van hart- en vaatziekten op latere leeftijd.

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About the author

Miranda Geelhoed was born on April 20th 1982 in Gouda, The Netherlands. In 2000 she graduated from secondary school at the "Coornhert Gymnasium" in Gouda. She studied medicine at the Erasmus University Rotterdam. During her study she spent four months at the Department of Child Neurology at the Dalhousie University and the Queen Health Sciences Centre in Halifax, Canada (head Prof. P. Camfield). She worked there on her final thesis entitled "The accuracy of outcome prediction models for childhood onset epilepsy". In June 2005 she obtained her Master of Science degree in Clinical Epidemiology at the Netherlands Institute for Health Sciences. That same year, she started working as PhD student at the Department of Epidemiology (head Prof. A. Hofman) and the Department of Pediatrics (head Prof. A.J. van der Heijden) at the Erasmus University Rotterdam. Her research was embedded in the Generation R Study and focused on the early influences on cardiovascular development (co-promotor Dr. V.W.V. Jaddoe). From November 2006 until May 2008, she completed her internships, while she continued working on her thesis. In 2009, she worked for 3 months as research fellow at the Department of Social Medicine (head Prof. G. Davey Smith, Prof. D.A. Lawlor) at the University of Bristol. In Bristol, she performed research on maternal determinants of cardiovascular function in children in the Avon Longitudinal Study of Parents and Children (ALSPAC). February 2010 she will start as resident (ANIOS) at the Department of Pediatrics at the Erasmus MC - Sophia Children's Hospital in Rotterdam (Prof. A.J. van der Heijden, Dr. M. de Hoog).

PhD Portfolio Summary
Summary of PhD training and teaching activities

Name PhD student:	J.J.M. Geelhoed
Erasmus MC Departments:	Epidemiology and Pediatrics
Research School:	NIHES
PhD period:	Sept 2005 - Oct 2006 and June 2008 - Dec 2009
Promotor(s):	Prof.dr. A. Hofman and Prof.dr. A.J. van der Heijden
Supervisor:	Dr. V.W.V. Jaddoe

1. PhD training

	Year	Workload (ECTS)
General academic skills		
Biomedical English Writing and Communication, Erasmus MC Rotterdam	2008	1.4
Cursus presenteren, Erasmus Universiteit Rotterdam	2008	1.4
Instellingsgebonden regelgeving en stralingshygiëne niveau 5R, Erasmus MC Rotterdam	2009	0.7
Training Solliciteren naar een opleidingsplaats, KNMG, Leids Universitair Medisch Centrum	2009	1.4
Research skills		
- MSc Clinical Epidemiology, NIHES:	2004-2005	120
- Principles of Research in Medicine and Epidemiology	2005	0.7
- Introduction to Data-Analysis	2005	0.7
- Methods of Clinical Research	2005	0.7
- Pharmaco-epidemiology	2005	0.7
- Topics in evidence-based medicine	2005	0.7
- Study design	2005	4,3
- Classical Methods for Data-analysis	2005	5.7
- Clinical Epidemiology	2005	5.7
- Methodological topics in Epidemiological Research	2005	1.4
- Modern statistical Methods	2005	4.3
- Epidemiology of Major Diseases and Major Determinants	2005	0.7
- Advanced Diagnostic Research	2005	1.4
- Analysis of Time-varying Exposures	2005	1.4
- Advanced Analysis of Prognosis Studies	2005	1.4
In-depth courses		
Repeated measurements in clinical studies, NIHES	2005	1.4
Missing values in clinical research, NIHES	2005	1.4
SNPs and human disease, Molmed	2005	1.4
Genetic Epidemiology of Complex Diseases, NIHES	2008	1.4
Genome-wide Association Studies, NIHES	2008	1.4
Advances in Genome-wide Association Studies, NIHES	2009	1.4

(Inter)national conferences		
Symposium Generation R 2006 Maternal and fetal origins of cardiovascular disease, Rotterdam, The Netherlands. Oral: *Fetal growth and left cardiac structures.*	2006	0.7
Europediatrics Congress 2006, Barcelona, Spain. Oral: *Fetal growth and left cardiac structures.*	2006	1.4
DOHaD 2006, Utrecht, The Netherlands. Poster: *Maternal anthropometrics in pregnancy and left ventricular mass in infancy.*	2006	0.7
Symposium Generation R 2007 Fetal growth and development, Rotterdam, The Netherlands. Oral: *Fetal growth and kidney growth and development.*	2007	0.7
Europaediatrics congress 2008, Nice, France. Posters: *Tracking and determinants of kidney size from fetal life until the age of 2 years and Tracking and determinants of left cardiac structures from fetal life until the age of 2 years.*	2008	1.4
Nederlands Kindergeneeskunde Congres 2008, Veldhoven, The Netherlands. Oral: *Factoren van invloed op niergroei tot de leeftijd van 2 jaar.*	2008	0.7
Symposium Metabolomics of the Obese 2009, Rotterdam, The Netherlands. Oral: *The Generation R Study: studies on growth and body composition.*	2009	0.7
Europaediatrics congress 2009, Hamburg, Germany. Oral: *Glucocorticoid receptor gene polymorphisms do not affect growth in fetal and early postnatal life .and posters Glucocorticoid receptor gene-9 beta polymorphism is associated with systolic blood pressure and heart growth in early childhood and Maternal smoking during pregnancy, fetal arterial resistance adaptations and cardiovascular function in childhood.*	2009	1.4
DOhad 2009, Santiago de Chili, Chili.	2009	1.4
Seminars and workshops		
PhD day, Erasmus MC Rotterdam, The Netherlands.	2005	0.3
Symposium ABCD study, Amsterdam, The Netherlands.	2006	0.3
Symposium Stichting Kind en Groei 2008: Small for gestational age, Erasmus Universiteit Rotterdam, The Netherlands.	2008	0.3
Jonge onderzoekersdag Nederlandse Vereniging voor Kindergeneeskunde, Velthoven, The Netherlands.	2008	0.4
Symposium Generation R 2008: Prenatal and early Postnatal Brain development, De Doelen, Rotterdam, The Netherlands.	2008	0.3
Lof der Geneeskunst 2008: Virussen vogelvrij, De Doelen, Rotterdam, The Netherlands.	2008	0.3
Generation R retraite: Developmental origins of Disease, Rotterdam, The Netherlands.	2009	0.3

2. Teaching activities

	Year	Workload (ECTS)
Supervising practicals and excursions		
Supervising practicals study design, NIHES	2006	0.7
Supervising Master's theses		
Supervised Kimberley Meyers, student Health Sciences, VU Amsterdam. Thesis title: Arteriële vaatstijfheid bij kinderen. Is Pulse Wave Velocity een goede meetmethode?	2009	2.0