Microvasculature and Cardiovascular Risk Factors in Childhood

The Generation R Study

Olta Gishti

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Acknowledgements

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Microvasculature and Cardiovascular Risk Factors in Childhood

The Generation R Study

Microvasculatuur en cardiovasculair risicofactoren in de kindertijd

Het Generation R Onderzoek

Thesis

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Erasmus University Rotterdam
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Rector Magnificus

Prof.dr. H.A.P. Pols

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Manuscripts based on this thesis

Chapter 2.1
Gishti O*, Yesil GD*, Felix JF, Reiss I, Ikram MK, Steegers EA, Hofman A, Jaddoe VW, Gaillard R. Influence of maternal gestational hypertensive disorders on microvasculature in school-age children. The Generation R Study. *Submitted. *Authors contributed equally*

Chapter 2.2
Gishti O, Jaddoe VW, Felix JF, Reiss I, Hofman A, Ikram MK, Steegers EA, Gaillard R. Influence of maternal angiogenic factors during pregnancy on microvascular structure in school-age children. *Hypertension. 2015;65:722-8.*

Chapter 2.3
Gishti O, Gaillard R, Manniesing R, Abrahamse-Berkeveld M, van der Beek EM, Heppe DH, Steegers EA, Hofman A, Duijts L, Durmuş B, Jaddoe VW. Fetal and infant growth patterns associated with total and abdominal fat distribution in school-age children. *J Clin Endocrinol Metab. 2014;99:2557-66*

Chapter 2.4
Gishti O, Jaddoe VW, Duijts L, Steegers EA, Reiss I, Hofman A, Wong TY, Ikram MK, Gaillard R. Impact of birth parameters and early life growth patterns on retinal microvascular structure in children. The Generation R Study. *J Hypertension. doi:10.1097/HJH.0000000000000561*

Chapter 3.1
Gishti O, Gaillard R, Durmuş B, Hofman A, Duijts L, Franco OH, Jaddoe VW. Infant diet and metabolic outcomes in school age children. *Eur J Clin Nutr. 2014;68:1008-15*

Chapter 3.2
Gishti O, Jaddoe VW, Duijts L, Franco OH, Hofman A, Ikram MK, Gaillard R. Influence of breastfeeding on retinal vessel calibers in school-age children. The Generation R Study. *Eur J Clin Nutr. In Press*

Chapter 3.3
Gishti O, Gaillard R, Durmuş B, Abrahamse-Berkeveld M, van der Beek EM, Hofman A, Franco OH, De Jonge LL, Jaddoe VW. Body mass index, total and abdominal fat distribution and cardiovascular risk factors in school-age children. *Pediatric Research. doi:10.1038/pr.2015.29*

Chapter 3.4
Gishti O, Jaddoe VW, Hofman A, Wong TY, Ikram MK, Gaillard R. Body fat distribution, metabolic and inflammatory markers and retinal microvasculature in school-age children. The Generation R Study. *Submitted*

Chapter 3.5
Gishti O, Jaddoe VW, Felix JF, Klaver CC, Hofman A, Wong TY, Ikram MK, Gaillard R. Retinal microvasculature and cardiovascular health in childhood. The Generation R Study. *Pediatrics. 2015;135:678-85*

Chapter 1 | Introduction

General introduction

Introduction

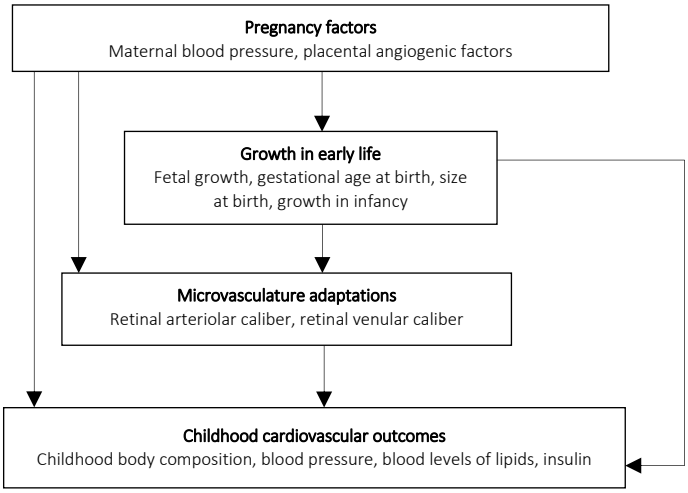
Cardiovascular disease is the leading cause of mortality, morbidity and hospitalization worldwide, and is a major public health problem in adult populations.[1] The developmental-origins hypothesis suggests that cardiovascular disease might originate from early life.[2] Adverse exposures, acting in different periods of fetal and early postnatal life might lead to permanent adaptations in the cardiovascular system, which are beneficial for short term survival, but increase the susceptibility of cardiovascular disease in later life.[2] This hypothesis is supported by experimental studies in animals showing that growth restriction in early life leads to developmental adaptations in cardiovascular structure and function, which leads to an increase in vulnerability to cardiovascular disease.[3, 4]

In line with this hypothesis, large observational studies in humans have shown that fetal growth restriction and rapid infant growth are associated with cardiac and vascular changes in childhood and an increased risk of cardiovascular disease in adulthood.[5] Also, observational studies using more detailed adverse exposures during fetal life suggested that, among other maternal factors, higher maternal blood pressure during pregnancy and the presence of gestational hypertensive disorders are associated with increased risks of fetal growth restriction and a higher blood pressure in childhood.[6, 7] Postnatally, suboptimal infant nutrition and increased adiposity levels throughout childhood are also shown to be associated with the development of cardiovascular disease in later life.[8, 9] Thus, previous research suggests that a restricted nutritional in utero environment and abundant postnatal environment may lead to cardiovascular disease in later life.

The mechanisms relating adverse maternal, fetal and infant factors with an increased risk of cardiovascular diseases in later life are not fully understood. Early microvasculature adaptations, in response to adverse exposures in early life, might be part of the underlying mechanisms in the development of cardiovascular disease.[10] Animal studies have shown that alterations in the microvascular structure and, hence, increased peripheral resistance, precede the development of hypertension.[11] In humans, the microvasculature can non-invasively be assessed by using retinal vascular imaging.[12] Several longitudinal studies among adults have shown that retinal arteriolar narrowing, likely indicative of increased peripheral vascular resistance, is associated with increased risks of hypertension and stroke in later life, whereas wider retinal venular caliber is associated with an increased risk of metabolic syndrome and inflammation.[13-15] Thus, these studies suggest that alterations in retinal vessel calibers can be used as early markers of cardio-metabolic disease risk.

In summary, cardiovascular disease might already originate in early life. Identifying risk factors and potential mechanisms influencing the development of cardiovascular diseases from early life onwards, is important for future preventive strategies that aim to improve cardiovascular health throughout the life course. Therefore, studies presented in this thesis were designed to identify maternal, fetal and infant factors associated with microvasculature alterations and cardiovascular health outcomes in childhood. The overall hypothesis of this thesis is given in the **Figure 1.1**.

Figure 1.1. Overview of hypothesis for the associations of maternal, fetal and infant factors with microvasculature adaptations and cardiovascular health in childhood studied in this thesis



Maternal, fetal and infant factors

Various maternal physical and lifestyle related characteristics during pregnancy are associated with an adverse cardiovascular profile in the offspring. Impaired fetal growth and accelerated infant growth are also associated with an increased risk of adverse cardiovascular outcomes in later life. Not much is known about the mechanisms underlying these observed associations. Alterations in microvasculature due to adverse exposures in early life, may be part of the underlying mechanisms. Therefore, the studies presented in this thesis are focused on the associations of maternal blood pressure development and angiogenic factors during pregnancy with childhood microvasculature. We also studied the associations of fetal and infant growth, infant diet and childhood body fat distribution with childhood microvasculature and cardiovascular risk factors.

Maternal blood pressure

Maternal gestational hypertensive disorders are associated with long-term offspring cardiovascular consequences.[6, 16] Offspring of mothers who develop preeclampsia during pregnancy have an increased risk of fetal growth restriction, higher blood pressure levels and increased risk of stroke in later life.[7, 17, 18] Gestational hypertensive disorders represent the extreme of the spectrum of maternal blood pressure development during pregnancy.[19] Several studies have also shown that higher maternal blood pressure levels during pregnancy across the full spectrum are associated with higher blood pressure levels in their offspring.[6, 20] The mechanisms underlying these associations might involve early microvasculature adaptations in the offspring.[21] It has been shown that maternal gestational hypertensive disorders are associated with endothelial dysfunction in the offspring.[22] However, not much is known about the influence of maternal cardiovascular status during pregnancy on structural microvasculature alterations in the offspring.

From an etiological perspective it is important to obtain a better understanding of the associations of maternal blood pressure development during pregnancy with offspring microvasculature structure alterations.

Maternal angiogenic factors

Suboptimal fetal and placental vascular development may have a persistent influence on cardiovascular function in childhood.[23] Placenta growth factor (PlGF) and soluble fms-like tyrosine kinase (sFlt-1) are important angiogenic factors that play a key role in the development of an adequate placental circulation.[24, 25] It has been suggested that lower levels of PlGF can disrupt the normal process of neo-angiogenesis and higher levels of sFlt-1 are associated with systemic endothelial dysfunction, which can result in a suboptimal feto-placental vascular development.[26-28] To study whether angiogenic factors during pregnancy not only affect placental and fetal vessel development, but may also persistently influence microvasculature structures postnatally, might give further insight into the mechanisms underlying the development of hypertension in later life.

Fetal and infant growth

Low and high birth weight are associated with a higher body mass index and higher blood pressure in childhood and adulthood.[29-33] Most studies used birth weight as proxy for fetal growth. However, birth weight is the result of various fetal growth patterns and the starting point of different infant growth patterns. Studies using more detailed fetal growth measures have also shown that impaired fetal growth, already from first trimester onwards, is associated with an adverse cardiovascular profile in later life.[34] The rate of infant weight gain is also associated with an increased risk of adverse cardiovascular risk factors in childhood, independent of birth weight.[35] Thus, previous studies suggest that there might be critical periods of growth in fetal life and infancy for the development of overweight and an adverse cardiovascular profile in later life. Therefore, it is important from a biological and preventive perspective to identify these critical periods in early life.

Infant diet

Early nutritional exposures may chronically affect cardiovascular development and lead to cardiovascular diseases in later life.[8] Previous studies suggest that breastfeeding during infancy may have a protective effect on the development of cardio-metabolic diseases and their risk factors in adulthood.[36-38] Studies in children also suggested that shorter breastfeeding duration and an early age of introduction of solid foods is associated with a higher body mass index, higher cholesterol and insulin levels, but results are inconsistent.[39, 40] The associations of breastfeeding on the risk of cardiovascular disease in later life might be explained by early cardiovascular structural and functional adaptations in response to early nutritional exposures.[41, 42] However, the previously suggested associations may also be explained by family related socio-demographic characteristics and maternal life style related factors. Thus far, no previous study has examined the associations of infant breastfeeding with microvasculature adaptations in later life.

Childhood body fat distribution

Childhood obesity is associated with cardiovascular diseases in later life.[43] Among adults, it has been shown that total body fat mass and waist circumference are, independent from body mass index, associated with adverse cardiovascular risk factors and the risk of all-cause mortality. These findings suggest that total body fat mass and abdominal fat mass might be more strongly associated with adverse health outcomes than body mass index.[44, 45] Thus far, studies focused on the associations of detailed total and abdominal fat mass with cardiovascular risk factors in children show inconsistent results.[9, 46] From an etiological perspective, it is important to obtain further insight in the associations of more detailed fat mass measures with microvasculature alterations and cardiovascular risk factors in childhood.

Childhood retinal vessel calibers and cardiovascular health

The development of hypertension might originate in early life.[2] Early alterations in the microvasculature structure might be part of the underlying mechanism leading to the development of hypertension.[10] Experimental studies in rats have shown that alterations in the microvascular structure precede the development of hypertension.[11] Studies in adults have shown that retinal arteriolar narrowing is strongly associated with higher blood pressure, and independently predicted the risk of stroke.[14, 47] Wider retinal venular caliber in adulthood is associated with an increased risk of the metabolic syndrome and measures of inflammation.[13] However, it remains unclear whether microvascular abnormalities are a result of cardiovascular disease or are part of the factors that relate to the development of these diseases. Thus, examining the associations retinal microvasculature with cardiovascular risk factors among children, without clinical cardiovascular disease, may provide further insight in the pathways underlying these associations.

General aim of this thesis

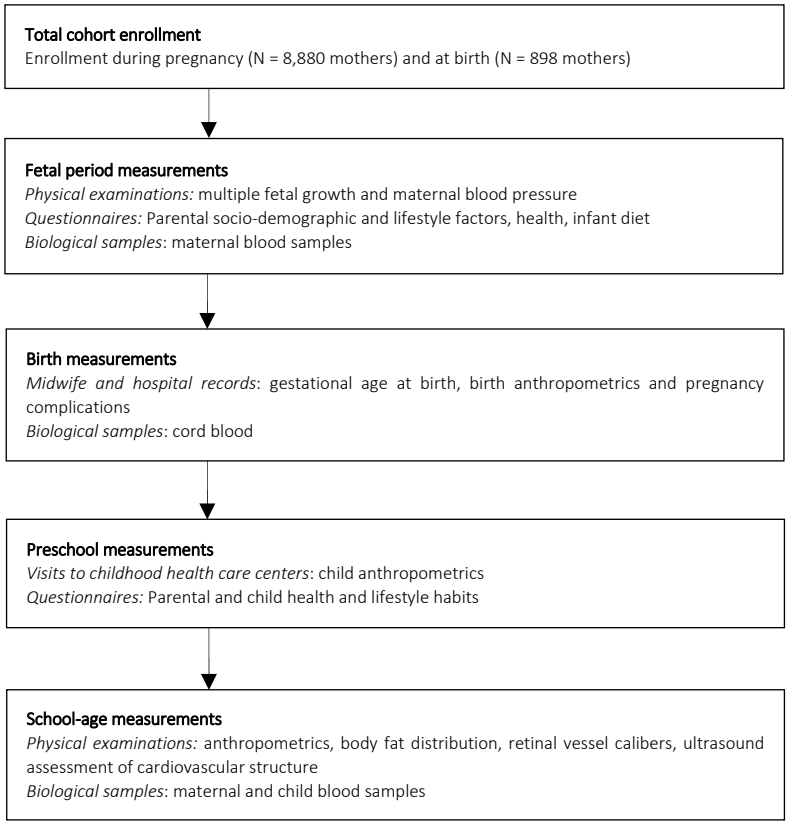
The general aim of this thesis was to identify maternal hemodynamic factors, fetal and infant factors leading to retinal vessel alterations and adverse cardiovascular outcomes in children.

General design

The studies presented in this thesis were embedded in the Generation R Study, a population based prospective cohort study from fetal life until young adulthood in Rotterdam, The Netherlands.[48] The Generation R Study is designed to identify early environmental and genetic 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 this study. Enrolment was aimed at early pregnancy, but was possible until the birth of the child. In total, 9,778 mothers were enrolled in the study, of whom 8,880 (91%) were included during pregnancy (**Figure 1.2**). Assessments were planned in early pregnancy (<18 weeks of gestation), mid-pregnancy (18 - 25 weeks of gestation) and late pregnancy (≥25 weeks of

gestation), and included parental physical examinations, maternal blood and urine collection, fetal ultrasound examinations, and self-administered questionnaires. In the preschool period, from birth to 4 years of age, data collection was performed in all children by questionnaires and visits to the routine child health care centers. All children were invited to a dedicated research center in the Erasmus MC – Sophia Children's Hospital to participate in detailed body composition and cardiovascular follow-up measurements at the age of 6 years. Measurements during this visit included anthropometrics, body composition, cardiovascular development and body fluid specimen collection.

Figure 1.2. Design and data collection in the Generation R Study



Outline of this thesis

The objectives are addressed in several studies presented in this thesis. In **Chapter 2**, studies on pregnancy influences on childhood microvasculature and cardiovascular outcomes are described. In **Chapter 2.1**, we examined whether maternal blood pressure in different pregnancy periods and gestational hypertensive disorders are associated with childhood microvasculature adaptations. The influence of angiogenic factors dur-

ing pregnancy on childhood retinal vessel calibers is described in **Chapter 2.2.** We studied the associations of fetal and infant growth patterns with childhood body fat distribution and childhood retina vessel calibers in **Chapter 2.3** and **2.4**, respectively.

In **Chapter 3**, we present studies focused on the associations of infant and childhood factors with cardiovascular risk factors in school-age children. The influence of breastfeeding patterns and the age at introduction of solid foods on childhood metabolic outcomes and microvasculature are given in **Chapter 3.1** and **Chapter 3.2**, respectively. In **Chapter 3.3** and **3.4**, we examined whether childhood body fat distribution and inflammatory markers are associated with cardiovascular risk factors or microvasculature alterations in children at 6 years old, respectively. **Chapter 3.5** describes the associations of retinal vessel calibers with cardiovascular risk factors in childhood.

Finally, **Chapter 4** provides a general discussion in which the studies described in this thesis are described in broader context, and implications and suggestions for future research are discussed.

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Chapter 2 | Pregnancy factors

Chapter 2.1

Maternal gestational hypertensive disorders and childhood microvasculature

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Abstract

Background: Gestational hypertensive disorders may lead to persistent vascular changes in the offspring. We examined the associations of maternal blood pressure development and hypertensive disorders during pregnancy with microvasculature adaptations in childhood, measured as retinal vessel calibers.

Methods and Results: In a population-based prospective cohort study from early pregnancy onwards among 3,748 pregnant mothers and their children, we measured maternal blood pressure in different periods of pregnancy. Information about gestational hypertensive disorders was obtained from medical records. At the age of 6 years, retinal arteriolar and venular calibers were measured from retinal photographs. We observed that higher maternal systolic and diastolic blood pressure in early pregnancy were associated with childhood retinal arteriolar narrowing (differences: -0.08 SDS (95% CI -0.12, -0.04) and -0.05 SDS (95% CI -0.09, -0.01), per SDS increase in systolic and diastolic blood pressure, respectively). These associations were independent from maternal blood pressure in mid- or late pregnancy. Maternal blood pressure in mid-pregnancy was not independently associated with childhood retinal vessel calibers, whereas higher maternal systolic blood pressure in late pregnancy was associated with childhood narrower retinal venular caliber only (difference: -0.05 SDS (95% CI -0.08, -0.01), per standardized residual increase in systolic blood pressure). Children of mothers with gestational hypertensive disorders tended to have narrower retinal arteriolar caliber (difference: -0.13 SDS (95% CI -0.27, 0.01)).

Conclusions: Higher maternal blood pressure during pregnancy is associated with persistent microvasculature adaptations in their children. Further studies are needed to replicate these observations and to examine the long-term consequences.

Introduction

Maternal gestational hypertensive disorders are associated with long-term offspring cardiovascular consequences.[1, 2] Gestational hypertensive disorders represent the extreme of the spectrum of maternal blood pressure development during pregnancy.[3] Several studies have also shown that higher maternal blood pressure levels during pregnancy across the full spectrum are associated with higher blood pressure levels in their offspring.[1, 4] Early fetal and placental microvasculature adaptations may explain the associations of maternal blood pressure levels during pregnancy with blood pressure levels in the offspring.[5] Microvascular adaptations can be assessed by retinal vessel caliber measurements.[6] Retinal arteriolar narrowing has been shown to precede the development of high blood pressure levels and is related to cardiovascular diseases.[7, 8] Thus far, no studies have examined detailed data on maternal blood pressure at different time points during pregnancy to identify critical periods for offspring microvasculature structure alterations.[9] Also, no previous study examined the associations of both maternal and paternal blood pressure during pregnancy with childhood microvasculature alterations, which could aid in further disentangling underlying mechanisms.[10] Stronger associations for maternal blood pressure suggest direct intra-uterine mechanisms, whereas similar or stronger associations for paternal blood pressure suggest a role for shared family-based lifestyle-related factors or genetic factors.

Therefore, we examined, among 3,748 mothers and their children participating in a population-based prospective cohort study from early pregnancy onwards, the associations of maternal blood pressure in different periods of pregnancy and gestational hypertensive disorders with childhood retinal vessel calibers.

Methods

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[11] All children were born between 2002 and 2006. Response rate at baseline was 61%.[11] The study protocol was approved by the Medical Ethical Committee of the Erasmus University Medical Center, Rotterdam. Written consent was obtained from all parents. Assessments during pregnancy were planned in early, mid and late pregnancy. In total 8,879 mothers were enrolled during pregnancy, of whom 8,860 had at least one maternal blood pressure measurement available. In total 5,910 mothers and their children participated in detailed cardiovascular follow-up studies. Retinal vessel measurements were available in 3,748 children. Missing retinal vessel measurements were mainly due to the later start of these measurements during the follow-up visits (Supplemental **Figure S2.1.1**). Results from non-response analysis showed preeclampsia was more frequent among mothers who were not included in the analyses, as compared to mothers included in our analyses, whereas no differences were present for mean systolic and diastolic blood pressure. Birth weight and gestational age at birth were lower among children who were not included in the analyses (Supplemental **Table S2.1.1**).

Maternal and paternal blood pressure during pregnancy

Maternal blood pressure was measured with the validated Omron 907 automated digital oscillometric sphygmanometer (OMRON Healthcare Europe B.V. Hoofddorp, the Netherlands).[12] All participants were seated in upright position with back support, and were asked to relax for 5 min. A cuff was placed around the non-dominant upper arm, which was supported at the level of the heart, with the bladder midline over the brachial artery pulsation. In case of an upper arm circumference exceeding 33 cm, a larger cuff (32–42 cm) was used. The mean value of 2 blood pressure readings over a 60 second interval was documented for each participant. In total, blood pressure was measured in 2,870 mothers in early pregnancy (median 13.2 weeks of gestation, 95% range 9.6–17.4), in 3,516 mothers in mid-pregnancy (median 20.4 weeks of gestation, 95% range 18.5–23.5), and in 3,606 mothers in late pregnancy (median 30.2 weeks of gestation, 95% range 28.5–32.8). In total, 9,992 blood pressure measurements were available. Blood pressure of the father was measured once and available for 2,645 fathers.

Gestational hypertensive disorders

Information about gestational hypertensive disorders was obtained from medical records. For women suspected of pregnancy complications based on these records, the information was checked with the original hospital charts. Details of these procedures have been described elsewhere.[13] Briefly, the following criteria were used to identify women with gestational hypertension: systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg after 20 weeks of gestation in previously normotensive women. These criteria plus the presence of proteinuria (defined as two or more dipstick readings of 2+ or greater, one catheter sample reading of 1+ or greater, or a 24 h urine collection containing at least 300 mg of protein) were used to identify women with preeclampsia.[14] Information on pregnancy complications was available for 3,630 mothers.

Retinal microvasculature assessment

At the age of 6 years retinal photographs were taken by a well-trained staff in a dedicated research center. Digital retinal photographs centered on the optic disc were taken of one eye with images resolution 4096 and 3072 pixels, using a Topcon digital retinal camera (model TRC, NW300). We used the same methods and protocols to measure retinal vascular caliber from the digitized retinal photographs, as described in previous studies among adults and children.[15-18] Briefly, a semi-automatic computer-imaging program was used to measure the caliber of the 6 largest retinal arterioles and venules located one half to 1 disc diameter from the optic disc margin.[19] These measurements were summarized as central retinal arteriolar and venular equivalents, representing the average arteriolar and venular caliber of that eye, respectively.[20] Graders, masked to birth parameters and other participant characteristics, operated the computer program and monitored all retinal measurements. We constructed grader specific standard deviation scores for both central retinal and arteriolar equivalents. Interclass correlation coefficients between graders were 0.79 for retinal arteriolar caliber and 0.89 for retinal venular caliber, which suggests adequate reproducibility.

Covariates

We obtained information about maternal and paternal age, education level and from questionnaires at enrollment.[11] Parental height (cm) and weight (kg) were assessed at enrollment. Body mass index (kg/m2) was calculated. Maternal parity, folic acid supplement use and smoking during pregnancy was obtained by questionnaires. Gestational age was established by fetal ultrasound examination during the first ultrasound visit.[11] Birth weight was obtained from medical records. Child's ethnicity (European, Non-European) was classified by the countries of birth of the parents.[21] Information on breastfeeding and average TV watching time was assessed by questionnaire.[22] At the age of 6 years, we measured childhood height to the nearest 0.1 cm and weight to the nearest grams using an electronic scale (SECA 888, Almere, The Netherlands), and body mass index (kg/m2) was calculated. Systolic and diastolic blood pressure was measured at the right brachial artery, four times with one-minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus TM (Paramus, NJ, USA). We used the mean of the last three blood pressure measurements for the analyses.[23]

Statistical analyses

First, we used conditional regression analyses to examine the independent associations of maternal blood pressure development in each period of pregnancy with retinal vessel calibers in childhood, taking into account the correlation between maternal blood pressure in early-, mid- and late pregnancy.[24] For these analyses, we constructed new systolic and diastolic blood pressure variables, which are statistically independent from each other, by using standardized residuals obtained from linear regression models of maternal systolic and diastolic blood pressure regressed on all prior corresponding blood pressure measurements.[24] This allows simultaneous inclusion of all maternal systolic and diastolic blood pressure measures into one regression model. Second, we examined the associations of paternal blood pressure in early pregnancy with childhood retinal caliber and tested whether the magnitude of these associations were statistically significantly different from the associations for maternal early pregnancy blood pressure with childhood retinal vessel caliber, by assessing the heterogeneity between the effect estimates. Finally, we assessed the associations of gestational hypertensive disorders with childhood retinal vessel calibers. For all analyses, we constructed different models: a basic model which was adjusted for age and sex of the child only; a confounder model which was additionally adjusted for maternal age, pre-pregnancy body mass index, education, parity, folic acid supplementation and smoking during pregnancy, child's ethnicity, breastfeeding and TV watching. The paternal models included paternal age, educational level, ethnicity and paternal body mass index instead of maternal age, educational level, ethnicity and maternal body mass index. Next, these models were additionally adjusted for gestational age at birth and birth weight to explore whether the observed associations were explained by birth outcomes (birth model) and for child's current body mass index and blood pressure to explore whether any association was explained by current childhood factors (childhood model). We considered the confounder model as the main model. For all analyses, we constructed standard deviation scores (SDS (observed value – mean) / SD)) for blood pressure and retinal vessel measures. We performed multiple imputations for missing values of covariates (<25%

missing values). Five datasets were created and analyzed together.[25] Analyses were performed using the Statistical Package of Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Table 2.1.1 shows the participant characteristics. The mean (SD) childhood arteriolar and venular calibers were 159.2 µm (15.0) and 218.9 µm (20.1), respectively.

Table 2.1.1. Characteristics of study population (N = 3,748)

Characteristics	Values
Maternal characteristics	
Age, median (95% range), y	30.7 (19.6, 39.4)
Gestational age at intake, median (95% range), weeks	13.9 (9.9, 24.8)
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.7 (18.1, 34.9)
Education, higher (%)	45.3
Parity, nulliparous (%)	56.6
Folic acid use, never (%)	26.1
Smoked during pregnancy, yes (%)	26.5
Early pregnancy blood pressure	
Gestational age at measurement, median (95% range), weeks	13.2 (9.6, 17.4)
Systolic blood pressure, mean (SD), mmHg	115.6 (12.1)
Diastolic blood pressure, mean (SD), mmHg	68.3 (9.6)
Mid pregnancy blood pressure	
Gestational age at measurement, median (95% range), weeks	20.4 (18.5, 23.5)
Systolic blood pressure, mean (SD), mmHg	116.6 (12.1)
Diastolic blood pressure, mean (SD), mmHg	67.1 (9.5)
Late pregnancy blood pressure	
Gestational age at measurement, median (95% range), weeks	30.2 (28.5, 32.8)
Systolic blood pressure, mean (SD), mmHg	118.3 (12.1)
Diastolic blood pressure, mean (SD), mmHg	69.2 (9.2)
Gestational diabetes, Yes, (%)	1.0
Gestational hypertension, Yes, (%)	4.3
Preeclampsia, Yes, (%)	1.8
Paternal characteristics	
Age, median (95% range), y	33.0 (22.2, 46.0)
Education, higher (%)	51.9
Ethnicity, European (%)	69.1
Body mass index, median (95% range), kg/m^2	24.9 (19.5, 32.9)
Systolic blood pressure, mean (SD), mmHg	130.0 (13.1)
Diastolic blood pressure, mean (SD), mmHg	73.2 (10.3)
Birth and Infant characteristics	
Sex, Boys (%)	49.7
Gestational age at birth, median (95% range), weeks	40.1 (36.0, 42.3)
Birth weight, mean (SD), grams	3442 (537)
Ethnicity of the child, European (%)	62.0
Ever breastfeed (%)	92.8
TV watching >= 2 hours per day (%)	19.6
Childhood characteristics	
Age at visit, median (95% range), years	6.0 (5.7, 8.1)
Height, mean (SD), cm	119.9 (6.2)
Weight, mean (SD), kg	23.6 (4.5)
Body mass index, median (95% range), kg/m^2	15.9 (13.6, 21.5)
Systolic blood pressure, mean (SD), mmHg	103.1 (8.2)
Diastolic blood pressure, mean (SD), mmHg	60.9 (6.9)
Retinal arteriolar caliber, mean (SD), µm	159.2 (15.0)
Retinal venial caliber, mean (SD), µm	218.9 (20.1)

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

Maternal blood pressure and childhood retinal vessel calibers

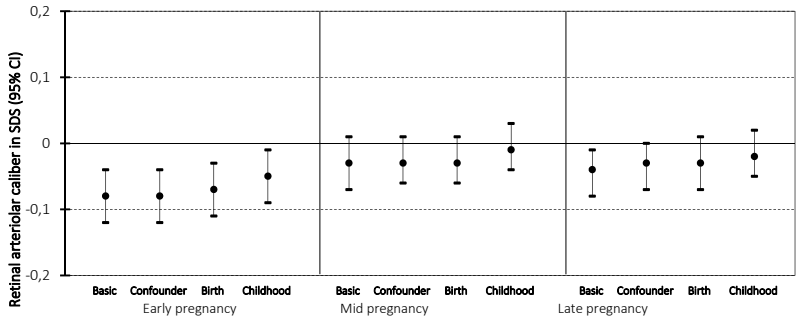
Figure 2.1.1 gives the results from conditional regression models. In the confounder models, a higher maternal systolic and diastolic blood pressure in early pregnancy were

independently associated with narrower retinal arteriolar caliber in childhood (differences: -0.08 SDS (95% CI -0.12, -0.04) and -0.05 SDS (95% CI -0.09, -0.01), per SDS increase in maternal systolic and diastolic blood pressure, respectively). These associations were not materially influenced by birth characteristics. After adjustment for current childhood body mass index and blood pressure, only the associations of maternal systolic blood pressure with narrower retinal arteriolar remained significant (difference: -0.05 SDS (95% CI 0.09, -0.01) per SDS increase in systolic blood pressure). No independent associations of maternal systolic and diastolic blood pressure in mid- or late pregnancy with childhood retinal arteriolar caliber were present (Figure 2.1.1A). Figure 2.1.1B shows that maternal systolic and diastolic blood pressure in early- and mid-pregnancy were not associated with childhood retinal venular caliber. Higher maternal systolic blood pressure, but not diastolic blood pressure, in late pregnancy was associated with narrower retinal venular caliber, independent from blood pressure in other pregnancy periods (difference: -0.05 SDS (95% CI -0.08, -0.01) per standardized residual increase in systolic blood pressure in the confounder model). These associations were not materially influenced by birth or childhood characteristics. Results from regular multiple linear regression models are given in the Supplemental Table S2.1.2 and shows similar results as the conditional models.

Figure 2.1.1. Effects estimates for the associations of maternal blood pressure levels with retinal vessel calibers in childhood from conditional regression models (N = 3,748)

A. **Childhood retinal arteriolar caliber**

A1. Maternal systolic blood pressure



A2. Maternal diastolic blood pressure

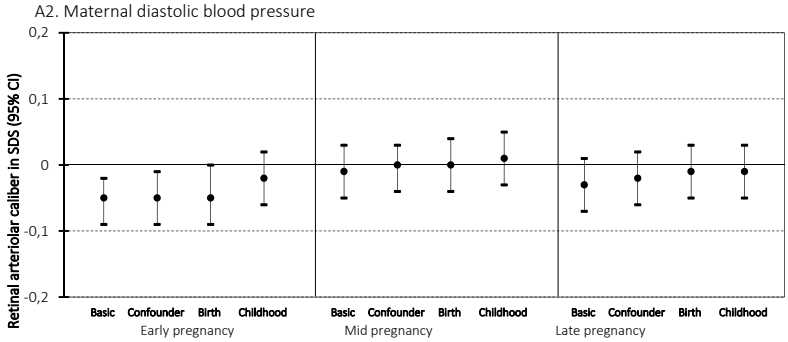
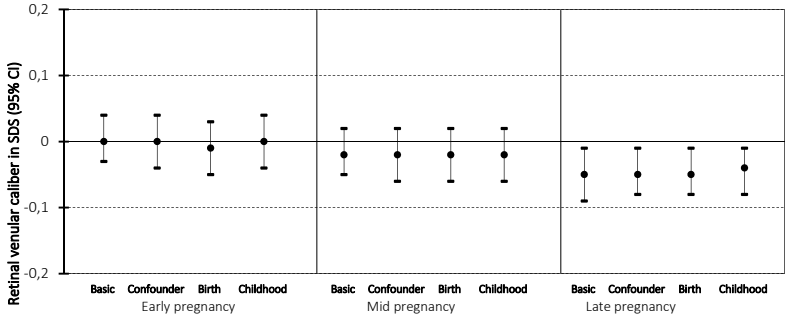


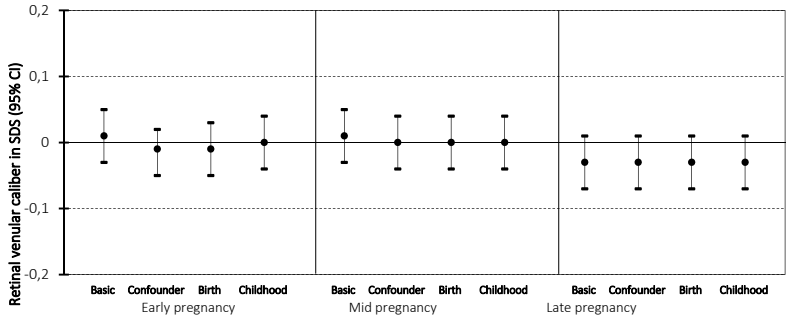
Figure 2.1.1. Effects estimates for the associations of maternal blood pressure levels with retinal vessel calibers in childhood from conditional regression models (N = 3,748)

B. **Childhood retinal venular caliber**

B1. Maternal systolic blood pressure



B2. Maternal diastolic blood pressure



The estimates are coefficients from conditional linear regression models. They represent the difference in retinal vessel calibers in SDS per standardized residual change in maternal systolic and diastolic blood pressure in the mid- and late pregnancy. The analyses in the early pregnancy was considered and starting point and shows the difference in retinal vessel calibers in SDS per SDS change in maternal systolic and diastolic blood pressure. Basic model is adjusted for age and sex of the child. Confounder model is additionally adjusted for maternal age, pre-pregnancy body mass index, education, parity, folic acid supplementation and smoking during pregnancy, and child's ethnicity, breastfeeding and TV watching. Birth model is additionally adjusted for gestational age at birth and birth weight. Childhood model is additionally adjusted for body mass index and blood pressure of the children at 6 years.

Maternal and paternal blood pressure and childhood retinal vessel calibers

Table 2.1.2 shows the comparison of associations of maternal and paternal blood pressure in early pregnancy with childhood retinal vessel calibers. In the confounder model, higher maternal early pregnancy systolic and diastolic blood pressure was associated with narrower childhood retinal arteriolar caliber. No association for paternal early pregnancy blood pressure was present (P-value for statistical difference between these associations: 0.07). In the fully adjusted model, with both maternal and paternal systolic blood pressure in the same model, maternal early pregnancy systolic blood pressure, but not paternal early pregnancy systolic blood pressure, still tended to be associated with narrower childhood retinal arteriolar caliber (difference: -0.04 SDS (95% CI -0.09, 0.01). No associations of parental early pregnancy blood pressure with childhood retinal venular caliber were present. Models adjusted for age and sex of the child only, are given in Supplemental Table S2.1.3.

Table 2.1.2. Maternal and paternal blood pressure with childhood retinal vessel calibers (N = 3,748)

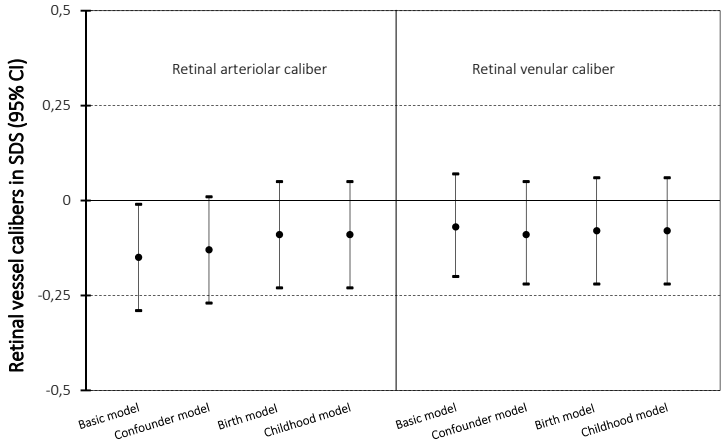
Parental blood pressure (SDS)	Childhood retinal vessel calibers in SDS (95% Confidence Interval)					
	Arteriolar caliber			Venular caliber		
	Confounder model	Birth model	Childhood model	Confounder model	Birth model	Childhood model
Maternal model	*N = 2,870*	*N = 2,870*	*N = 2,870*	*N = 2,870*	*N = 2,870*	*N = 2,870*
Systolic blood pressure	-0.08 (-0.12, -0.04)	-0.07 (-0.11, -0.03)	-0.05 (-0.09, -0.01)	-0.00 (-0.04, 0.04)	-0.00 (-0.04, 0.04)	0.00 (-0.04, 0.04)
Diastolic blood pressure	-0.05 (-0.09, -0.01)	-0.04 (-0.08, -0.00)	-0.02 (-0.06, 0.02)	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.04)	0.00 (-0.04, 0.04)
Paternal model	*N = 2,645*	*N = 2,645*	*N = 2,645*	*N = 2,645*	*N = 2,645*	*N = 2,645*
Systolic blood pressure	-0.03 (-0.07, 0.01)	-0.03 (-0.08, 0.01)	-0.02 (-0.06, 0.02)	0.00 (-0.04, 0.04)	0.00 (-0.04, 0.04)	0.00 (-0.04, 0.04)
Diastolic blood pressure	-0.03 (-0.08, 0.01)	-0.03 (-0.08, 0.01)	-0.03 (-0.07, 0.02)	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)
Both parents	*N = 2,182*	*N = 2,182*	*N = 2,182*	*N = 2,182*	*N = 2,182*	*N = 2,182*
Maternal systolic blood pressure	-0.07 (-0.11, -0.02)	-0.06 (-0.11, -0.01)	-0.04 (-0.09, 0.01)	0.02 (-0.02, 0.07)	0.02 (-0.02, 0.07)	0.03 (-0.02, 0.07)
Maternal diastolic blood pressure	-0.03 (-0.08, 0.02)	-0.02 (-0.07, 0.02)	-0.01 (-0.05, 0.04)	0.01 (-0.04, 0.05)	0.01 (-0.04, 0.05)	0.01 (-0.03, 0.06)
Both parents	*N = 2,182*	*N = 2,182*	*N = 2,182*	*N = 2,182*	*N = 2,182*	*N = 2,182*
Paternal systolic blood pressure	-0.03 (-0.07, 0.02)	-0.03 (-0.07, 0.01)	-0.01 (-0.05, 0.03)	0.00 (-0.04, 0.04)	-0.00 (-0.04, 0.04)	0.00 (-0.04, 0.05)
Paternal diastolic blood pressure	-0.04 (-0.08, 0.01)	-0.04 (-0.08, 0.00)	-0.03 (-0.07, 0.01)	-0.02 (-0.06, 0.03)	-0.02 (-0.06, 0.03)	-0.02 (-0.06, 0.03)

The estimates are coefficients from linear regression models. They represent the differences in retinal vessel calibers in SDS per standard deviation score increase in systolic and diastolic blood pressure of the mothers and fathers. Confounder model is adjusted for age and sex of the child, maternal age, pre-pregnancy body mass index, education, child's ethnicity, parity, folic acid supplementation and smoking during pregnancy, breastfeeding and TV watching. Birth model is additionally adjusted for gestational age at birth and birth weight. Childhood model is additionally adjusted for body mass index and blood pressure of the children at 6 years. Paternal models include paternal age, educational level, ethnicity and paternal body mass index instead of maternal age, educational level, ethnicity and maternal body mass index. Both parents models contain both maternal and paternal blood pressure in the same model. Models adjusted only for age and sex of the child are given in Supplemental Table S2.1.3. All P-values for heterogeneity between maternal and paternal associations were not significant.

Gestational hypertensive disorders with retinal vessel calibers in childhood

Figure 2.1.2 shows that gestational hypertensive disorders were associated with narrower childhood retinal arteriolar caliber in the model adjusted for sex and age (difference: -0.15 SDS (95% CI -0.29, -0.01)). These associations partly attenuated after additional adjustment for potential confounders (difference: -0.13 SDS (95% CI -0.27, 0.01)). Additional adjustment for gestational age, birth weight, childhood body mass index and blood pressure further attenuated these associations into non-significant. Gestational hypertensive disorders were not associated with childhood retinal venular caliber (**Figure 2.1.2**).

Figure 2.1.2. Associations of gestational hypertensive disorders with retinal vessel calibers in childhood (N= 3,748)



The estimates are coefficients from linear regression models, and represent the difference in childhood retinal vessel calibers in SDS among children from mothers with gestational hypertensive disorders as compared to children from mothers with non-complicated pregnancies. Basic model is adjusted for age and sex of the child. Confounder model is additionally adjusted for maternal age, pre-pregnancy body mass index, education, parity, folic acid supplementation and smoking during pregnancy, and child's ethnicity, breastfeeding and TV watching. Birth model is additionally adjusted for gestational age at birth and birth weight. Childhood model is additionally adjusted for body mass index and blood pressure of the children at 6 years.

Discussion

In this population-based prospective cohort study, we observed that higher maternal systolic blood pressure in pregnancy is associated with childhood retinal calibers. These associations were not explained by maternal socio-demographic and lifestyle-related characteristics, birth or childhood characteristics.

Methodological considerations

We used a population-based prospective cohort design including a large number of subjects whom we studied from early fetal life onwards. Sixty-nine percent of children from mothers with information about blood pressure and pregnancy complications participated in the follow-up measurements at the age of 6 years. Of these, 63% participated in the retinal vascular measurements. This lower rate is mainly explained by the later start of these measurements during the follow-up visits. Selection bias in follow-up studies mainly arises from loss to follow-up rather than from non-response at baseline.[26] Loss to follow-up would lead to selection bias if the associations of maternal blood pressure with childhood retinal vessel calibers would be different between those included and those not included in the final analyses. Non-response analysis showed that preeclampsia was less frequent among mothers included in the analyses compared to those not included, whereas no differences were present for mean systolic and diastolic blood pressure. Also birth weight and gestational age at birth were lower among children who were not included in the analyses compared to those who were included. It is hard to speculate whether these differences would have underestimated the observed associations materially, but we consider this unlikely. Blood pressure has a large within subject-variation and is also liable to measurement error. This measurement

error may have led to an underestimation of the observed effect estimates.[3] Furthermore, we had a relatively small number of cases of gestational hypertensive disorders, which might have led to lack of power to assess the associations of gestational hypertensive disorders with childhood microvasculature. We used validated techniques to measure retinal vessel calibers. We did not take into account other ocular factors that might affect retinal vessels measurement, such as axial length and refractive error.[27, 28] However, it has been previously shown in adults that these factors have only a small impact on the measurement of retinal vessel calibers.[29] We adjusted the analyses for a large number of potential confounders, but residual confounding might still occur, as in any observational study. For example, no detailed information was available about parental and childhood dietary intake.

Interpretation of main findings

Gestational hypertensive disorders are associated with an adverse cardiovascular profile in the offspring.[30] Offspring of mothers who develop preeclampsia during pregnancy have an increased risk of fetal growth restriction, higher blood pressure levels, vascular abnormalities and increased risk of stroke in later life.[5, 31, 32] A systemic review, which included 18 studies with data on 45,249 individuals, showed that children born to mothers with complicated pregnancies have a higher blood pressure in childhood and early adulthood.[30]

Gestational hypertensive disorders form the extreme of the spectrum of blood pressure development during pregnancy.[3] Higher maternal blood pressure levels within the normal range during pregnancy are also associated with offspring cardiovascular risk factors.[33, 34] A study among 4,675 mothers and their children showed that increased maternal blood pressure during early- and mid-pregnancy, but not in late pregnancy, was associated with increased body mass index and blood pressure in children aged 10 years.[1] In the same study, no associations of maternal blood pressure with other offspring vascular outcomes including flow-mediated dilatation, pulse wave velocity and brachial artery diameter, were present.[1] Another study among 5,573 mothers observed that higher blood pressure in offspring of mothers with de novo hypertension during early-pregnancy compared with offspring of normotensive mothers.[35]

Several intrauterine mechanisms have been hypothesised to underlie the association of maternal blood pressure levels during pregnancy with blood pressure levels in offspring.[5] Fetal microvasculature vasoconstriction or anatomic adaptations such as increased intimal thickness, medial hyperplasia and hyalinization of the microvasculature might lead to an increased the risk of hypertension in later life.[36] Thus far, large studies have shown that preterm birth and low birth weight are related to retinal arteriolar narrowing in later life.[18, 37, 38] To the best of our knowledge, no other studies have examined the associations of maternal blood pressure development in different pregnancy periods and gestational hypertensive disorders with offspring alterations in microvasculature.

We observed that higher maternal blood pressure in early pregnancy was associated with childhood retinal arteriolar narrowing, whereas higher maternal blood pressure in late pregnancy was associated with childhood retinal venular narrowing. These associations were independent of maternal blood pressure in other pregnancy periods and

independent of birth characteristics or childhood body mass index and blood pressure. Maternal hypertensive disorders tended to be associated with retinal arteriolar narrowing in childhood, but these associations were largely explained by birth and childhood factors. In line with our findings, a cross-sectional study among 768 mother-offspring pairs found a significant correlation between higher maternal blood pressure and childhood retinal arteriolar narrowing at a median age of 11.5 years.[9] Another study among 102 newborns showed that maternal pre-existing hypertension and development of hypertension during pregnancy was inversely associated with endothelial vasodilatation in offspring.[31] A study among 109 participants showed that offspring of mothers with hypertensive pregnancy complications had endothelial dysfunction and an increased carotid intima-media thickness.[39] These findings suggest that the higher blood pressure among children from mothers with higher blood pressure levels during pregnancies might be a result of endothelial dysfunction, which can also affect vascular development already during fetal development.[39]

Both higher maternal and paternal blood pressure levels during pregnancy are shown to be associated with an increased risk for higher blood pressure among children, with some studies suggesting a stronger effect for maternal than paternal blood pressure.[40, 41] A stronger association for maternal blood pressure during pregnancy with childhood retinal caliber would suggest direct intra-uterine mechanisms, whereas similar or stronger associations for paternal blood pressure suggest a role for shared family-based lifestyle-related factors or genetic factors. We observed that higher maternal blood pressure in early pregnancy tended to be more strongly associated with childhood retinal arteriolar narrowing, as compared to paternal blood pressure in early pregnancy, which suggests that at least part of the association may be explained by direct intra-uterine mechanisms.

Thus, our results suggest that higher maternal blood pressure levels, especially in early and late pregnancy, are related to childhood microvasculature adaptations. Based on the parental-offspring comparison, at least part of this association may be explained by direct intra-uterine mechanisms. The biological mechanisms underlying these associations remain unclear. In early pregnancy, the fetal cardiovascular system rapidly develops, whereas late pregnancy is characterized by increased placental blood flow and rapid fetal growth.[42] Thus, different mechanisms may explain the observed associations. Higher maternal blood pressure levels during pregnancy are a marker of a suboptimal placental development and function, which may affect fetal vascular development and fetal growth restriction.[43] This may lead to alterations in microvasculature in later life.[18] Additionally, offspring of mothers with higher blood pressure levels during pregnancy are at increased risk of endothelial dysfunction, which may also lead to alterations in microvasculature.[44] Results from this study should be considered as hypothesis generating further observational and experimental studies to explore the underlying mechanisms and potential critical periods during pregnancy.

Conclusions

Our study suggest that higher maternal blood pressure in pregnancy is associated with childhood microvasculature structures. Further studies are needed to replicate these

observations and to examine the long-term consequences of these microvasculature adaptations.

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Supplemental Material

Figure S2.1.1. Flow chart of participants in study

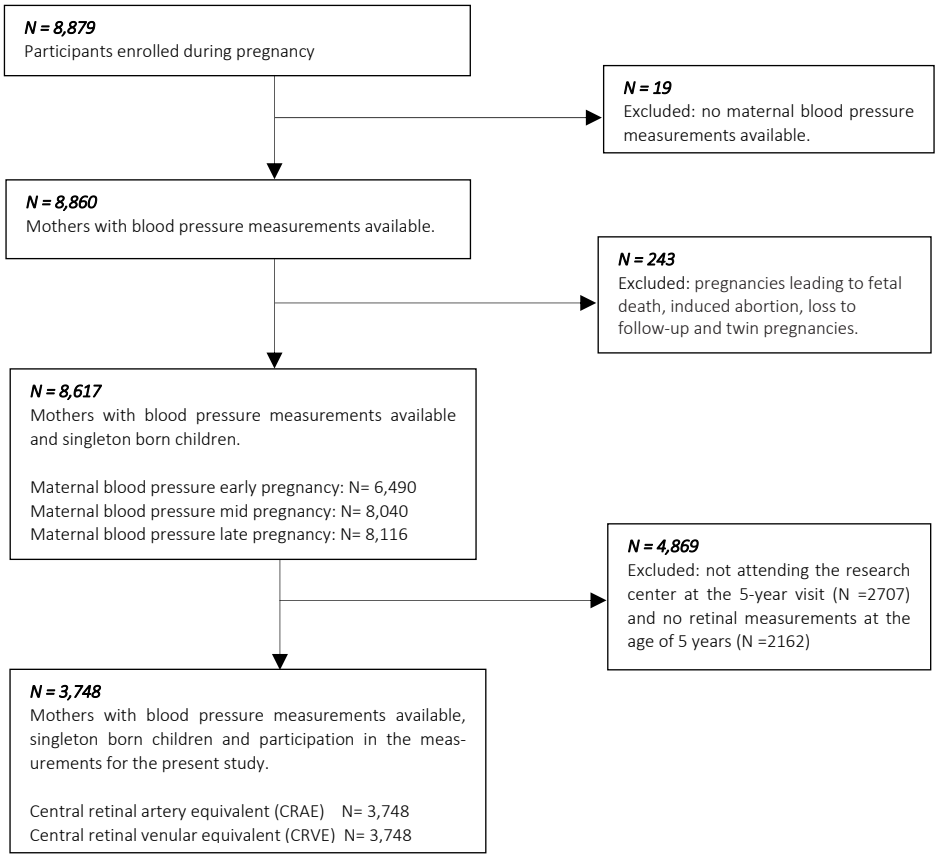

Table S2.1.1. Comparison of subject characteristics between children included and not included in the analyses

Characteristics	Participation	Non-participation	P value
Maternal characteristics			
Age, median (95% range), y	30.7 (19.6, 39.4)	29.9 (18.9, 39.0)	*< 0.01*
Gestational age at intake, median (95% range), weeks	13.9 (9.9, 24.8)	14.2 (10.1, 24.9)	*< 0.01*
Pre-pregnancy body mass index, median (95% range), kg/m2	22.7 (18.1, 34.9)	22.6 (17.8, 35.0)	*0.10*
Education, higher (%)	45.3	39.2	*< 0.01*
Parity, nulliparous (%)	56.6	55.0	*0.14*
Folic acid use, never (%)	26.1	31.9	*< 0.01*
Smoked during pregnancy, yes (%)	26.5	27.8	*0.24*
Early pregnancy blood pressure			
Gestational age at measurement, median (95% range), weeks	13.2 (9.6, 17.4)	13.4 (9.7, 17.6)	*0.01*
Systolic blood pressure, mean (SD), mmHg	115.6 (12.1)	115.4 (12.5)	*0.52*
Diastolic blood pressure, mean (SD), mmHg	68.3 (9.6)	68.1 (9.6)	*0.35*
Mid pregnancy blood pressure			
Gestational age at measurement, median (95% range), weeks	20.4 (18.5, 23.5)	20.4 (18.5, 23.8)	*0.11*
Systolic blood pressure, mean (SD), mmHg	116.6 (12.1)	116.6 (12.0)	*0.94*
Diastolic blood pressure, mean (SD), mmHg	67.1 (9.5)	67.2 (9.3)	*0.61*
Late pregnancy blood pressure			
Gestational age at measurement, median (95% range), weeks	30.2 (28.5, 32.8)	30.2 (28.4, 32.9)	*0.72*
Systolic blood pressure, mean (SD), mmHg	118.3 (12.1)	118.1 (12.0)	*0.34*
Diastolic blood pressure, mean (SD), mmHg	69.2 (9.2)	69.0 (9.5)	*0.35*
Gestational diabetes, Yes, (%)	1.0	1.1	*0.45*
Gestational hypertension, Yes, (%)	4.3	3.4	*0.02*
Preeclampsia, Yes, (%)	1.8	2.4	*0.04*
Paternal characteristics			
Age, median (95% range), y	33.0 (22.2, 46.0)	32.4 (21.6, 44.8)	*< 0.01*
Education, higher (%)	51.9	49.6	*0.01*
Ethnicity, European (%)	69.1	66.6	*0.04*
Body mass index, median (95% range), kg/m2	24.9 (19.5, 32.9)	25.1 (19.4, 33.5)	*0.10*
Systolic blood pressure, mean (SD), mmHg	130.0 (13.1)	130.2 (13.8)	*0.59*
Diastolic blood pressure, mean (SD), mmHg	73.2 (10.3)	73.3 (11.0)	*0.79*
Birth and Infant characteristics			
Sex, Boys (%)	49.7	51.1	*0.22*
Gestational age at birth, median (95% range), weeks	40.1 (36.0, 42.3)	40.1 (34.9, 42.3)	*< 0.01*
Birth weight, mean (SD), grams	3442 (537)	3387 (579)	*< 0.01*
Ethnicity of the child, European (%)	62.0	59.1	*0.01*
Ever breastfeeding (%)	92.8	91.5	*0.06*
TV watching >= 2 hours per day (%)	19.6	19.7	*0.94*

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

Table S2.1.2. Associations of maternal blood pressure during different periods of pregnancy with childhood retinal vessel calibers from regular linear regression models (N = 3,748)

Maternal blood pressure at different periods of pregnancy (SDS)	Childhood retinal vessel calibers in SDS (95% Confidence Interval)							
	Arteriolar caliber				Venular caliber			
	Basic model	Confounder model	Birth model	Childhood model	Basic model	Confounder model	Birth model	Childhood model
Early pregnancy	*N = 2,870*	*N = 2,870*	*N = 2,870*	*N = 2,870*	*N = 2,870*	*N = 2,870*	*N = 2,870*	*N = 2,870*
Systolic blood pressure	-0.08 (-0.12, -0.04)*	-0.08 (-0.12, -0.04)*	-0.07 (-0.11, -0.03)*	-0.05 (-0.09, -0.01)*	0.00 (-0.03, 0.04)	-0.00 (-0.04, 0.04)	-0.01 (-0.05, 0.03)	-0.00 (-0.04, 0.04)
Diastolic blood pressure	-0.05 (-0.09, -0.02)*	-0.05 (-0.09, -0.01)*	-0.05 (-0.09, -0.00)*	-0.02 (-0.06, 0.02)	0.01 (-0.03, 0.05)	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)	-0.00 (-0.04, 0.04)
Mid pregnancy	*N = 3,516*	*N = 3,516*	*N = 3,516*	*N = 3,516*	*N = 3,516*	*N = 3,516*	*N = 3,516*	*N = 3,516*
Systolic blood pressure	-0.05 (-0.09, -0.02)*	-0.05 (-0.09, -0.01)*	-0.05 (-0.08, -0.01)*	-0.02 (-0.05, 0.02)	-0.00 (-0.04, 0.03)	-0.01 (-0.05, 0.02)	-0.01 (-0.05, 0.02)	-0.01 (-0.04, 0.03)
Diastolic blood pressure	-0.03 (-0.06, 0.00)	-0.03 (-0.06, 0.01)	-0.02 (-0.06, 0.01)	0.00 (-0.04, 0.04)	0.01 (-0.02, 0.04)	-0.00 (-0.04, 0.03)	-0.00 (-0.04, 0.03)	0.00 (-0.03, 0.04)
Late pregnancy	*N = 3,606*	*N = 3,606*	*N = 3,606*	*N = 3,606*	*N = 3,606*	*N = 3,606*	*N = 3,606*	*N = 3,606*
Systolic blood pressure	-0.08 (-0.11, -0.04)*	-0.07 (-0.10, -0.03)*	-0.07 (-0.10, -0.03)*	-0.03 (-0.07, 0.00)	-0.04 (-0.07, -0.01)*	-0.05 (-0.08, -0.01)*	-0.05 (-0.08, -0.01)*	-0.04 (-0.07, -0.01)*
Diastolic blood pressure	-0.06 (-0.09, -0.02)*	-0.05 (-0.09, -0.02)*	-0.04 (-0.08, -0.01)*	-0.02 (-0.05, 0.02)	-0.01 (-0.04, 0.02)	-0.37 (-1.04, 0.30)	-0.02 (-0.05, 0.02)	-0.02 (-0.05, 0.02)

The estimates are coefficient from linear regression models. They represent the difference in retinal vessel calibers in SDS per standard deviation score increase in systolic and diastolic blood pressure of the mothers. Basic model is adjusted for age and sex of the child. Confounder model is additionally adjusted for age and sex of the child, maternal age, pre-pregnancy body mass index, education, parity, folic acid supplementation and smoking during pregnancy, and child's ethnicity, breastfeeding and TV watching. Birth model is additionally adjusted for gestational age at birth and birth weight. Childhood model is additionally adjusted for body mass index and blood pressure of the children at 6 years. *P<0.05

Table S2.1.3. Associations of maternal and paternal blood pressure with childhood retinal vessel calibers in basic models (N=3,748)

Parental blood pressure at first trimester of pregnancy (SDS)	Retinal vessel calibers in SDS	
	Arteriolar caliber	Venular caliber
Maternal	*N = 2,870*	*N = 2,870*
Systolic blood pressure	-0.08 (-0.12, -0.04)*	0.00 (-0.03, 0.04)
Diastolic blood pressure	-0.05 (-0.09, -0.02)*	0.01 (-0.03, 0.05)
Paternal	*N = 2,645*	*N = 2,645*
Systolic blood pressure	-0.04 (-0.08, -0.00)*	-0.00 (-0.04, 0.04)
Diastolic blood pressure	-0.04 (-0.07, 0.00)	-0.01 (-0.05, 0.03)
Maternal and paternal	*N = 2,182*	*N = 2,182*
Maternal systolic blood pressure	-0.07 (-0.11, 0.03)	0.03 (-0.01, 0.07)
Maternal diastolic blood pressure	-0.03 (-0.08, 0.01)	0.03 (-0.02, 0.07)
Paternal and maternal	*N = 2,182*	*N = 2,182*
Paternal systolic blood pressure	-0.03 (-0.08, 0.01)	-0.01 (-0.05, 0.04)
Paternal diastolic blood pressure	-0.04 (-0.08, 0.01)	-0.02 (-0.06, 0.02)

The estimates are coefficient from linear regression models. They represent the differences in retinal vessel calibers in SDS per standard deviation score increase in systolic and diastolic blood pressure of the mothers and fathers. Model is adjusted for age and sex of the child. Maternal and paternal models contain both maternal and paternal blood pressure in the same model. All p-values for heterogeneity between maternal and paternal associations not significant. *P<0.05

Chapter 2.2

Maternal angiogenic factors and childhood microvasculature

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Abstract

Background: Reduced PlGF levels and higher sFlt-1 levels in mothers during pregnancy may have persistent effects on vascular structures in their offspring. We examined whether angiogenic factors during pregnancy also affect childhood retinal microvasculature in a population-based prospective cohort study among 3,505 mothers and their children.

Methods and Results: We measured in the first and second trimester of pregnancy, maternal placenta growth factor (PlGF) and soluble fms-like tyrosine kinase (sFlt-1) levels. At the age of 6 years, we measured childhood retinal arteriolar and venular calibers from digitized retinal photographs. We performed multiple linear regression models, taking account for maternal and childhood socio-demographic and lifestyle-related characteristics, birth characteristics and childhood current body mass index and blood pressure into account. We observed that first trimester maternal PlGF and sFlt-1 levels were not associated with childhood retinal arteriolar caliber. Lower second trimester maternal PlGF levels, but not sFlt-1 levels, were associated with narrower childhood retinal arteriolar caliber (difference: -0.09 SDS (95% CI: -0.16, -0.01), per SDS decrease in PlGF). This association was not explained by maternal and childhood socio-demographic and lifestyle-related characteristics, birth characteristics or childhood current body mass index and blood pressure. Maternal PlGF and sFlt-1 levels in the first or second trimester were not associated with childhood retinal venular caliber.

Conclusions: Our results suggest that lower maternal second trimester PlGF levels affect the microvascular development in the offspring, leading to narrower retinal arteriolar caliber in childhood. Further studies are needed to confirm these findings and to explore the underlying mechanisms and long-term cardiovascular consequences.

Introduction

Impaired fetal growth is associated with cardiovascular disease in adulthood.[1] The mechanisms underlying these associations are not known, but may include developmental adaptations in the cardiovascular system during fetal life in response to suboptimal fetal environment.[1, 2] Recently, we observed that suboptimal fetal and placental vascular development may have a persistent influence on cardiovascular function in later life.[3] Placental and fetal vascular development is a complex process in which several angiogenic factors are involved.[4] Placenta growth factor (PlGF), a pro-angiogenic factor also secreted by cytotrophoblast, plays a key role in normal angiogenesis. This process is necessary for the development of the fetal vascular system in the placental villous tree and development of an adequate utero-placental circulation.[5] Soluble fms-like tyrosine kinase (sFlt-1), is an anti-angiogenic factor secreted by endothelial cells which can block the effect of PlGF.[4] Previous studies showed that lower PlGF levels can disrupt the normal process of neo-angiogenesis, whereas higher sFlt-1 levels during pregnancy can lead to systemic endothelial dysfunction.[6] Both pathways may result in a suboptimal feto-placental vascular development.[7, 8] For the current study, we hypothesized that angiogenic factors during pregnancy may not only affect placental and fetal vessel development, but also persistently influence microvasculature structures postnatally. Early childhood developmental adaptations in microvasculature may subsequently increase the risk of development of hypertension in later life.[9]

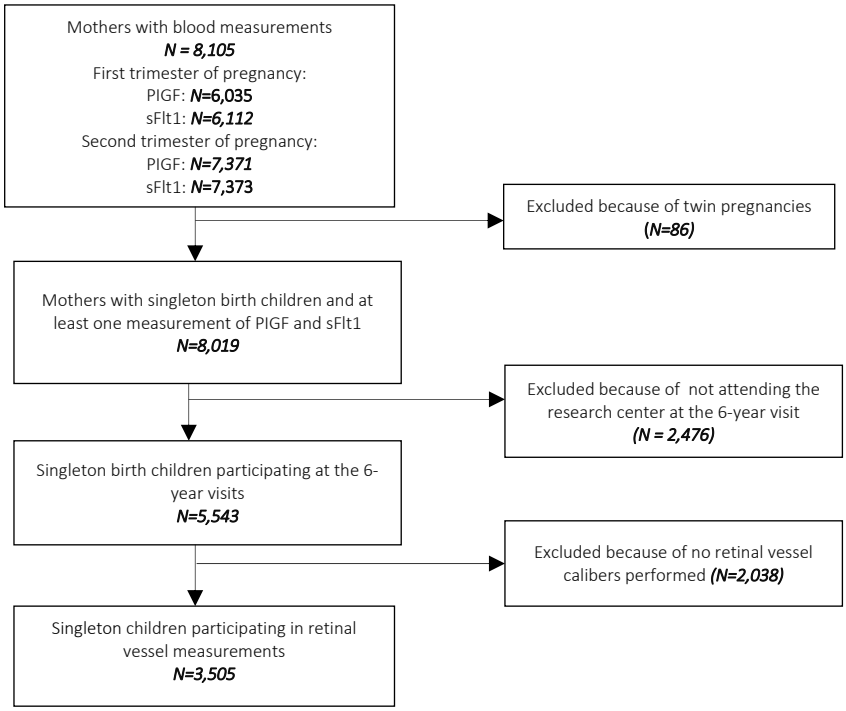
Therefore, we examined among 3,506 mothers and their children participating in a population-based prospective cohort study from early fetal life onwards the associations of maternal PlGF and sFlt-1 levels during the first and second trimester of pregnancy with retinal vessel calibers at the age of 6 years.

Methods

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[10] All children were born between 2002 and 2006. Response rate at baseline was 61%.[10] The study protocol was approved by the Medical Ethical Committee of the Erasmus MC, Rotterdam. Written informed consent was obtained from all parents. In total, 8,879 mothers were included prenatally. Maternal blood samples were available among 8,019 singleton births. In total, 5,544 children participated in the follow-up measurements at a median age of 6.0 years (90% range 5.7 – 8.1). Retinal vessel measurements were available in 3,505 children. Missing retinal vessel measurements were mainly due to the later start of these measurements during the follow-up visits (**Figure 2.2.1**). PlGF and sFlt-1 levels during first trimester of pregnancy were lower among mothers included, compared to those not included in the analyses. Also children who were not included in the analyses had a lower birth weight and were less often breastfed, as compared to children who were included (Supplemental **Table S2.2.1**).

Figure 2.1.1. Flow chart of the study population



Angiogenic factors during pregnancy

Maternal non-fasting venous blood samples were drawn in the first trimester (13.4 weeks of gestational age, 90% range 10.5–17.2) and second trimester (20.4 weeks of gestational age, 90% range 18.8–22.9). Details of processing procedures have been described previously.[10] Blood samples were stored at -80°C. PlGF and sFlt-1 levels were analyzed using an immunoelectrochemoluminence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands). The between-run coefficients of variation for plasma PlGF were 4.7% at 24 pg/mL and 3.8% at 113 pg/mL. The coefficients for sFlt-1 were 2.8% at 5.5 ng/mL and 2.3% at 34.0 ng/mL. Because these measurements were not normally distributed we log-transformed them for further analyses.

Retinal microvasculature assessment

At the age of 6 years retinal photographs were taken by a well-trained staff in a dedicated research center. Digital retinal photographs centered on the optic disc were taken in one eye with images resolution 4096 and 3072 pixels, using Topcon digital retinal camera (model TRC, NW300). We use the same methods and protocols to measure retinal vascular caliber from the digitized retinal photographs, as described in previous studies among adults and children.[11, 12] Briefly, a semi-automatic computer imaging program was used to measure the caliber of the 6 largest retinal arterioles and venules located one half to 1 disc diameter from the optic disc margin.[13] Using the revised Knudtson-Parr-Hubbard formula, absolute arteriolar and venular diameter were esti-

mated in micrometers and subsequently were summarized as central retinal arteriolar and venular equivalents, representing the average arteriolar and venular caliber of that eye, respectively.[14] Graders, masked to birth parameters and other participant characteristics, operated the computer program and monitored all retinal measurements. We constructed grader specific standard deviation scores for both central retinal and arteriolar equivalents. Interclass correlation coefficients between graders were 0.79 for retinal arteriolar caliber and 0.89 for retinal venular caliber.

Covariates

We obtained information about maternal age, parity, educational level, pre-pregnancy body mass index, smoking during pregnancy and folic acid supplement use by questionnaires. Maternal blood pressure was assessed at enrollment and information on pregnancy complications (hypertensive disorders, gestational diabetes) was obtained from medical records. Information on ethnicity was obtained from the first questionnaire at enrolment in the study.[10] Gestational age was established by fetal ultrasound examination during the first ultrasound visit and birth weight was obtained from medical records. We obtained information on breastfeeding and average TV watching time by questionnaire. At the age of 6 years, we measured childhood height and weight and calculated body mass index (kg/m^2). Childhood systolic and diastolic blood pressure was measured at the right brachial artery, four times with one-minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus TM (Paramus, NJ, USA) and we used the mean of the last three blood pressure measurements for the analyses.[15]

Statistical analyses

First, we used linear regression models to examine the continuous associations of maternal PlGF and sFlt-1 levels with childhood retinal vessel calibers. These models were first adjusted for child's age and sex and gestational age at blood collection (basic model) and were subsequently additionally adjusted for potential confounders, including maternal and childhood socio-demographic and lifestyle-related characteristics. These covariates were selected based on their associations with placental function or the outcomes of interest based on previous studies or a change in effect estimate of >10%.[12, 16-18] Supplemental **Table S2.2.2** shows the associations of each covariate with the outcomes of interest. Next, these models were additionally adjusted for gestational age at birth, birth weight, child's current body mass index and blood pressure to explore whether the observed associations were explained by birth or childhood characteristics. For all analyses we constructed standard deviation scores (SDS) ((observed value – mean) / SD)) for PlGF and sFlt-1 levels. Second, because lower PlGF and higher sFlt-1 levels have been associated with an increased risk of adverse pregnancy outcomes [19], we defined low PlGF as the lower 25 percent and a high sFlt-1 as the upper 25 percent within our study population. We used similar models to assess the associations of low PlGF and high sFlt-1 levels with childhood retinal vessel calibers. We tested potential interactions between PLGF and sFlt-1 levels and gestational age at birth, birth weight and child's sex in relation to retinal vessel calibers. Since no significant interactions were present, no further stratified analyses were performed. In order to reduce potential bias

due to missing data, we performed multiple imputations for missing values of covariates (<25% missing values). Five datasets were created and analyzed together.[20] Imputations were based on the relationships between covariates, determinants and outcomes. Analyses were performed using the Statistical Package of Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Table 2.2.1 shows characteristics of the study population. The mean (SD) arteriolar and venular calibers were 159.1 µm (14.9) and 219.0 µm (20.0), respectively.

Table 2.2.1. Characteristics of study population (N = 3,505)

Characteristics	Values
Maternal characteristics	
Age, median (95% range), y	30.7(19.8, 39.4)
Height, mean (SD), cm	167.6 (7.4)
Pre-pregnancy weight, mean (SD), kg	66.8 (12.9)
Pre-pregnancy body mass index, median (95% range), kg/m^2	23.8 (18.8, 36.0)
Systolic blood pressure, mean (SD), mm/Hg	115.3 (12.0)
Diastolic blood pressure, mean (SD), mm/Hg	67.9 (9.5)
Parity, nulliparous (%)	56.7
Education, higher (%)	62.1
Folic acid use, never (%)	25.3
Smoked during pregnancy, Yes (%)	26.8
Gestational diabetes, Yes, (%)	0.9
Gestational hypertension, Yes, (%)	4.3
Preeclampsia, Yes, (%)	1.9
PlGF first trimester, median (95% range), pg/mL	41.8 (14.8, 192.8)
PlGF second trimester, median (95% range), pg/mL	199.2 (75.9, 592.8)
sFlt-1 first trimester, median (95% range), ng/mL	5.0 (1.9, 18.0)
sFlt-1 second trimester, median (95% range), ng/mL	5.0 (1.5, 17.3)
Birth and infant characteristics	
Gender, boys (%)	49.7
Gestational age at birth, median (95% range), weeks	40.1 (36.0, 42.2)
Birth weight, mean (SD), grams	3446 (538)
Ethnicity, European (%)	62.7
Ever breastfeeding (%)	92.8
TV watching >= 2 hours per day (%)	19.0
Childhood characteristics	
Age at visit, median (95% range), years	6.0 (5.7, 8.1)
Body mass index, mean (SD), kg/m^2	16.3 (2.0)
Systolic blood pressure, mean (SD), mm/Hg	103.0 (8.2)
Diastolic blood pressure, mean (SD), mm/Hg	60.8 (6.9)
Retinal arteriolar caliber, mean (SD), µm	159.1 (14.9)
Retinal venial caliber, mean (SD), µm	219.0 (20.0)

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

Angiogenesis markers and retinal vessel calibers

Table 2.2.2 shows that in the models adjusted for child's age at visit, sex and gestational age at blood collection, lower maternal first trimester PlGF levels, but not sFlt-1 levels, were associated with narrower retinal arteriolar caliber in childhood (difference: -0.06 SDS (95% CI: -0.12, 0.00), per SDS decrease in PlGF). This association was fully explained by maternal and childhood socio-demographic and lifestyle-related characteristics. Lower maternal second trimester PlGF and sFlt-1 levels were associated with narrower

childhood retinal arteriolar caliber (differences: -0.12 SDS (95% CI: -0.19, -0.04) and -0.04 SDS (95% CI: -0.07, -0.01) per SDS decrease in PlGF and sFlt-1, respectively). After additional adjustment for confounder factors, lower maternal PlGF levels, but not sFlt-1 levels, were still associated with narrower retinal arteriolar caliber in childhood (P value = 0.01). This association was not further influenced by adjustment for birth and childhood characteristics (difference: -0.09 SDS (95% CI: -0.16, -0.01), per SDS decrease in PlGF levels in the fully adjusted model). Maternal PlGF and sFlt-1 levels in first or second trimester were not associated with childhood retinal venular caliber.

Table 2.2.2. Maternal and paternal blood pressure with childhood retinal vessel calibers (N = 3,748)

Angiogenesis markers in SDS	Childhood retinal vessel calibers in SDS (95% Confidence Interval)					
	Arteriolar caliber			Venular caliber		
	Basic model	Confounder model	Childhood model	Basic model	Confounder model	Childhood model
First trimester						
PlGF (N=2,634)	0.06 (0.00, 0.12)*	0.06 (0.00, 0.11)	0.05 (-0.01, 0.10)	0.01 (-0.05, 0.07)	0.02 (-0.04, 0.07)	0.01 (-0.05, 0.07)
sFlt-1 (N=2,633)	0.07 (-0.08, 0.21)	0.04 (-0.11, 0.18)	0.02 (-0.12, 0.17)	-0.01 (-0.16, 0.13)	-0.02 (-0.17, 0.12)	-0.03 (-0.17, 0.12)
Second trimester						
PlGF (N=3,226)	0.12 (0.04, 0.19)||	0.10 (0.02, 0.18)§	0.09 (0.01, 0.16)‡	0.07 (0.00, 0.15)	0.07 (-0.01, 0.15)	0.07 (-0.01, 0.015)
sFlt-1 (N=3,240)	0.04 (0.01, 0.07)‡	0.02 (-0.01, 0.06)	0.02 (-0.01, 0.06)	0.04 (0.01, 0.07)†	0.03 (0.00, 0.07)	0.03 (0.00, 0.07)

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal vessel calibers per SDS change in PlGF and sFlt-1 levels. Basic model is adjusted for child's age at visit, sex and gestational age at blood collection. Confounder model is additionally adjusted for lifestyle and socio-demographic confounders (maternal age, education, pre-pregnancy body mass index, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, pregnancy complications (gestational hypertension, preeclampsia and gestation diabetes), plus child's ethnicity, breastfeeding and TV watching). Childhood factors model is additionally adjusted for child' gestational age at birth and birth weight and childhood body mass index and blood pressure. *P = 0.05; †P-value = 0.03; ‡P-value = 0.02; §P-value = 0.01; ||P-value = 0.003.

Table 2.2.3 shows the associations of low maternal PlGF levels and high maternal sFlt-1 level, defined as the lower and upper 25 percent of the distribution, respectively, with childhood retinal arteriolar caliber. No associations were present for first trimester levels. As compared to normal maternal second trimester PlGF levels, low PlGF levels were associated with narrower childhood retinal arteriolar caliber (difference: -0.11 SDS (95% CI: -0.19, -0.03)). This association was only partly explained by maternal and childhood factors. In the fully adjusted model, low maternal PlGF levels tended to be associated with narrower childhood retinal arteriolar caliber (difference: -0.08 SDS (95% CI: -0.16, 0.00) compared to normal PlGF levels). Compared to the normal sFlt-1 levels, high second trimester sFlt-1 levels were associated with wider childhood retinal arteriolar caliber (difference: 0.10 SDS (95% CI: 0.02, 0.17)), but this association was fully explained by maternal and childhood characteristics.

Table 2.2.4 shows that no associations of first and second trimester maternal PlGF levels with childhood retinal venular caliber were present. High second trimester sFlt-1 levels were associated with wider childhood retinal venular caliber (difference: 0.09 SDS (95% CI: 0.01, 0.17)), but these associations were fully explained by maternal and childhood factors.

Table 2.2.3. Associations between PlGF and sFlt-1 during pregnancy with retinal arteriolar caliber in childhood (N = 3,505)

Angiogenesis markers	No.	Retinal arteriolar caliber in SDS (95% Confidence Interval (CI))		
		Basic model	Confounder model	Childhood model
First trimester				
PlGF				
< 28.30 pg/mL	655	-0.08 (-0.18, 0.02)	-0.08 (-0.19, 0.03)	-0.07 (-0.17, 0.03)
> 28.30 pg/mL	1,979	Reference	Reference	Reference
sFlt-1				
< 7.75 ng/mL	1,975	Reference	Reference	Reference
> 7.75 ng/mL	658	-0.07 (-0.15, 0.02)	-0.03 (-0.12, 0.06)	-0.01 (-0.10, 0.08)
Second trimester				
PlGF				
< 145.80 pg/mL	806	-0.11 (-0.19, -0.03)†	-0.09 (-0.17, -0.01)‡	-0.08 (-0.16, 0.00)
> 145.80 pg/mL	2,434	Reference	Reference	Reference
sFlt-1				
< 7.35 ng/mL	2,432	Reference	Reference	Reference
> 7.35 ng/mL	808	0.10 (0.02, 0.17)*	0.07 (-0.01, 0.15)	0.07 (-0.01, 0.14)

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal arteriolar caliber. Basic model is adjusted for child's age at visit, sex and gestational age at blood collection. Confounder model is additionally adjusted for lifestyle and socio-demographic confounders (maternal age, education, pre-pregnancy body mass index, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, pregnancy complications (gestational hypertension, preeclampsia and gestation diabetes), plus child's ethnicity, breastfeeding and TV watching). Childhood factors model is additionally adjusted for child' gestational age at birth and birth weight and childhood body mass index and blood pressure. Low PlGF is defined as the lower 25 percent and a high sFlt-1 as the upper 25 percent within our study population.* P-value = 0.02; †P-value = 0.008; ‡P-value = 0.036.

Table 2.2.4. Associations between PlGF and sFlt-1 during pregnancy with retinal venular caliber in childhood (N = 3,505)

Angiogenesis markers	No.	Retinal venular caliber in SDS (95% Confidence Interval (CI))		
		Basic model	Confounder model	Childhood model
First trimester				
PlGF				
< 28.30 pg/mL	655	0.01 (-0.10, 0.11)	0.00 (-0.10, 0.10)	0.01 (-0.09, 0.11)
> 28.30 pg/mL	1,979	Reference	Reference	Reference
sFlt-1				
< 7.75 ng/mL	1,975	Reference	Reference	Reference
> 7.75 ng/mL	658	-0.01 (-0.10, 0.08)	0.00 (-0.09, 0.09)	0.01 (-0.08, 0.09)
Second trimester				
PlGF				
< 145.80 pg/mL	806	-0.07 (-0.15, 0.01)	-0.07 (-0.15, 0.02)	-0.06 (-0.14, 0.02)
> 145.80 pg/mL	2,434	Reference	Reference	Reference
sFlt-1				
< 7.35 ng/mL	2,432	Reference	Reference	Reference
> 7.35 ng/mL	808	0.09 (0.01, 0.17)*	0.08 (0.00, 0.16)	0.08 (-0.00, 0.16)

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal venular caliber. Basic model is adjusted for child's age at visit, sex and gestational age at blood collection. Confounder model is additionally adjusted for lifestyle and socio-demographic confounders (maternal age, education, pre-pregnancy body mass index, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, pregnancy complications(gestational hypertension, preeclampsia and gestation diabetes), plus child's ethnicity, breastfeeding and TV watching). Childhood factors model is additionally adjusted for child' gestational age at birth and birth weight and childhood body mass index and blood pressure. *P = 0.03.

Discussion

Results from this prospective cohort study suggest that lower maternal second trimester PlGF levels are associated with narrower retinal arteriolar caliber in childhood. This association was not explained by maternal or childhood factors. No associations of first and second trimester sFlt-1 level with childhood retinal vessel caliber were present.

Methodological considerations

We used a population-based cohort study design with a large number of subjects. The response rate at baseline was 61%. Of all children participating in follow-up measurements at the age of 6 years, 63% participated in the retinal vessels follow-up studies. Loss to follow-up could lead to biased effect estimates if the associations of angiogenic factors with retinal vessel calibers would be different between children included and not included in the analyses. Non-response analyses showed that maternal PlGF and Sflt-1 levels during first trimester and child's birth weight was lower in mothers and children who were not included in the current analyses compared to those who were included. This could have led to an underestimation of the observed associations. We used validated techniques to measure retinal vessel calibers. We did not take into account other ocular factors, such as axial length and refractive error that might affect retinal vessels measurement.[21, 22] However, it has been previously shown among adults that these factors have only a small impact on the measurement of retinal vessel calibers and that they do not influence the associations between retinal vessel calibers and cardiovascular disease.[23] Although, we used a population based prospective cohort of healthy children and we performed extensive adjustments for a large number of potential confounders, residual confounding in the observed associations might still occur, as in any observational study. For example, no information was available about intraocular blood pressure and glucose levels in children, which can influence retinal vessel structure.

Interpretation of main findings

It has been suggested that developmental adaptations to the heart and blood vessels in response to a suboptimal fetal environment may lead to increased risks for cardiovascular diseases in adulthood.[1] Previously, we observed that suboptimal fetal and placental vascular development may have a persistent influence on cardiovascular function in childhood.[24] A prospective study among 44 adolescents found smaller diameters of large arteries among adolescents born with intrauterine growth restriction caused by an abnormal feto-placental blood flow, compared to those born with normal fetal growth and normal feto-placental blood flow.[25] Another study among 13,273 mothers and their children showed that placental vascular lesions, which might reduce feto-placental blood flow and increase feto-placental vascular resistance, are associated with higher blood pressure in infancy.[26] We have also previously reported that a higher feto-placental vascular resistance, which is an important component of the fetal circulation, is associated with lower left ventricular mass and a higher systolic blood pressure in childhood.[3] Thus, inadequate feto-placental vascular development has an adverse influence on offspring cardiovascular structure and function.

Feto-placental vascular development is dependent on several angiogenic factors.[4] PlGF is a pro-angiogenic factor, which plays an important role in aggregation and growth of endothelial precursors necessary for the formation of fetal vascular system in the placental villous tree. PlGF also contributes to the development of an adequate utero-placental circulation, by remodeling the maternal endothelium of the spiral arteries. sFlt-1, an anti-angiogenic factor secreted by endothelial cells, blocks the effect of PlGF.[4] Previous studies have reported that lower levels of PlGF and higher levels of sFlt-

1 in first and second trimester of pregnancy are associated with increased risk of preeclampsia, preterm birth and fetal growth restriction.[7] In the current study, we observed that lower second trimester maternal PlGF levels are associated with narrower retinal arteriolar caliber in childhood. This association was not explained by maternal socio-demographic and lifestyle related characteristics, pregnancy complications, birth characteristics or childhood factors. No associations of sFlt-1 with childhood retinal vessel caliber were present. Several longitudinal studies among adults have shown that retinal arteriolar narrowing, likely indicative of increased peripheral vascular resistance, is associated with increased risks of hypertension in later life, and can therefore be used as an early marker of cardiovascular disease risk.[11, 27] Also, studies among children aged 6-8 and 12.7 years have shown that narrower retinal arterioles were significantly correlated with increased risks of hypertension.[12]

Our results suggest that reduced maternal second trimester PlGF levels, which are associated with a suboptimal placenta development and the risk of adverse pregnancy outcomes [7, 8], might also lead to narrowing of microvasculature in later life. Reduced PlGF levels can disrupt normal growth of the vessels and the process of neo-angiogenesis, which might lead to permanent alterations in micro vessels structure.[28] Furthermore, lower PlGF levels reflect inadequate placental development, which affects oxygen levels in the feto-placental environment.[29] In humans, hypoxia during pregnancy increases sFlt-1 circulating levels and its biological sequelae, which can lead to altered in utero endothelial development by inhibiting endothelial cell proliferation and tubule formation [30], and by stimulating a systemic endothelial dysfunction.[6] Higher sFlt-1 levels block the normal function of pregnancy-related pro-angiogenic factors such as VEGF and PlGF, leading to lower PlGF circulating levels. Lower pro-angiogenic factors concentrations are shown to decrease nitric oxide (NO) availability, which could lead to endothelial dysfunction and impaired endothelium dependent vasodilatation.[31] Finally, altered PlGF and sFlt-1 levels inhibit transforming growth factor –β (TGF-β).[32] TGF-β is required for inhibition of sprouting endothelial cells angiogenesis and plays a role in vessel maturation and stabilization, and lower TGF-β concentrations can lead to endothelial dysfunction.[33] Thus, retinal arteriolar narrowing in childhood might reflect structural microvasculature changes and pathophysiological processes related to endothelial dysfunction.[34]

To the best of knowledge the current study is the first that examined the relation of maternal angiogenic factors during pregnancy with childhood microcirculation. Therefore, these results should be considered as hypothesis generating and need further replication. Also, further studies are needed to examine the potential mechanisms underlying the observed associations and whether these early microvasculature maladaptation contribute to an increased risk of cardiovascular disease in later life.

Conclusions

Maternal angiogenic factors during pregnancy are important for normal placental and fetal vascular development and function. Not much is known whether angiogenic factors during pregnancy might also affect childhood retinal microvasculature. Our results suggest that lower maternal second trimester PlGF levels influence microvascular development in the offspring, leading to narrower retinal arteriolar

caliber in childhood. This association was not explained by maternal or childhood factors. No associations of first and second trimester sFlt-1 level with childhood retinal vessel caliber were present. Further studies are needed to replicate these observations, to explore the underlying mechanisms and to examine the long long-term cardiovascular consequences.

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Supplemental Material

Table S2.2.1. Comparison of subject characteristics between children included and not included in the analyses

Characteristics	Participation	No participation	P value
Maternal characteristics			
Age, median (95% range), y	30.7(19.8, 39.4)	29.9 (19.0, 39.0)	*< 0.01*
Height, mean (SD), cm	167.6 (7.4)	166.9 (7.4)	*< 0.01*
Pre-pregnancy weight, mean (SD), kg	66.8 (12.9)	66.0 (12.9)	*0.01*
Pre-pregnancy body mass index, median (95% range), kg/m^2	23.8 (18.8, 36.0)	23.8 (18.6, 36.6)	*0.91*
Systolic blood pressure, mean (SD), mm/Hg	115.3 (12.0)	115.3 (12.5)	*0.60*
Diastolic blood pressure, mean (SD), mm/Hg	67.9 (9.5)	67.8 (9.6)	*0.34*
Parity, nulliparous (%)	56.7	54.2	*0.12*
Education, higher (%)	62.1	62.7	*0.88*
Folic acid use, never (%)	25.3	31.1	*< 0.01*
Smoked during pregnancy, Yes (%)	26.8	27.9	*0.32*
Gestational diabetes, Yes, (%)	0.9	1.2	*0.32*
Gestational hypertension, Yes, (%)	4.3	3.5	*0.06*
Preeclampsia, Yes, (%)	1.9	2.5	*0.09*
PlGF first trimester, median (95% range), pg/mL	41.8 (14.8, 192.8)	44.0 (14.5, 198.7)	*< 0.01*
PlGF second trimester, median (95% range), pg/mL	199.2 (75.9, 592.8)	202.7 (71.3, 595.6)	*0.05*
sFlt-1 first trimester, median (95% range), ng/mL	5.0 (1.9, 18.0)	5.1 (1.8, 14.7)	*0.03*
sFlt-1 second trimester, median (95% range), ng/mL	5.0 (1.5, 17.3)	4.9 (1.6, 18.1)	*0.38*
Birth and infant characteristics			
Gender, boys, (%)	49.7	50.9	*0.29*
Gestational age at birth, median (95% range), weeks	40.1 (36.0, 42.2)	40.1 (34.9, 42.3)	*0.01*
Birth weight, mean (SD), grams	3446 (538)	3386 (578)	*< 0.01*
Ethnicity, European (%)	62.7	59.8	*0.01*
Ever breastfeeding (%)	92.8	91.3	*0.03*
TV watching >= 2 hours per day (%)	19.0	19.8	*0.53*

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

Table S2.2.2. Associations of covariates with childhood retinal vessel calibers

Characteristics of the study population	Retinal vessel calibers in SDS	
	Arteriolar caliber	Venular caliber
Maternal characteristics		
Age	0.001 (-0.005, 0.008)	-0.007 (-0.014, -0.01)*
Systolic blood pressure	-0.006 (-0.008, -0.003)*	0.001 (-0.002, 0.003)
Diastolic blood pressure	-0.005 (-0.009, -0.002)*	0.001 (-0.003, 0.004)
Parity		
Nulliparous	*Reference*	*Reference*
Multiparous	-0.067 (-0.134, 0.001)	-0.049 (-0.116, 0.019)
Education		
Primary	-0.165 (-0.441, 0.112)	0.007 (-0.269, 0.283)
Secondary	-0.111 (-0.209, -0.014)*	-0.093 (-0.191, 0.004)
High	*Reference*	*Reference*
Folic acid use		
No	-0.045 (-0.141, 0.051)	0.027 (-0.069, 0.122)
Start 1st 10 weeks of pregnancy	0.012 (-0.078, 0.102)	-0.022 (-0.111, 0.067)
Start periconceptional	*Reference*	*Reference*
Smoked during pregnancy		
Yes	-0.083 (-0.163, -0.003)*	-0.066 (-0.145, 0.014)
No	*Reference*	*Reference*
Gestational diabetes		
Yes	0.030 (-0.316, 0.377)	0.040 (-0.306, 0.386)
No	*Reference*	*Reference*
Gestational hypertension		
Yes	-0.203 (-0.369, -0.037)*	-0.151 (-0.317, 0.015)
No	*Reference*	*Reference*
Preeclampsia		
Yes	-0.037 (-0.292, 0.217)	0.120 (-0.132, 0.372)
No	*Reference*	*Reference*
Birth and infant characteristics		
Gender		
Boys	*Reference*	*Reference*
Girls	0.172 (0.106, 0.238)*	0.208 (0.142, 0.274)*
Gestational age at birth	0.049 (0.030, 0.069)*	-0.004 (-0.023, 0.016)
Birth weight	0.112 (0.051, 0.174)*	0.004 (-0.058, 0.066)
Ethnicity		
Yes	0.03 (-0.038, 0.101)	0.077 (0.008, 0.146)*
No	*Reference*	*Reference*
Ever breastfeeding		
Yes	-0.164 (-0.310, -0.018)*	-0.179 (-0.325, -0.033)*
No	*Reference*	*Reference*
TV watching		
>= 2 hours per day	-0.071 (-0.167, 0.024)	0.099 (0.002, 0.196)
< 2 hours per day	*Reference*	*Reference*
Childhood characteristics		
Age at visit	-0.159 (-0.215, -0.103)*	-0.068 (-0.125, -0.012)
Body mass index	-0.035 (-0.052, -0.018)*	-0.09 (-0.008, 0.026)
Systolic blood pressure	-0.026 (-0.30, -0.022)*	-0.06 (-0.010, -0.002)*
Diastolic blood pressure	-0.020 (-0.025, -0.015)*	-0.001 (-0.006, 0.004)

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal vessel calibers in SDS per unit change of each covariate and for different categories of each covariate as compared to the reference group.
* P-value <0.05.

Chapter 2.3

Early growth patterns and childhood body fat distribution

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Abstract

Background: Higher infant growth rates are associated with an increased risk of obesity in later life. We examined the associations of longitudinally measured fetal and infant growth patterns with total and abdominal fat distribution in childhood.

Methods and Results: We performed a population-based prospective cohort study among 6,464 children. We measured growth characteristics in second and third trimester of pregnancy, at birth, and at 6, 12, and 24 months. Body mass index, fat mass index (body fat mass/height^2), lean mass index (body lean mass/height^2) and android/gynoid fat ratio measured by Dual-energy X-ray Absorptiometry, and subcutaneous and preperitoneal abdominal fat measured by ultrasound at the median age of 6.0 years (90% range 5.7 – 7.4). We observed that weight gain in second and third trimester of fetal life, and in early, mid and late infancy were independently and positively associated with childhood body mass index (p-values<0.05). Only infant weight gain was associated with higher fat mass index, android/gynoid fat ratio, and abdominal fat in childhood (p-values<0.05). Children with both fetal and infant growth acceleration had the highest childhood body mass index, fat mass index and subcutaneous abdominal fat, whereas children with fetal growth deceleration and infant growth acceleration had the highest value for android/gynoid fat ratio and the lowest value for lean mass index (p-values<0.05).

Conclusions: Both growth in fetal life and infancy affects childhood body mass index, whereas only infant growth affects directly measured total body and abdominal fat. Fetal growth deceleration followed by infant growth acceleration may lead to an adverse body fat distribution in childhood.

Introduction

Fetal life and infancy are critical periods for the development of overweight and cardiometabolic diseases.[1, 2] Both children with a high birth weight and those with a low birth weight followed by infant growth acceleration have an increased risk of overweight in later life.[3-5] Most studies used birth weight as proxy for fetal growth. However, birth weight is the result of various fetal growth patterns and the starting point of different infant growth patterns. Although higher infant weight gain is a well-established risk factor of childhood overweight, studies focused on directly measured fetal growth effects on adiposity are scarce.[6, 7] Also, body mass index is a widely accepted outcome measure, but more detailed body fat distribution measures might be stronger related to cardio-metabolic risk factors.[8, 9] In young adults, abdominal visceral fat is a risk factor for a diabetegonic and atherogenic profile[9], while abdominal subcutaneous fat is related to circulating leptin concentrations.[10] In adults, waist circumference is in addition to body mass index, associated with higher overall mortality rates.[8] We have previously shown that third trimester fetal growth restriction, followed by a high postnatal weight gain is related to higher fat mass in infancy.[11]

For the present study, we aimed to identify critical periods during fetal life and infancy that might be important for development of an adverse total and abdominal fat profile in childhood. We examined in 6,464 children participating in a population-based prospective cohort study from early fetal life onwards the associations of repeatedly measured fetal and infant growth characteristics with childhood body mass index, and fat mass index and abdominal fat distribution measured by Dual-energy X-ray absorptiometry (DXA) and abdominal ultrasound at the age of 6 years.

Methods

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[12] All children were born between 2002 and 2006. Response rate at birth was 61%.[12] The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all parents. In total, 8,305 children participated in the follow-up measurements at the median age of 6.0 years (90% range 5.7 – 7.4), of whom 6,464 participated in the detailed measurements for the current study (Supplemental **Figure S2.3.1** gives a detailed flow chart).

Fetal and infant growth assessments

Fetal ultrasound examinations were carried out in first (median 13.2 weeks (90% range 11.1 to 17.0)), second (median 20.5 weeks (90% range 18.9 to 22.8)), and third (median 30.4 weeks (90% range 28.8 to 32.4)) trimester.[12] First trimester ultrasound was used for establishing gestational age because this method is better than using last menstrual period.[13] Second and third trimester ultrasounds were used to assess fetal growth. Fetal head circumference (HC), abdominal circumference (AC) and femur length (FL) were measured to the nearest millimeter using standardized ultrasound procedures. Estimated fetal weight (EFW) was calculated using the formula by Hadlock *et al.*[14] (log10

EFW=1.5662-0.0108 (HC) +0.0468 (AC) +0.171 (FL) +0.00034 (HC)^2-0.003685 (AC x FL)). Standard deviation scores (SDS) for all fetal growth characteristics were constructed based on the data from the study group.

Birth length and weight were obtained from community midwife and hospital registries. We created gestational age- and sex-adjusted birth length and weight (SDS) within our study population by using Growth Analyser 3.5 (Dutch Growth Research Foundation, Rotterdam, the Netherlands) based on North-European growth charts.[15] Preterm birth was defined as a gestational age of <37 weeks. Small for gestational age and large for gestational age were defined as the lowest and highest 10th percentiles of gestational age- and sex-adjusted birth weight in the cohort.

Infant length and weight were measured in community health centers using standardized methods at the ages of 6 months (median 6.2 months (90% range 5.5 to 7.5)), 12 months (median 11.1 months (90% range 10.2 to 12.3)) and 24 months (median 24.8 months (90% range 23.5 to 27.5)).[12] Length was measured in a supine position to the nearest millimeter with a neonatometer. Weight was measured with a mechanical personal scale and body mass index was calculated. We created age- and sex-adjusted SDS of these infant anthropometrics within our study population using Dutch reference growth charts (Growth Analyser 3.5, Dutch Growth Research Foundation, Rotterdam, The Netherlands).[16]

We defined growth intervals for each growth characteristic at different time points. Second trimester fetal growth was defined as the period between second and third trimester fetal life; third trimester fetal growth as the period between third trimester to birth; early, mid and late infancy as the periods between birth to 6 months, 6 to 12 and 12 to 24 months of age, respectively. As previously described, a change in SDS greater or smaller than 0.67 between each growth characteristics measured at different time points was considered as growth acceleration and growth deceleration, respectively.[6, 16] A change of 0.67 SDS represents the width of each percentile band on standard growth charts, which helps to indicate growth acceleration and deceleration in clinical practice.[6, 16]

Total and abdominal fat distribution assessments in childhood

At the median age of 6 years (median 6.0 years (95% range 5.7, 7.4 years)) we performed follow-up measurements of childhood height and weight without shoes and heavy clothing. All measurements were performed in a dedicated research center by research staff, who were trained to perform the measures according to specific research protocols. Height was measured to the nearest millimeter by a stadiometer (Holtain Limited, Crosswell, Crymych, UK). Weight was measured to the nearest gram using an electronic scale (SECA 888, Almere, The Netherlands), and body mass index (kg/m2) was calculated.

Total body and regional fat mass was measured using a Dual-energy X-ray absorptiometry (DXA) scanner (iDXA, GE-Lunar, 2008, Madison, WI, USA) and analyzed with the enCORE software v.12.6.[17] DXA can accurately detect whole-body fat mass within less than 0.25% coefficient of variation. Children were placed without shoes, heavy clothing and metal objects in supine position on the DXA table. Total fat mass (kg) was calculated as percentage of total body weight (kg) measured by DXA. The fat mass index

(body fat mass/height^2), lean mass index (body lean mass/height^2) and the android/gynoid fat mass ratio were calculated. The android/gynoid fat ratio reflects the central body fat distribution in the abdomen and hip regions, respectively and was used as a marker of waist/hip fat distribution.[18]

Abdominal ultrasound examinations were performed with ultrasound, as described in detail.[19, 20] Briefly, subcutaneous and pre-peritoneal fat thicknesses were measured with a linear transducer[20], which was placed perpendicular to the skin surface on the median upper abdomen. We scanned longitudinally just below the xiphoid process to the navel along the midline (linea alba). All measurements were performed off-line. Subcutaneous fat mass distance (SC-distance) was measured as distance of the inner surface of subcutaneous tissue to the linea alba. Preperitoneal fat mass distance (PP-distance) was measured as distance of the linea alba to the peritoneum on top of the liver. Subcutaneous and preperitoneal fat mass areas were measured as areas of 2 cm length along the midline starting from the maximum preperitoneal distance in direction of the navel (SC-area, PP-area). We measured three times the areas of 2 cm length along midline, and we used the mean value of these measures. The intra-observer reproducibility and the intra-class correlation coefficients ranged from 0.93 to 0.97.

Covariates

We collected information about maternal age, parity, educational level, pre-pregnancy body mass index, smoking during pregnancy, and folic acid supplement use by questionnaires. Child's ethnicity (European, Non-European) was classified by the countries of birth of the parents.[21] Information on breastfeeding and average TV watching time was assessed by questionnaire.[22]

Statistical analysis

First, differences in subject characteristics between boys and girls were examined with ANOVA and Chi-square tests. Second, we used linear regression models to assess the associations of growth measures in different fetal and infant growth intervals with adiposity outcomes at the age of 6 years. These analyses were adjusted for the corresponding age interval. We tested for potential interaction of child's sex with gain in length or weight in different growth intervals. Third, to take account for the correlation between fetal and infant growth characteristics we used conditional regression analyses to examine associations of these measures with childhood outcomes. We constructed length, weight and body mass index gain variables, which are statistically independent from each other, using standardized residuals resulting from the linear regression model of length, weight and body mass index regressed on the prior corresponding growth measurements.[23] This allows simultaneous inclusion of all growth measures in a regression model.[23] Fourth, we used multiple linear regression models to explore the associations of fetal and infant anthropometrics with childhood fat measures. These models did not take growth measured at other ages into account. Finally, we categorized fetal (second trimester to birth) growth and infant (birth to 24 months) growth, each into three groups defined as growth deceleration, normal growth and growth acceleration. We used stratified multiple linear regression models to assess whether the associations of fetal growth with adiposity outcomes were modified by infant growth. We tested

potential interactions between fetal growth and infant growth measures. We additionally explored the associations of children born small and large for their gestational age with childhood fat outcomes. For all analyses, we included covariates in the models based on their associations with body fat distribution in previous studies, an association with outcomes, or a change in effect estimates of >10%. As abdominal fat measures had skewed distributions, we applied natural log-transformation. To enable comparison of effect estimates, results are presented in outcome SDS ((observed value – mean) / SD) per change growth SDS. Tests for trends were performed by treating growth characteristics as a continuous term. In order to reduce potential bias due to missing data, we performed multiple imputations of missing covariates (<25% missing values) by generating five independent datasets using the Markov Chain Monte Carlo method, and the pooled effect estimates (95% Confidence Interval (CI)) are presented.[24] Imputations were based on the relationships between covariates, determinants and outcomes. Analyses were performed using the Statistical Package of Social Sciences version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Table 2.3.1 gives the subject characteristics. Table 2.3.2 presents the fetal, birth, infant and childhood anthropometrics for boys and girls. At the age of 6 years, girls had higher fat mass index and abdominal body fat measures than boys (p-values <0.01).

Fetal and infant growth and childhood body fat outcomes

Table 2.3.3 shows that length gain from second trimester life until mid-infancy was not consistently associated with adiposity outcomes in childhood, whereas length gain in late infancy was positively associated with body mass index and lean mass index, and inversely with fat mass index and preperitoneal fat mass in childhood (p-values <0.05). Estimated fetal weight and abdominal circumference growth in second and third trimester of fetal life, and weight gain in early, mid and late infancy were all positively associated with childhood body mass index (p-values <0.05), with the strongest effect estimates at the oldest age. Estimated fetal weight gain and abdominal circumference gain in fetal life were not associated with childhood general and abdominal fat, but weight and body mass index gain in all infancy periods were associated with higher total mass, android/gynoid fat mass ratio, subcutaneous and preperitoneal abdominal fat mass and lower lean mass index in childhood (p-values <0.05). These effect estimates were smaller than those for childhood body mass index. Supplemental Tables S2.3.1 and S2.3.2 show the associations of fetal and infant growth characteristics with childhood fat measures using regular linear regression analyses. Supplemental Table S2.3.4 gives the associations of size at birth with body fat outcomes at the age of 6 years.

We further explored whether the associations of growth characteristics with childhood body fat outcomes were independent from growth measures at other ages using conditional growth analyses. Figure 2.3.1A shows that independent from growth in length at other ages, length in fetal life was inversely, and length in infancy was positively associated with childhood body mass index (p-values<0.05). We observed stronger

effect estimates for weight than for length. Figures 2.3.1B-F show that, at 20 weeks of gestational age, length and estimated fetal weight growth were not independently associated with total and abdominal fat measures, whereas at 30 weeks of gestational age fetal femur length was inversely associated with fat mass index, android/gynoid fat mass ratio,and subcutaneous and preperitoneal abdominal fat mass (p-value< 0.05). Weight and body mass index at 6, 12 and 24 months were all independently and positively associated with these fat outcomes, whereas were inversely associated with lean mass index, with stronger effect estimates at older ages (p-value< 0.05). Similar results were present in the crude models (Supplemental Figure S2.3.2).

Table 2.3.1. Maternal and child characteristics

Characteristics	Boys	Girls	P value
Maternal characteristics			
Age (years)	31.1 (21.1, 38.7)	31.0 (21.3, 38.5)	*0.02*
Height (cm)	167.6 (7.2)	167.4 (7.6)	*0.03*
Weight (kg)	67.0 (53.0, 94.0)	67.0 (52.0, 95.0)	*0.05*
Body mass index(kg/m^2)	23.8 (19.4, 33.2)	23.9 (19.5, 33.7)	*0.01*
Parity (%)			
0	55.6	57.1	*<0.01*
>=1	44.4	42.9	
Education (%)			
Lower	53.8	55.6	*<0.01*
Higher	46.2	44.4	
Folic acid use (%)			
No use	27.4	25.5	*<0.01*
Start in the first 10 weeks	31.9	31.2	
Start periconceptional (%)	40.7	43.3	
Smoked during pregnancy (%)			
Never	72.9	75.1	*<0.01*
Ever	27.1	24.9	
Childhood characteristics			
Ethnicity (%)			
Dutch or European	63.1	64.6	*0.01*
Non-European	36.9	35.4	
Ever breastfeeding (%)	92.4	92.4	*0.40*
Breastfeeding duration (months)	3.5 (0.5, 12.0)	3.5 (0.5, 12.0)	*0.13*
TV watching (%)			
>= 2 hours per day	20.8	18.1	*<0.01*
< 2hours per day	79.2	81.9	

Values are means (SD), percentages (%), or medians (90% range) for variables with skewed distribution. The values represent the pooled results after multiple imputations (see methods). Differences characteristics for boys and girls were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables.

Combined effects of fetal and infant growth patterns and childhood body fat outcomes

As compared to children with normal fetal and infant growth, children with both fetal and infant growth deceleration had the lowest childhood body mass index, fat mass index and subcutaneous abdominal fat mass and the highest childhood lean mass index. Children with both fetal and infant growth acceleration had the highest levels of childhood fat mass (Table 2.3.4). The highest value for android/gynoid fat mass ratio and the lowest value of lean mass index were observed among children with fetal growth deceleration followed by infant growth acceleration. Interaction terms between fetal and infant growth categories were significant for fat mass index, android/gynoid fat mass

ratio and subcutaneous abdominal fat mass. Sensitivity analyses without preterm children did not materially change the results (data not shown). Supplemental Table S2.3.3 gives the associations of size at birth, instead of fetal growth, and infant growth with body fat outcomes. These results were largely similar as those from the models with fetal growth.

Table 2.3.2. Fetal, infant and childhood anthropometrics

	Boys	Girls	P value
Second trimester (n = 5,614)			
Gestational age (weeks)	20.6 (18.9-22.9)	20.5 (18.9-22.7)	*<0.01*
Femur length (mm)	33.5 (3.6)	33.5 (3.6)	*0.81*
Abdominal circumference (mm)	158.2 (15.3)	155.9 (14.2)	*<0.01*
Estimated fetal weight (g)	387 (98)	378 (91)	*<0.01*
Third trimester (n = 5,752)			
Gestational age (weeks)	30.4 (28.9-32.4)	30.3 (28.8-32.3)	*<0.01*
Femur length (mm)	57.4 (3.1)	57.6 (3.1)	*<0.01*
Abdominal circumference (mm)	265.4 (16.6)	263.3 (16.8)	*<0.01*
Estimated fetal weight (g)	1632 (259)	1618 (268)	*0.05*
Birth (n = 6,464)			
Gestational age (weeks)	40.1 (37.0-42.1)	40.1 (36.9-42.0)	*0.11*
Length (cm)	50.6 (2.4)	49.9 (2.3)	*<0.01*
Birth weight (grams)	3486 (570)	3362 (533)	*<0.01*
Preterm birth (< 37 weeks) %	4.1	4.3	*0.60*
Low birth weight (<2500 g) %	4.9	5.2	*0.40*
Small for gestational age (%)	4.3	4.7	*0.71*
Large for gestational age (%)	10.2	9.8	*0.96*
6 months (n = 4,760)			
Age at visit (months)	6.2 (5.4-7.5)	6.2 (5.5-7.5)	*0.67*
Length (cm)	68.5 (2.5)	66.7 (2.5)	*<0.01*
Weight (kg)	8.2 (0.9)	7.6 (0.8)	*<0.01*
Body mass index (kg/m^2)	17.4 (1.4)	17.1 (1.4)	*<0.01*
12 months (n = 4,396)			
Age at visit (months)	11.1 (10.2-12.3)	11.1 (10.2-12.3)	*0.57*
Length (cm)	75.1 (2.5)	73.5 (2.5)	*<0.01*
Weight (kg)	10.0 (1.1)	9.3 (1.0)	*<0.01*
Body mass index (kg/m^2)	17.6 (1.4)	17.2 (1.4)	*<0.01*
2 years (n = 4,122)			
Age at visit (years)	24.8 (23.5-27.5)	24.8 (23.6-27.4)	*0.740*
Length (cm)	88.9 (3.3)	87.7 (3.4)	*<0.01*
Weight (kg)	13.2 (1.5)	12.7 (1.5)	*<0.01*
Body mass index (kg/m^2)	16.7 (1.4)	16.5 (1.5)	*<0.01*
6 years (n = 6,464)			
Age at visit (years)	6.0 (5.7-7.6)	6.0 (5.7-7.2)	*0.02*
Height (cm)	120.0 (6.1)	119.0 (6.1)	*<0.01*
Weight (kg)	23.4 (4.1)	23.2 (4.5)	*0.01*
BMI (kg/m^2)	15.9 (14.0-19.4)	15.9 (13.9-20.2)	*0.34*
Fat mass index	0.0015 (0.0011-0.0022)	0.0019 (0.0014-0.0026)	*< 0.01*
Lean mass index	0.0052 (0.0040, 0.0062)	0.005 (0.0037, 0.0060)	*< 0.01*
Android / gynoid fat ratio	0.24 (0.17-0.36)	0.24 (0.17-0.39)	*0.01*
Subcutaneous fat area (mm^2)	41.0 (19.0-125.0)	57.0 (25.3-171.0)	*< 0.01*
Preperitoneal fat area (mm^2)	35.0 (17.0-81.0)	44.0 (21.0-112.0)	*< 0.01*

Values are means (SD) or medians (90% range) for variables with skewed distribution. Differences in fetal, infant and child characteristics for boys and girls were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables. Small for gestational age was defined as age- and sex-adjusted birth weight < 10%. Large for gestational age was defined as age- and sex-adjusted birth weight > 10%.

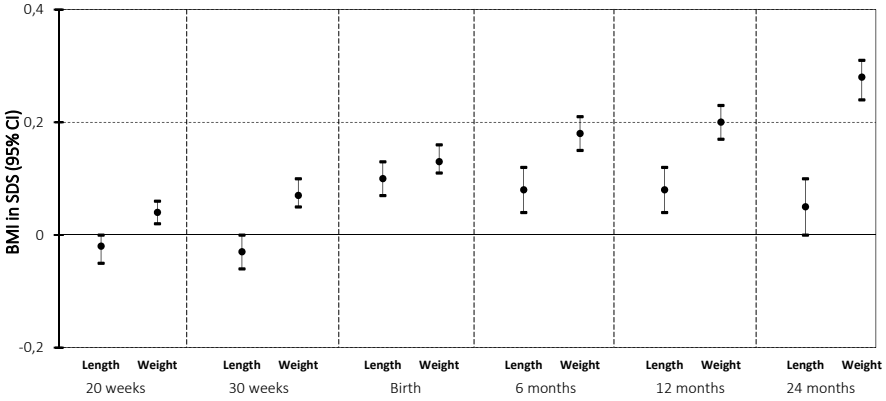
Table 2.3.3. Associations of fetal and infant length, weight, abdominal circumference and body mass index gain, with body fat outcomes at the age of 6 years

		Difference in body fat outcomes SDS (95% Confidence Interval)					
	N	Body mass index	Fat mass index	Lean mass index	Android/gynoid fat ratio	Subcutaneous fat area	Preperitoneal fat area
Length gain (SDS)							
Second trimester	N = 5,447	0.01 (-0.02, 0.03)	-0.01 (-0.03, 0.01)	0.01 (-0.01, 0.02)	-0.02 (-0.05, 0.01)	0.00 (-0.02, 0.03)	-0.02 (-0.04, 0.02)
Third trimester	N = 3,747	0.08 (0.05, 0.10)*	0.01 (-0.01, 0.03)	-0.01 (-0.02, 0.01)	0.02 (-0.00, 0.04)	-0.01 (-0.04, 0.01)	0.00 (-0.03, 0.03)
Early infancy	N = 2,695	0.00 (-0.03, 0.03)	0.00 (-0.03, 0.02)	0.00 (-0.01, 0.02)	0.00 (-0.03, 0.03)	0.03 (0.00, 0.06)	0.03 (-0.01, 0.06)
Mid infancy	N = 3,669	0.05 (0.00, 0.09)*	0.04 (-0.01, 0.08)	-0.02 (-0.04, 0.01)	0.04 (-0.01, 0.09)	0.02 (-0.03, 0.08)	0.06 (0.01, 0.12)
Late infancy	N = 3,419	0.05 (0.01, 0.10)*	-0.04 (-0.09, -0.01)*	0.02 (0.00, 0.09)*	0.00 (-0.05, 0.05)	-0.05 (-0.09, 0.01)	-0.05 (-0.10, 0.00)*
Weight gain (SDS)							
Second trimester	N = 5,402	0.05 (0.02, 0.07)*	-0.01 (-0.03, 0.02)	0.00 (-0.01, 0.02)	-0.04 (-0.01, 0.01)	0.00 (-0.03, 0.03)	0.00 (-0.03, 0.03)
Third trimester	N = 5,707	0.08 (0.06, 0.11)*	0.00 (-0.02, 0.03)	0.00 (-0.02, 0.01)	-0.03 (-0.06, 0.00)	0.02 (-0.01, 0.05)	0.01 (-0.02, 0.04)
Early infancy	N = 4,726	0.15 (0.13, 0.18)*	0.11 (0.09, 0.14)*	-0.07 (-0.08, -0.05)*	0.08 (0.05, 0.10)*	0.08 (0.06, 0.11)*	0.06 (0.03, 0.09)*
Mid infancy	N = 4,096	0.21 (0.16, 0.26)*	0.09 (0.04, 0.14)*	-0.05 (-0.08, -0.02)*	0.09 (0.03, 0.15)*	0.11(0.06, 0.18)*	0.11 (0.05, 0.17)*
Late infancy	N = 3,479	0.28 (0.23, 0.33)*	0.11 (0.06, 0.15)*	-0.07 (-0.09, -0.04)*	0.15 (0.10, 0.21)*	0.13 (0.08, 0.18)*	0.14 (0.09, 0.20)*
Abdominal circumference gain (SDS)							
Second trimester	N = 5,431	0.04 (0.01, 0.06)*	-0.01 (-0.03, 0.01)	0.00 (-0.01, 0.02)	-0.01 (-0.04, 0.02)	0.00 (-0.03, 0.02)	0.00 (-0.02, 0.03)
Body mass index (SDS)							
Mid infancy	N = 3,644	0.13 (0.09, 0.17)*	0.05 (0.01, 0.08)*	-0.03 (-0.05, -0.01)*	0.04 (0.00, 0.09)*	0.07 (0.03, 0.11)*	0.04 (0.00, 0.08)*
Late infancy	N = 3,396	0.18 (0.14, 0.21)*	0.10 (0.07, 0.13)*	-0.06 (-0.08, -0.04)*	0.11 (0.07, 0.15)*	0.12 (0.08, 0.15)*	0.12 (0.08, 0.16)*

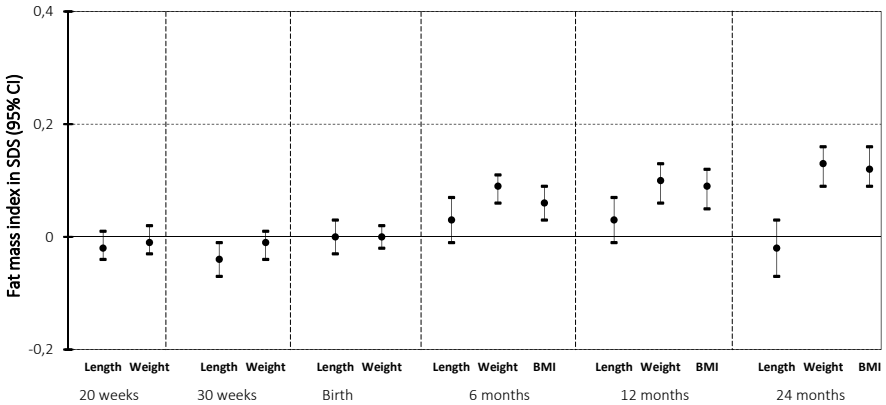
Values are standardized regression coefficients (95% confidence interval) and reflect the difference for each body fat measure per SDS increase of fetal and infant anthropometrics in different age intervals. All models are adjusted for child's age at visit, sex and height, plus maternal age, education, pre-pregnancy BMI, parity, folic acid use, maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching, and for the age interval between two measurements. All p-values > 0.05 for interaction of child's sex with gain in length or weight in different age intervals. * P < 0.01.

Figure 2.3.1. Associations of fetal and infant growth measures conditional on prior measures with body fat outcomes at the age of 6 years

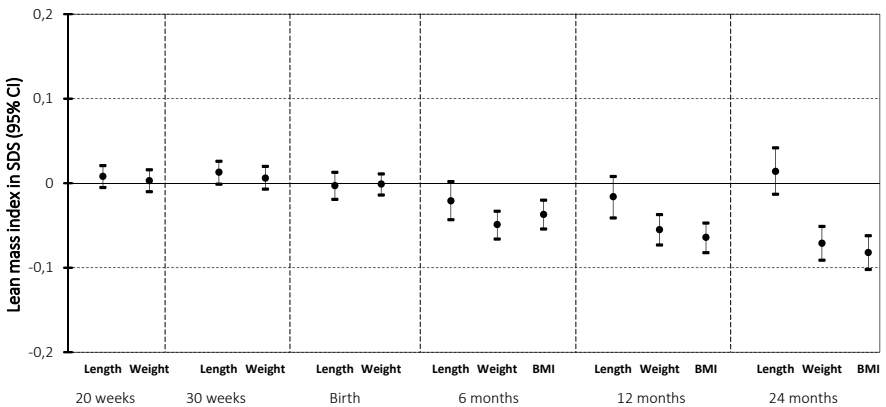
A. Body mass index



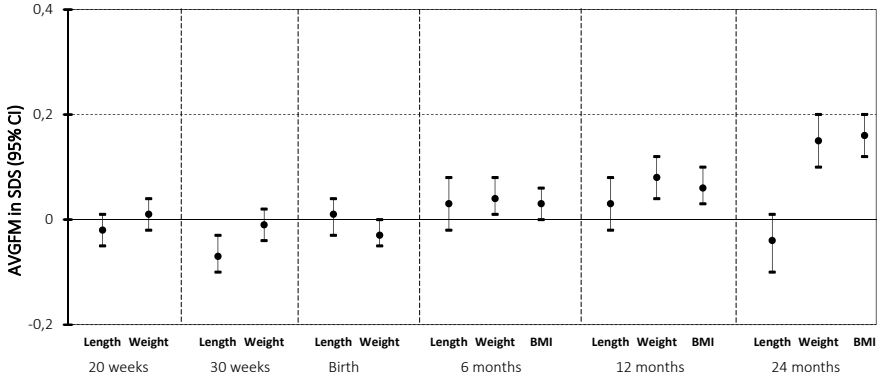
B. Fat mass index



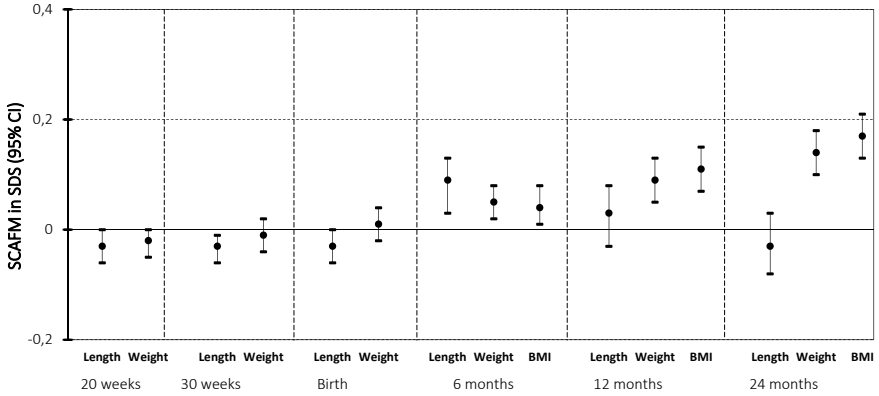
C. Lean mass index



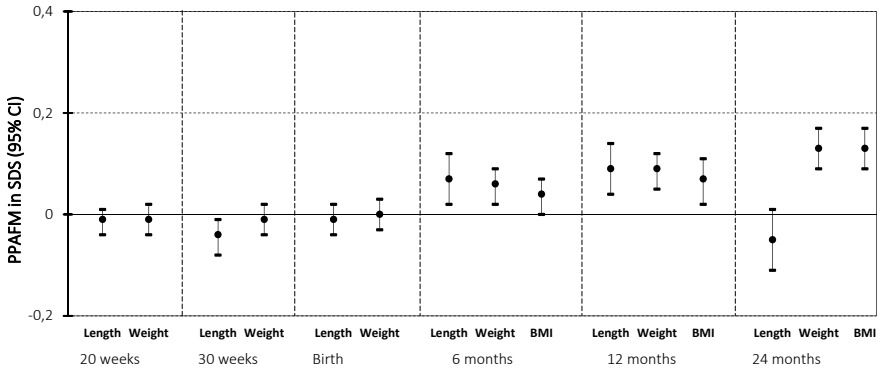
D. Android/Gynoid fat mass ratio



E. Subcutaneous area fat mass



F. Preperitoneal area fat mass



Abbreviations: SDS; Standard deviation scores; CI, confidence interval; BMI; body mass index; AVGFM; android/gynoid fat mass ratio; SCAFM; subcutaneous fat mass area; PPAFM; preperitoneal fat mass area; Values are standardized regression coefficients (95% CI) obtained from conditional analyses. The estimates represent differences in total and abdominal fat measures per standardized residual change of fetal and infant growth measures. Analyses with length and weight gain variables considered as starting point growth measures at 20 weeks of gestational age, whereas analysis with BMI considered as starting point BMI at the age of 6 months. Models are adjusted for child's age at visit, sex and height, plus maternal age, education, pre-pregnancy BMI, parity, folic acid use and maternal smoking during pregnancy, plus child's ethnicity, breast-feeding and TV watching.

Table 2.3.4. Associations of fetal and infant growth with body fat outcomes at the age of 6 years

Fetal growth	Infant growth	Difference in body fat outcomes SDS (95% Confidence Interval)					
		Body mass index	Fat mass index	Lean mass index	Android/gynoid fat ratio	Subcutaneous fat area	Preperitoneal fat area
Deceleration	Deceleration	-0.58 (-0.72, -0.43)* N=146	-0.26 (-0.41, -0.13)* N=144	0.18 (0.10, 0.26)* N=144	-0.06 (-0.23, 0.10) N=144	-0.18 (-0.34, -0.01)* N=119	-0.10 (-0.30, 0.08) N=120
	Normal	-0.20 (-0.30, 0.10) N=441	-0.03 (-0.12, 0.06) N=431	0.02 (-0.03, 0.07) N=431	-0.02 (-0.13, 0.04) N=431	-0.12 (-0.23, 0.00) N=349	-0.07 (-0.18, 0.05) N=348
	Acceleration	0.30 (0.20, 0.39)* N=358	0.28 (0.20, 0.38)* N=376	-0.16 (-0.21, -0.11)* N=376	0.35 (0.24, 0.46)* N=376	0.26 (0.15, 0.37)* N=315	0.20 (0.09, 0.32)* N=306
	Trend effect estimates	*0.34 (0.27, 0.41)**	*0.23 (0.16, 0.30)**	*-0.14 (-0.18, -0.10)**	*0.21 (0.12, 0.29)**	*0.23 (0.15, 0.31)**	*0.16 (0.08, 0.24)**
Normal	Deceleration	-0.35 (-0.45,-0.26)* N=888	-0.22 (-0.32, -0.13)* N=863	0.14 (0.09, 0.20)* N=888	-0.09 (-0.21, 0.02) N=863	-0.18 (-0.29, -0.07)* N=717	-0.17 (-0.20, 0.05)* N=709
	Normal	Reference N=968	Reference N=946	Reference N=946	Reference N=946	Reference N=783	Reference N=772
	Acceleration	0.51 (0.41, 0.60)* N=619	0.25 (0.16, 0.35)* N=600	-0.14 (-0.19, -0.08)* N=619	0.17 (0.06, 0.29)* N=600	0.28 (0.16, 0.39)* N=503	0.25 (0.14, 0.37)* N=498
	Trend effect estimates	*0.36 (0.31, 0.40)**	*0.24 (0.20, 0.29)**	*-0.14 (-0.17, -0.11)**	*0.16 (0.11, 0.21)**	*0.21 (0.16, 0.27)**	*0.16 (0.10, 0.22)**
Acceleration	Deceleration	-0.19 (-0.28, 0.01) N=282	-0.09 (-0.18, 0.00) N=273	0.05 (0.00, 0.11) N=273	-0.04 (-0.15, 0.07) N=273	-0.06 (-0.16, 0.05) N=215	0.04 (-0.07, 0.16) N=214
	Normal	0.14 (0.04, 0.25)* N=293	0.10 (0.01, 0.19)* N=280	-0.06 (-0.11, -0.01)* N=280	0.05 (-0.06, 0.15) N=280	0.14 (0.03, 0.24) N=233	0.06 (-0.05, 0.17) N=234
	Acceleration	0.66 (0.52, 0.81)* N=88	0.29 (0.15, 0.43)* N=85	-0.13 (-0.21, -0.05)* N=85	0.20 (0.03, 0.36)* N=85	0.33 (0.17, 0.49)* N=68	0.30 (0.13, 0.47)* N=67
	Trend effect estimates	*0.35 (0.28, 0.42)**	*0.23 (0.16, 0.29)**	*-0.13 (-0.16, -0.09)**	*0.15 (0.08, 0.22)**	*0.20 (0.13, 0.29)**	*0.13 (0.06, 0.22)**

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for each body fat measures compared to children with normal fetal and infant growth. Fetal growth was defined as the period between second trimester to birth. Infant growth was defined as the period between birth to 2 years. Models are adjusted for child's age at visit, sex and height, plus maternal age, education, pre-pregnancy BMI, parity, folic acid use and maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching. Trend tests represent the effect estimates of infant growth within each third trimester fetal growth group. * $P < 0.01$

Discussion

In this population-based prospective cohort study, we aimed to identify critical periods during fetal life and infancy that might be important for development of an adverse total and abdominal fat profile in childhood. We observed that weight gain in second and third trimester of fetal life and in early, mid and late infancy was independently and positively associated with childhood body mass index. Only infant weight gain was associated higher childhood fat mass index, android/gynoid fat mass ratio, and abdominal fat mass. Children who had fetal growth deceleration followed by infant growth acceleration had the highest value for android/gynoid fat mass ratio.

Methodological considerations

We used a population-based prospective cohort design including a large number of subjects whom we studied from early fetal life onwards. The large number of repeated fetal and infant anthropometric measurements enabled us to explore potential critical periods in early life for the development of an adverse body fat distribution. Selection bias in follow-up studies mainly arises from loss to follow-up rather than from non-response at baseline.[25] Of all children with information about birth weight and gestational age, 77% did participate in the follow-up measurements at the age of 6 years, of whom at least 80% participated in the body fat assessments. Loss to follow-up would lead to selection bias if the associations of fetal and infant anthropometrics with body fat outcomes would be different between children included and not included in the final analyses. Birth weight was lower in those who were not included in the current analyses than in those who were included (difference -50.4 g (95% CI -24.7, -76.1), $p<0.05$). It is hard to speculate whether this difference would affect the observed associations materially, but we consider this unlikely. We had low number of children with information on birth length available, because it was not measured at all delivery centers. Information on anthropometrics at the age of 24 months was missing in 32% of the study population. These missing values are mainly due to the design of the study, which included growth data collected from birth until the age of 4 years in only a subgroup of the total study population.[12] We performed detailed measurements of childhood body fat distribution. Both DXA and abdominal ultrasound have been validated against CT. The abdominal ultrasound measurement of preperitoneal abdominal fat, may be prone to relatively higher measurement error, which may subsequently lead to underestimation of the observed associations.[19] We could not adjust for detailed measures of childhood diet, as this information was available only in a small subgroup of the study population. Although, we performed an extensive adjustment for a large number of potential confounders, residual confounding in the observed associations might still occur, as in any observational study.

Comparison of main findings with other studies

Fetal and early childhood life has been recognized as critical periods for the development of overweight and cardio-metabolic diseases in later life.[26] Our findings suggest that both fetal and infant growth patterns influence body mass index and body fat distribution in childhood, with strongest effects present for growth in late infancy and for

body mass index as outcome. Previously, it has been shown that both children with a high and a low birth weight followed by infant growth acceleration tend to have a higher body mass index and are at increased risk of overweight in childhood and adulthood.[4-7, 27] A prospective cohort study in 27,899 full term children showed that, independent of birth weight, the rate of weight gain during the first 4 months was associated with an increased risk of overweight at the age of 7 years.[28] Another cohort study among 848 full term born children suggested that children who have infant growth acceleration in height and weight between birth and the age of 2 years, had a higher body mass index at the age of 5 years.[6]

Body fat distribution is stronger related with cardio-metabolic risk factors in childhood and adulthood than body mass index.[8, 9] We measured both total and abdominal fat mass using DXA and ultrasound. We did not observe consistent associations for independent fetal and early infant length with abdominal fat. We used preperitoneal fat as a measure of visceral fat.[19] Our results suggest that early growth patterns had stronger effects on fat mass index than on preperitoneal abdominal fat mass in childhood. The lack of associations may be explained by the relative narrow range of variation preperitoneal fat in children and a larger measurement error for this measure.[19] In the current study, only infant weight and body mass index gain, but not growth in the fetal period, were associated with higher childhood total and abdominal fat, and an adverse body fat distribution as reflected by a higher android/gynoid fat ratio. Previously, we also observed that infant weight gain from birth to the age of 2 years was associated with higher abdominal preperitoneal fat in children at the age of 2 years.[29] Various studies have identified rapid weight gain in infancy as predictor of higher body fat.[3, 30] A prospective cohort study among 561 children reported that rapid weight gain in infancy was associated with higher skinfold thicknesses at the age of 7 years.[31] Another study among 121 obese individuals aged 5 to 22 years, suggested that the variability in central adiposity was more strongly influenced by infant growth than by birth weight.[32] Thus, rapid infant growth might influence development of later body composition.

We have shown in a subgroup of 252 infants in the same study population that birth weight was positively associated with total fat at the age of 6 months.[11] Similarly, a large cohort study in 6,086 children, suggested that higher birth weight predicted higher DXA-derived total fat, but not truncal fat at the age of 9 years.[33] A study among 255 7-year old children suggested that birth weight was inversely associated with central fat.[34] In the current study, we observed that higher birth weight was associated with higher total and abdominal body fat, but after adjustment for current child's height, these associations attenuated. In a previous study we also did not observe associations between birth weight and abdominal body fat outcomes at the age of 2 years.[29] Our findings are in line with a small study among 242 overweight children that suggested that birth weight predicted body mass index and DXA-derived truncal and total fat, but not MRI-derived visceral or subcutaneous fat in children at the age of 11 years.[35] Differences in results between studies might be attributed to different outcome measures, adjustment for different potential confounders or adjustment for current height. We also observed that children with fetal growth deceleration followed by infant growth acceleration had increased body fat outcomes and lower lean mass index. These findings are in line with other studies showing that combination of fetal growth decelera-

tion and infant growth acceleration may lead to higher total and abdominal body fat than fetal growth deceleration without infant growth acceleration.[7, 32, 36] This might be one of the pathways underlying the previously observed associations of low birth weight with type 2 diabetes in later life.[37] Similarly, it has also been suggested that children born small for gestational age had higher abdominal subcutaneous fat in adolescence [38, 39] and higher total and abdominal fat, measured by DXA and MRI, between the ages of 2 to 6 years.[40] This findings are in line with the developmental and origins of health hypothesis which suggests that an adverse fetal environment leads to adaptations that program the metabolism of the fetus.[41] Further studies are needed to assess which factors influence infant growth acceleration.

Conclusions

This study suggests that both growth in fetal growth and infancy affects childhood body mass index, whereas only infant growth affects directly measured fat mass index, android/gynoid fat mass and subcutaneous and preperitoneal abdominal fat. Fetal growth deceleration followed by infant growth acceleration may lead to an adverse body fat distribution. Further studies are needed to explore whether these growth patterns affect body fat distribution and risk of diseases later in life.

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Supplemental Material

Figure S2.3.1. Flow chart of participants in study

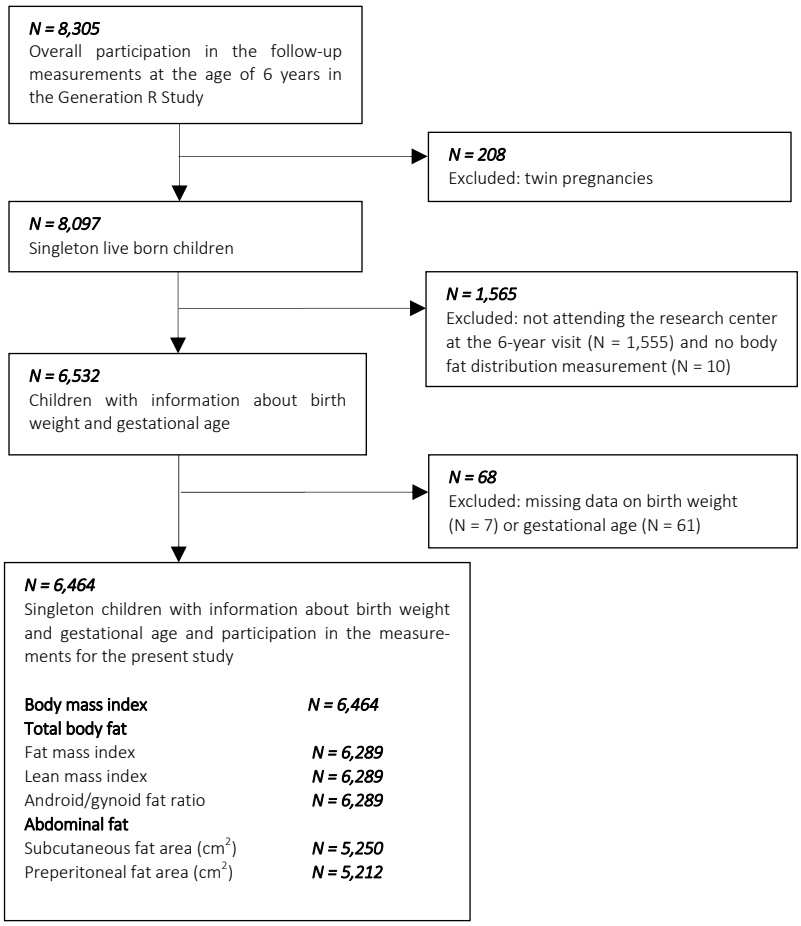
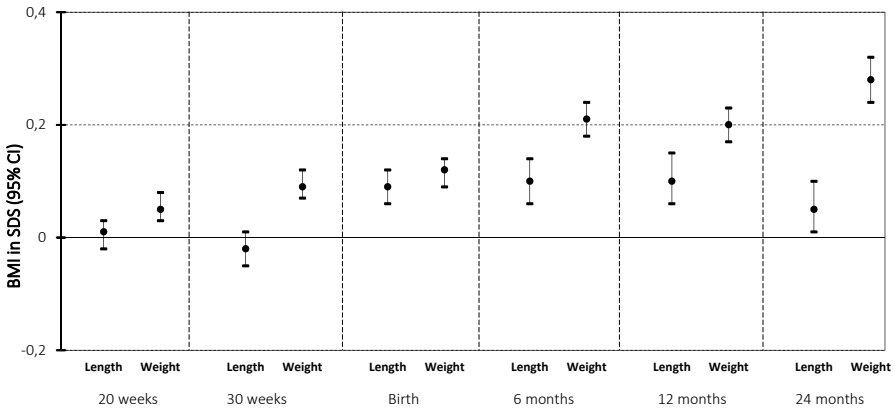
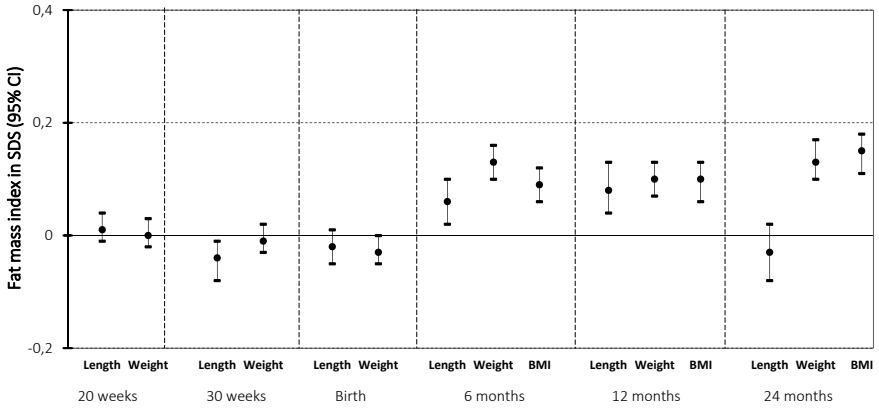


Figure S2.3.2. Associations of fetal and infant growth measures conditional on prior measures with body fat outcomes at the age of 6 years

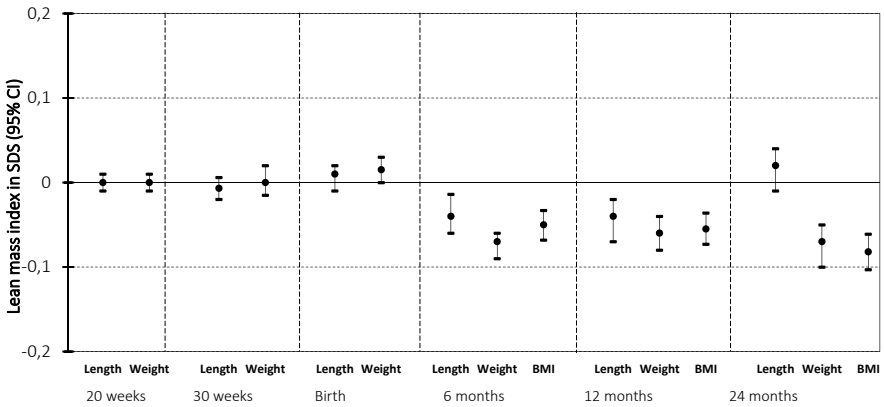
A. Body mass index



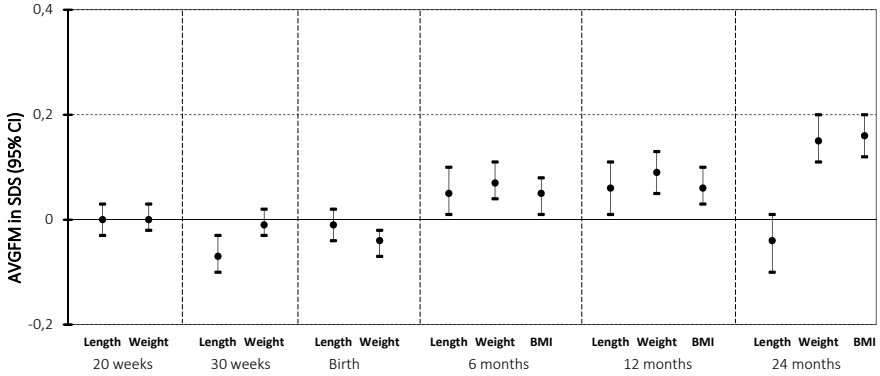
B. Fat mass index



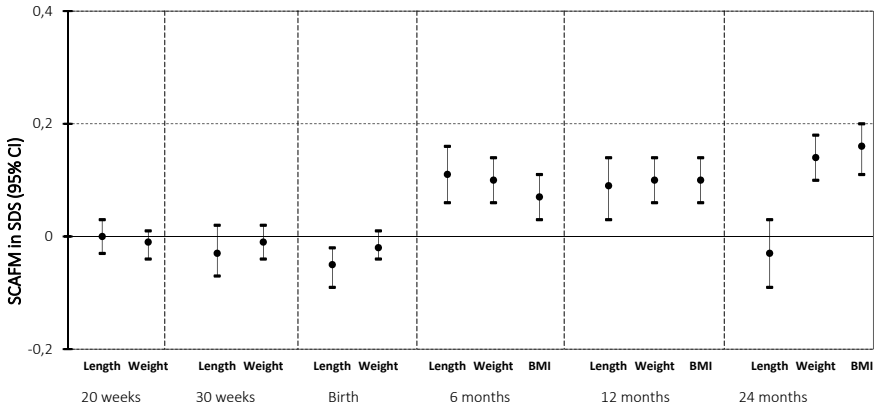
C. Lean mass index



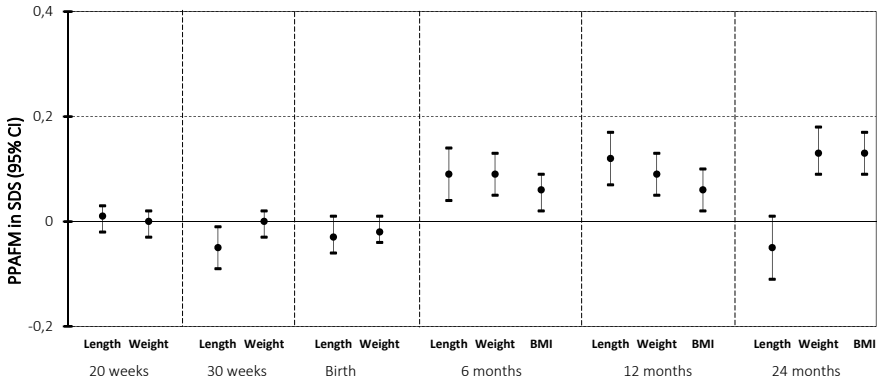
D. Android/Gynoid fat mass ratio



E. Subcutaneous area fat mass



F. Preperitoneal area fat mass



Abbreviations: SDS; Standard deviation scores; CI, confidence interval; BMI; body mass index; AVGFM; android/gynoid fat mass ratio; SCAFM; subcutaneous fat mass area; PPAFM; preperitoneal fat mass area; Values are standardized regression coefficients (95% CI) obtained from conditional analyses. The estimates represent differences in total and abdominal fat measures per standardized residual change of fetal and infant growth measures. Analyses with length and weight gain variables considered as starting point growth measures at 20 weeks of gestational age, whereas analysis with BMI considered as starting point BMI at the age of 6 months. Models are adjusted for child's age at visit, sex and height.

Table S2.3.1. Associations of fetal, birth and infant characteristics with body fat outcomes at the age of 6 years (crude models) (N = 6,464)

		Difference in body fat outcomes SDS (95% Confidence Interval)					
Growth characteristics (SDS)	**N**	**Body mass index**	**Fat mass index**	**Lean mass index**	**Android/gynoid fat ratio**	**Subcutaneous fat area**	**Preperitoneal fat area**
2nd trimester							
Length	N = 5,578	0.01 (-0.02, 0.03)	0.01(-0.01, 0.04)	-0.01 (-0.02, 0.01)	0.00 (-0.03, 0.03)	0.00 (-0.03, 0.03)	0.01 (-0.02, 0.03)
Weight	N = 5,548	0.05 (0.03, 0.08)*	0.00 (-0.02, 0.03)	0.00 (-0.01, 0.01)	0.02 (-0.01, 0.04)	-0.01 (-0.04, 0.01)	0.00 (-0.03, 0.02)
Abdominal circumference	N = 5,569	0.08 (0.05, 0.10)*	-0.01 (-0.03, 0.02)	0.00 (-0.01, 0.02)	0.02 (-0.01, 0.05)	-0.02 (-0.05, 0.01)	-0.01 (-0.04, 0.02)
3rd trimester							
Length	N = 5,734	0.01 (-0.02, 0.03)	-0.01 (-0.03, 0.02)	0.00 (-0.01, 0.02)	-0.03 (-0.06, -0.00)*	-0.01 (-0.04, 0.02)	-0.01 (-0.04, 0.02)
Weight	N = 5,715	0.11 (0.09, 0.14)*	0.00 (-0.02, 0.03)	0.00 (-0.02, 0.01)	0.01 (-0.02, 0.04)	-0.01 (-0.03, 0.02)	0.00 (-0.03, 0.03)
Abdominal circumference	N = 5,724	0.14 (0.12, 0.17)*	0.00 (-0.02, 0.03)	-0.01 (-0.02, 0.01)	0.02 (0.00, 0.05)*	-0.01 (-0.03, 0.02)	0.00 (-0.03, 0.03)
Birth							
Length	N = 3,976	0.07 (0.05, 0.09)*	-0.01 (-0.04, 0.01)	0.01 (-0.01, 0.02)	-0.01 (-0.04, 0.01)	-0.04 (-0.07, -0.01)*	-0.02 (-0.05, 0.01)
Weight	N = 6,464	0.17 (0.15, 0.19)*	-0.02 (-0.04, 0.00)	0.01 (0.00, 0.02)	-0.04 (-0.06, -0.01)*	-0.02 (-0.04, 0.01)	0.00 (-0.03, 0.02)
6 months							
Height	N = 4,239	0.12 (0.09, 0.15)*	-0.01(-0.04, 0.02)	0.00 (-0.02, 0.02)	-0.01 (-0.05, 0.03)	0.04 (-0.00, 0.08)	0.05 (0.01, 0.09)*
Weight	N = 4,730	0.39 (0.37, 0.42)*	0.20 (0.17, 0.23)*	-0.12 (-0.13, -0.10)*	0.11 (0.07, 0.14)*	0.19 (0.16, 0.23)*	0.13 (0.09, 0.16)*
Body mass index	N = 4,210	0.38 (0.36, 0.41)*	0.20 (0.18, 0.23)*	-0.12 (-0.13, -0.10)*	0.11 (0.08, 0.14)*	0.17 (0.14, 0.20)*	0.10 (0.07, 0.13)*
12 months							
Height	N = 4,365	0.15 (0.12, 0.18)*	0.07 (0.03, 0.10)*	-0.05 (-0.07, -0.02)*	0.05 (0.01, 0.09)*	0.10 (0.06, 0.15)*	0.14 (0.09, 0.18)*
Weight	N = 4,373	0.44 (0.42, 0.47)*	0.25 (0.22, 0.28)*	-0.15 (-0.16, -0.13)*	0.15 (0.12, 0.19)*	0.25 (0.22, 0.29)*	0.18 (0.15, 0.22)*
Body mass index	N = 4,342	0.44 (0.41, 0.46)*	0.20 (0.18, 0.23)*	-0.12 (-0.13, -0.10)*	0.12 (0.09, 0.15)*	0.19 (0.16, 0.22)*	0.11 (0.08, 0.14)*
24 months							
Height	N = 4,055	0.16 (0.14, 0.19)*	0.01 (-0.04, 0.05)	-0.01 (-0.04, 0.01)	0.02 (-0.03, 0.07)	0.06 (0.01, 0.12)*	0.07 (0.02, 0.12)*
Weight	N = 4,113	0.52 (0.49, 0.54)*	0.34 (0.31, 0.37)*	-0.20 (-0.22, -0.18)*	0.25 (0.21, 0.29)*	0.35 (0.31, 0.38)*	0.26 (0.23, 0.30)*
Body mass index	N = 4,074	0.54 (0.52, 0.56)*	0.27 (0.25, 0.30)*	-0.15 (-0.17, -0.14)*	0.19 (0.17, 0.22)*	0.26 (0.23, 0.29)*	0.19 (0.16, 0.22)*

Values are standardized regression coefficients (95% confidence interval) and reflect the associations of fetal and infant anthropometrics at different ages with each body fat outcome measurement. Models are adjusted for child's age at visit, sex and height. *P < 0.01

Table S2.3.2. Associations of fetal, birth and infant characteristics with body fat outcomes at the age of 6 years (fully adjusted models) (N = 6,464)

		Difference in body fat outcomes SDS (95% Confidence Interval)					
Growth characteristics (SDS)	**N**	**Body mass index**	**Fat mass index**	**Lean mass index**	**Android/gynoid fat ratio**	**Subcutaneous fat area**	**Preperitoneal fat area**
2nd trimester							
Length	N = 5,578	-0.02 (-0.05, 0.00)	-0.02 (-0.04, 0.01)	0.01 (-0.00, 0.02)	-0.02 (-0.05, 0.01)	-0.03 (-0.06, 0.00)	-0.01 (-0.04, 0.01)
Weight	N = 5,548	0.04 (0.02, 0.06)*	-0.01 (-0.03, 0.02)	0.01 (-0.01, 0.02)	0.01 (-0.02, 0.04)	-0.02 (-0.05, 0.00)	-0.01 (-0.04, 0.02)
Abdominal circumference	N = 5,569	0.08 (0.06, 0.11)*	0.00 (-0.02, 0.003)	0.00 (-0.02, 0.01)	0.03 (-0.00, 0.05)	-0.01 (-0.03, 0.02)	-0.00 (-0.03, 0.03)
3rd trimester							
Length	N = 5,734	-0.01 (-0.04, 0.01)	-0.03 (-0.05, 0.00)	0.01 (-0.00, 0.02)	-0.04 (-0.07, -0.01)*	-0.02 (-0.05, 0.00)	-0.02 (-0.05, 0.01)
Weight	N = 5,715	0.09 (0.07, 0.11)*	-0.01 (-0.03, 0.01)	0.00 (-0.01, 0.02)	0.00 (-0.02, 0.03)	-0.01 (-0.04, 0.01)	-0.01 (-0.03, 0.02)
Abdominal circumference	N = 5,724	0.13 (0.10, 0.15)*	-0.00 (-0.03, 0.02)	0.00 (-0.01, 0.01)	0.02 (-0.02, 0.05)	0.00 (-0.03, 0.02)	0.00 (-0.03, 0.03)
Birth							
Length	N = 3,976	0.07 (0.05, 0.10)*	0.00 (-0.02, 0.03)	0.00 (-0.02, 0.01)	-0.00 (-0.03, 0.02)	-0.02 (-0.05, 0.01)	0.00 (-0.03, 0.03)
Weight	N = 6,464	0.17 (0.15, 0.20)*	-0.01 (-0.03, 0.01)	0.00 (-0.01, 0.01)	-0.03 (-0.06, 0.03)	0.01 (-0.02, 0.03)	0.00 (-0.02, 0.03)
6 months							
Height	N = 4,239	0.12 (0.09, 0.15)*	-0.00 (-0.04, 0.03)	0.00 (-0.02, 0.02)	-0.01 (-0.04, 0.03)	0.03 (0.00, 0.07)	0.05 (0.01, 0.09)
Weight	N = 4,730	0.36 (0.34, 0.39)*	0.16 (0.13, 0.18)*	-0.09 (-0.11, 0.08)*	0.08 (0.05, 0.11)*	0.14 (0.10, 0.17)*	0.09 (0.06, 0.13)*
Body mass index	N = 4,210	0.35 (0.33, 0.38)*	0.16 (0.14, 0.19)*	-0.09 (-0.11, -0.08)*	0.09 (0.06, 0.11)*	0.13 (0.10, 0.15)*	0.07 (0.04, 0.10)*
12 months							
Height	N = 4,365	0.14 (0.11, 0.16)*	0.02 (-0.01, 0.06)	-0.02 (-0.04, 0.00)*	0.02 (-0.02, 0.06)	0.06 (0.02, 0.10)*	0.11 (0.07, 0.15)*
Weight	N = 4,373	0.42 (0.39, 0.44)*	0.21 (0.18, 0.24)*	-0.12 (-0.14, -0.11)*	0.12 (0.09, 0.16)*	0.21 (0.17, 0.24)*	0.15 (0.12, 0.19)*
Body mass index	N = 4,342	0.42 (0.39, 0.44)*	0.18 (0.16, 0.21)*	-0.10 (-0.12, -0.09)*	0.10 (0.07, 0.13)*	0.16 (0.14, 0.19)*	0.09 (0.06, 0.12)*
24 months							
Height	N = 4,055	0.15 (0.13, 0.18)*	-0.03 (-0.07, 0.02)	0.01 (-0.02, 0.03)	0.00 (-0.05, 0.05)	0.02 (-0.03, 0.07)	0.05 (0.00, 0.10)
Weight	N = 4,113	0.50 (0.47, 0.52)*	0.29 (0.26, 0.32)*	-0.17 (-0.19, -0.15)*	0.22 (0.18, 0.26)*	0.30 (0.26, 0.34)*	0.23 (0.19, 0.27)*
Body mass index	N = 4,074	0.52 (0.49, 0.54)*	0.25 (0.22, 0.27)*	-0.14 (-0.15, -0.13)*	0.18 (0.15, 0.21)*	0.23 (0.20, 0.26)*	0.17 (0.14, 0.20)*

Values are standardized regression coefficients (95% confidence interval) and reflect the associations of fetal and infant anthropometrics at different ages with each body fat outcome measurement. Models are adjusted for child's age at visit, sex and height, plus maternal age, education, pre-pregnancy BMI, parity, folic acid use and maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching. * $P < 0.01$.

Table S2.3.3. Associations of size at birth with body fat outcomes at the age of 6 years

	N	Difference in body fat outcomes SDS (95% Confidence Interval)				
		Body mass index	Fat mass index	Android/gynoid fat ratio	Subcutaneous fat area	Preperitoneal fat area
SGA	N = 643	-0.28 (-0.36, -0.21)*	0.02 (-0.05, 0.09)	0.15 (0.06, 0.23)*	0.01 (-0.07, 0.09)	0.01 (-0.08, 0.09)
AGA	N =5,161	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
LGA	N = 645	0.28 (0.21, 0.36)*	-0.01 (-0.08, 0.06)	-0.01 (-0.09, 0.08)	-0.00 (-0.08, 0.08)	-0.05 (-0.13, 0.04)

Abbreviations: SGA: small size for gestation age; AGA: appropriate for gestational age; LGA; large size for gestation age. Values are standardized regression coefficients (95% confidence interval) and reflect the difference for each body fat outcome measurement among children born small and large size for gestational age. Models are adjusted for child's age at visit, sex and height, plus maternal age, education, pre-pregnancy BMI, parity, folic acid use and maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching.

Table S2.3.4. Associations of size at birth and infant growth with body fat outcomes at the age of 6 years

Gestational age- and sex-adjusted birth weight	Infant growth	Difference in body fat outcomes SDS (95% Confidence Interval)					
		Body mass index	Fat mass index	Lean mass index	Android/gynoid fat ratio	Subcutaneous fat area	Preperitoneal fat area
Small size for gestational age	Catch-down	-1.64 (-2.05, -1.22)* N=14	-0.54 (-0.95, -0.14)* N=14	0.41 (0.18, 0.64)* N=14	-0.42 (-0.92, 0.06) N=14	-0.80 (-1.30, -0.26)* N=10	-0.43 (-0.95, 0.09) N=11
	Normal	-0.64 (-0.79, -0.49)* N=118	-0.10 (-0.25, 0.05) N=113	0.15 (0.06, 0.34)* N=441	-0.02 (-0.20, 0.16) N=113	-0.11 (-0.29, 0.07) N=98	-0.03 (-0.22, 0.16) N=93
	Catch-up	0.00 (-0.11, 0.11) N=234	0.09 (-0.02, 0.20) N=223	-0.04 (-0.11, 0.02) N=223	0.22 (0.09, 0.35)* N=223	0.06 (-0.07, 1.19) N=188	0.07 (-0.07, 0.20) N=187
	Trend effect estimates	*0.72 (0.56, 0.90)**	*0.24 (0.07, 0.42)**	*-0.15 (-0.26, -0.05)**	*0.28 (-0.03, 0.58)*	*0.29 (0.08, 0.50)**	*0.08 (-0.13, 0.29)*
Appropriate size for gestational	Catch-down	-0.40 (-0.47, -0.33)* N=775	-0.19 (-0.26, -0.13)* N=759	0.13 (0.09, 0.17)* N=888	-0.06 (-0.14, 0.02)* N=759	-0.15 (-0.23, -0.07)* N=630	-0.06 (-0.15, 0.02) N=625
	Normal	Reference N=1,688	Reference N=1,464	Reference N=1,464	Reference N=1,464	Reference N=1,349	Reference N=1,333
	Catch-up	0.55 (0.45, 0.59)* N=846	0.31 (0.24, 0.38)* N=826	-0.16 (-0.20, -0.12)* N=826	0.26 (0.18, 0.34)* N=826	0.33 (0.25, 0.41)* N=683	0.28 (0.19, 0.36)* N=673
	Trend effect estimates	*0.44 (0.39, 0.48)**	*0.25 (0.21, 0.29)**	*-0.14 (-0.17, -0.12)**	*0.16 (0.11, 0.20)**	*0.23 (0.18, 0.28)**	*0.17(0.11, 0.22)*
Large size for gestational age	Catch-down	0.10 (-0.01, 0.20) N=289	-0.09 (-0.19, 0.01) N=277	0.07 (0.01, 0.12)* N=277	-0.06 (-0.18, 0.06) N=277	-0.08 (-0.20, 0.04) N=227	-0.03 (-0.15, 0.10) N=227
	Normal	0.67 (0.53, 0.81) N=133	0.28 (0.14, 0.42)* N=131	-0.11 (-0.19, -0.03)* N=293	0.21 (0.04, 0.38)* N=131	0.22 (0.05, 0.39)* N=107	0.16 (-0.02, 0.33) N=108
	Catch-up	1.11 (0.64, 1.58)* N=11	0.73 (0.26, 1.19)* N=11	-0.30 (-0.56, -0.03)* N=11	0.44 (-0.11, 0.99) N=11	0.68 (0.17, 1.20)* N=10	0.84 (0.29, 1.37)* N=10
	Trend effect estimates	*0.53 (0.38, 0.67)**	*0.43 (0.28, 0.58)**	*-0.22 (-0.31, -0.14)**	*0.31 (0.15, 0.46)*	*0.36 (0.18, 0.54)**	*0.32 (0.14, 0.51)**

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for each body fat outcome measurement compared to appropriate size for gestational age children with a normal infant growth. Models are adjusted for child's age at visit, sex and height, plus maternal age, education, pre-pregnancy BMI, parity, folic acid use and maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching. Trend tests represent the effect estimates of infant growth within each birth weight group. * P < 0.01

Chapter 2.4

Early growth patterns and childhood retinal microvascular structure

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Abstract

Background: Preterm birth and low birth weight are associated with increased risks of cardiovascular diseases. Early microvasculature adaptions may be part of the underlying mechanisms. We examined the associations of birth outcomes and longitudinally measured fetal and infant growth patterns with retinal vessel calibers in childhood.

Methods and Results: In a population-based prospective cohort study among 4,122 children we measured growth characteristics in second and third trimester of pregnancy, at birth, and at 6, 12, 24, 36, 48 and 72 months. At the age of 6 years, we measured retinal arteriolar and venular calibers from digitized retinal photographs. We observed that compared to term born children, those born preterm had narrower retinal arteriolar caliber (differences: –0.46 SDS (95% CI: -0.77, -0.15) and -0.24 SDS (95% CI: -0.42, -0.05) for children born <34 and 34-37 weeks of gestation, respectively). Children born with a low birth weight (< 2500 grams) had narrower retinal arteriolar caliber than children with a normal birth weight, but this association was fully explained by gestational age at birth. Accelerated infant growth until 24 months was associated with narrow retinal arteriolar caliber, especially among preterm born children (p-value<0.05). Early growth measures were not associated with retinal venular caliber.

Conclusions: Preterm birth and accelerated infant growth are associated with narrower retinal arteriolar caliber in childhood. Whether these microvascular adaptations explain the well-known associations of fetal and infant characteristics with cardiovascular disease in later life should be further studied.

Introduction

Fetal life and infancy are critical periods for the development of higher blood pressure levels in later life.[1] Low birth weight and preterm birth are associated with higher cardiovascular complications in later life.[2] A study among 6,576 children observed that growth during infancy was associated with higher systolic blood pressure in childhood, independent from growth in weight at previous ages.[3] Similarly, another study among 346 young adults, reported that both low birth weight and weight gain between 1 and 5 years of age were associated with a higher blood pressure at the age of 22 years.[2] The mechanisms underlying these associations are not known, but may include early adaptations in the microvasculature.[4, 5] Vasoconstriction and anatomic adaptations such as increased intimal thickness, medial hyperplasia and hyalinization of the microvasculature might lead to higher blood pressure in later life.[6]

The microvasculature can be non-invasively assessed by retinal photography.4 Retinal arteriolar narrowing is associated with the occurrence and development of hypertension in childhood and adulthood.[7, 8] Thus far, previous studies performed in children and adults suggested that a lower birth weight is associated with retinal vascular narrowing in later life.[9, 10] A study among 1,067 children showed that body mass index change between 5-5.5 and 8.5-10 years in childhood was associated with wider retinal venular calibers at the age of 12 years.[11] Thus far, no studies have examined the detailed associations of both longitudinal fetal and infant growth patterns with retinal vessel caliber in childhood. The influence of early growth characteristics on retinal vessels may provide further information about critical periods associated with alterations in the microvasculature.

Therefore, we examined in 4,122 children participating in a population-based prospective cohort study from early fetal life onwards the associations of birth outcomes and repeatedly measured fetal and infant growth characteristics with retinal vessel calibers at the age of 6 years. We also explored whether these associations were explained by maternal and infant socio-demographic and lifestyle-related characteristics and childhood body mass index and blood pressure.

Methods

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[12] All children were born between 2002 and 2006. Response rate at baseline was 61%.[12] The study protocol was approved by the Medical Ethical Committee of the Erasmus MC, Rotterdam. Written informed consent was obtained from all parents. In total, 8,305 children participated in the follow-up measurements at the median age of 6.2 years (90% range 5.8 - 7.6). Information on weight and gestational age at birth was available in 8,029 children. In total 6,474 children participated in detailed cardiovascular follow-up studies. Retinal vessel measurements were available in 4,122 children. Missing retinal vessel measurements were mainly due to the later start of these measurements during the follow-up visits (Supplemental **Figure S2.4.1**). Children who were not included in the analyses had

smaller gestational age and birth and were less often breastfeed, as compared to children who were included (Supplemental **Table S2.4.1**).

Fetal and infant growth assessments

Fetal ultrasound examinations were carried out in first (median 13.2 weeks (90% range 11.1 to 17.0)), second (median 20.5 weeks (90% range 18.9 to 22.8)), and third (median 30.4 weeks (90% range 28.8 to 32.4)) trimester.[12, 13] First day of the last menstrual period, in mothers with a known and reliable first day of the last menstrual period, a regular menstrual cycle of 28 days (range 24–32 days), was used to define gestational age.[13] The first day of the last menstrual period came from the referring letter from the community midwife or hospital. We confirmed this date with the mother at the ultrasound visit and obtained additional information on the regularity and duration of the menstrual cycle. For mothers without this information, first trimester ultrasound was used for establishing gestational age.[14] We measured first trimester fetal crown to rump length in the gestational age range of 10 weeks 0 days to 13 weeks 6 days in a true mid-sagittal plane with the genital tubercle and the fetal spine longitudinally in view.[15] Intra-class correlation coefficients for intra-observer and inter-observer reproducibility of crown to rump length measurements were 0.998 and 0.995.[16] Second and third trimester ultrasounds were used to assess fetal growth. Fetal head circumference (HC), abdominal circumference (AC) and femur length (FL) were measured to the nearest millimeter using standardized ultrasound procedures. Estimated fetal weight (EFW) was calculated using the formula by Hadlock *et al.*[17]: (log10 EFW=1.5662-0.0108 (HC) +0.0468 (AC) +0.171 (FL) +0.00034 (HC) ^{2}-0.003685 (AC x FL)). Standard deviation scores (SDS) for all fetal growth characteristics were constructed based on the data from the study group.

Birth length and weight were obtained from community midwife and hospital registries. We created gestational age- and sex-adjusted birth length and weight (SDS) within our study population by using North-European growth charts.[18] Early and late preterm children were defined as a gestational age of <34 weeks and 34-37 weeks, respectively. Small for gestational age and large for gestational age were defined as the lowest and highest 10th percentiles of gestational age- and sex-adjusted birth weight in the cohort.

Childhood length and weight were measured in community health centers using standardized methods at the ages of 6 months (median 6.2 months (90% range 5.5 to 7.5)), 12 months (median 11.1 months (90% range 10.2 to 12.3)) and 24 months (median 24.8 months (90% range 23.5 to 27.5)), 36 months (median 36.6 months (90% range 35.6 to 39.6)), 48 months (median 45.8 months (90% range 44.7 to 48.0)).[12] Length was measured in a supine position to the nearest millimeter with a neonatometer. Weight was measured with a mechanical personal scale and body mass index was calculated. At the age of 6 years, height was measured to the nearest 0.1 cm and weight to the nearest grams in a dedicated research center by a well-trained staff. Weight was measured to the nearest gram using an electronic scale (SECA 888, Almere, The Netherlands), and body mass index (kg/m2) was calculated. We created age- and sex-adjusted SDS of these infant anthropometrics within our study population using Dutch reference growth charts.[19]

In line with previous studies, we considered a change in SDS greater or smaller than 0.67 between each growth characteristics measured at different time points as growth acceleration and growth deceleration, respectively.[19, 20] A change of 0.67 SDS represents the width of each percentile band on standard growth charts, which helps to indicate growth acceleration and deceleration in clinical practice.[19, 20]

Retinal microvasculature assessment

At the age of 6 years retinal photographs were taken by a well-trained staff in a dedicated research center. Digital retinal photographs centered on the optic disc were taken in one eye with images resolution 4096 and 3072 pixels, using Topcon digital retinal camera (model TRC, NW300). We use the same methods and protocols to measure retinal vascular caliber from the digitized retinal photographs, as described in previous studies among adults and children.[9, 21] Briefly, a semi-automatic computer imaging program was used to measure the caliber of the 6 largest retinal arterioles and venules located one half to 1 disc diameter from the optic disc margin.[22] Using the revised Knudtson-Parr-Hubbard formula, absolute arteriolar and venular diameter were estimated in micrometers and subsequently were summarized as central retinal arteriolar and venular equivalents, representing the average arteriolar and venular caliber of that eye, respectively.[23] Graders, masked to birth parameters and other participant characteristics, operated the computer program and monitored all retinal measurements. We constructed grader specific standard deviation scores for both central retinal and arteriolar equivalents. Interclass correlation coefficients between graders were 0.79 for retinal arteriolar caliber and 0.89 for retinal venular caliber, which suggest adequate reproducibility.

Covariates

We collected information about maternal age, parity, educational level, pre-pregnancy body mass index, smoking during pregnancy and folic acid supplement use by questionnaires. Maternal blood pressure was assessed at enrollment and pregnancy complications (hypertensive disorders, gestational diabetes) were obtained from medical records. Child's ethnicity (European, Non-European) was classified by the countries of birth of the parents.[24] Information on breastfeeding and average TV watching time was assessed by questionnaire. At the age of 6 years, systolic and diastolic blood pressure was measured at the right brachial artery, four times with one-minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus TM (Paramus, NJ, USA) and we used the mean of the last three blood pressure measurements for the analyses.[25]

Statistical analysis

First, we used linear regression models to examine the associations of birth outcomes with retinal vessel calibers at the age of 6 years. These models were first adjusted for image grader and age and sex of the child only (basic model) and subsequently additionally for confounders including mother and childhood socio-demographic and lifestyle-related characteristics, and for potential mediators including childhood body mass index and blood pressure. We considered the confounder model as the main model. We

included covariates in the models based on their associations with retinal vessel calibers in previous studies or a change in effect estimates of >10%. Second, to take account for the correlation between fetal and early childhood growth characteristics, we used conditional regression analyses to examine associations of these growth characteristics with retinal vessel calibers at the age of 6 years. We constructed length, weight and body mass index variables, which are statistically independent from each other, allowing simultaneous inclusion in multiple regression models.[26] Details of these models are given in the Supplemental **Method S2.4.1**. We also used regular multiple linear regression models to explore the associations of fetal and childhood growth with childhood retinal vessel calibers. Finally, we examined the combined effects gestational age at birth and infant growth deceleration, normal growth and growth acceleration between birth and 24 months. We used stratified multiple linear regression models to assess whether the associations of infant growth with retinal vessel calibers were modified by gestational age at birth. We tested potential interactions between gestational age at birth and birth weight with child's sex in relation to retinal vessel calibers. In order to reduce potential bias due to missing data, we performed multiple imputations of missing covariates (<25% missing values) by generating five independent datasets using the Markov Chain Monte Carlo method, and the pooled effect estimates (95% Confidence Interval (CI)) are presented.[27] Imputations were based on the relationships between covariates, determinants and outcomes. We also imputed missing growth variables to examine the conditional associations of length, weight and body mass index with retinal vessel calibers (data not shown). Analyses were performed using the Statistical Package of Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Table 2.4.1 shows the participants characteristics. The mean (SD) retinal arteriolar and venular calibers were 159.1µm (14.9) and 219.0 µm (20.2), respectively. Growth characteristics at different ages are given in **Table 2.4.2**.

Birth outcomes and childhood retinal microvasculature

Table 2.4.3 shows that in the models adjusted for image grader, and age and sex of the child and maternal and infant socio-demographic and lifestyle-related confounders, both early and late preterm born children had narrower retinal arteriolar caliber, compared to children born at term (differences: -0.46 SDS (95% CI: -0.77, -0.15), and -0.24 SDS (95% CI: -0.42, -0.05), respectively). Compared to children with a normal birth weight children (2500-3999 grams), children with a low birth weight (< 2499 grams) had narrower retinal arteriolar caliber (difference: -0.26 SDS (95%CI: -0.44, -0.08)). Additional adjustment for childhood body mass index and blood pressure only slightly changed the effect estimates. No associations of gestational age adjusted birth weight with retinal arteriolar caliber was present. We did not observe associations of birth outcomes with retinal venular caliber. Similar results were present in the models adjusted for image grader, and age and sex of the child only (Supplemental **Table S2.4.2**).

Table 2.4.1. Characteristics of study population (N = 4,122)

Characteristics	Values
Maternal characteristics	
Age, median (95% range), y	31.0 (19.7, 39.9)
Gestational age at intake, median (95% range), weeks	13.9 (10.8, 22.5)
Height, mean (SD), cm	168.0 (7.4)
Pre-pregnancy weight, mean (SD), kg	64.0 (12.8)
Pre-pregnancy body mass index, median (95% range), kg/m^2	23.8 (19.4, 33.2)
Systolic blood pressure, mean (SD), mm/Hg	115.3 (12.0)
Diastolic blood pressure, mean (SD), mm/Hg	67.9 (9.5)
Parity, nulliparous, N (%)	2238 (55.9)
Education, higher, N (%)	1720 (46.1)
Folic acid use, never, (N, %)	737 (26.1)
Smoked during pregnancy, Yes, N (%)	877 (26.5)
Gestational diabetes, Yes, N (%)	41 (1.0)
Gestational hypertension, Yes, N (%)	155 (4.2)
Preeclampsia, Yes, N (%)	63 (1.8)
Birth and infant characteristics	
Gestational age at birth, median (full range), weeks	39.9 (26.3, 43.6)
Birth weight, mean (SD), grams	3439 (542)
Early preterm birth (< 34 weeks), N (%)	47 (1.1)
Late preterm (34-37 weeks), N (%)	149 (3.6)
Low birth weight (<2500 g), N (%)	152 (2.7)
Small for gestational age, N (%)	371 (10.0)
Ethnicity, European, N (%)	2518 (63.0)
Ever breastfeeding, N (%)	2978 (92.6)
TV watching >= 2 hours per day, N (%)	605 (19.2)
Childhood characteristics	
Age at visit, median (95% range), years	6.2 (5.8, 7.6)
Body mass index, mean (SD), kg/m^2	16.3 (1.9)
Systolic blood pressure, mean (SD), mm/Hg	103.0 (8.2)
Diastolic blood pressure, mean (SD), mm/Hg	60.9 (6.8)
Retinal arteriolar caliber, mean (SD), µm	159.1 (14.9)
Retinal venular caliber, mean (SD), µm	219.0 (20.2)

Values are means (SD), number and percentages N (%), or medians (90% range). Values for gestation age at birth are median and full range (minimum, maximum). Small for gestational age was defined as the lowest 10th percentiles of gestational age- and sex-adjusted birth weight.

Table 2.4.2. Fetal and infant growth characteristics (N = 4,122)

Growth characteristics	Values
Second trimester (n = 3559)	
Gestational age, median (90% range), weeks	20.5 (18.9, 22.8)
Femur length, mean (SD), mm	33.5 (3.6)
Estimated fetal weight, mean (SD), grams	380.6 (94.5)
Third trimester (n = 3655)	
Gestational age, median (90% range), weeks	30.3 (28.8, 32.3)
Femur length, mean (SD), mm	57.5 (3.06)
Estimated fetal weight, mean (SD), grams	1622 (257)
Birth (n = 4122)	
Gestational age, median (90% range), weeks	39.9 (37.0, 42.0)
Length, mean (SD), cm	50.3 (2.3)
Birth weight, mean (SD), grams	3439 (542)
6 months (n = 3066)	
Age at visit, median (90% range), months	6.3 (5.5, 7.5)
Height, mean (SD), cm	67.7 (2.6)
Weight, mean (SD), kg	7.9 (0.9)
Body mass index, mean (SD), kg/m^2	17.3 (1.4)
12 months (n = 2810)	
Age at visit, median (90% range), months	11.1 (10.2, 12.2)
Height, mean (SD), cm	74.4 (2.7)
Weight, mean (SD), kg	9,7 (1.1)
Body mass index, mean (SD), kg/m^2	17.4 (1.4)

Table 2.4.2. Fetal and infant growth characteristics (N = 4,122)

Growth characteristics	Values
2 years (n = 2604)	
Age at visit, median (90% range), months	24.8 (23.5, 27.5)
Height, mean (SD), cm	88.3 (3.5)
Weight, mean (SD), kg	13.0 (1.5)
Body mass index, mean (SD), kg/m^2	16.6 (1.4)
3 years (n = 2447)	
Age at visit, median (90% range), months	36.6 (35.6, 39.6)
Height, mean (SD), cm	97.4 (3.8)
Weight, mean (SD), kg	15.3 (1.9)
Body mass index, mean (SD), kg/m^2	16.1 (1.3)
4 years (n = 2148)	
Age at visit, median (90% range), months	45.8 (44.7, 48.0)
Height, mean (SD), cm	103.3 (4.1)
Weight, mean (SD), kg	17.0 (2.2)
Body mass index, mean (SD), kg/m^2	15.9 (1.42)
6 years (n = 4122)	
Age at visit, median (90% range), months	72.6 (69.3, 91.5)
Height, mean (SD), cm	120 (6.2)
Weight, mean (SD), kg	23.5 (4.5)
Body mass index, mean (SD), kg/m^2	16.3 (1.9)

Values are means (SD), percentages (%), or medians (90% range) for variables with skewed distribution.

Table 2.4.3. Associations of birth outcomes with retinal vessel calibers at the age of 6 years (N=4, 122)

		Difference in retinal vessel calibers (95% Confidence Interval)			
		Arteriolar caliber (SDS)		Venular caliber (SDS)	
Birth characteristics	**No.**	Confounder Model	Mediator Model	Confounder Model	Mediator Model
Gestational age at birth					
Early preterm (<34 weeks)	47	-0.46 (-0.77, -0.15)*	-0.47 (-0.78, -0.17)*	-0.03 (-0.34, 0.28)	-0.04 (-0.35, 0.27)
Late preterm (34-37 weeks)	149	-0.24 (-0.42, -0.05)*	-0.21 (-0.39, -0.03)*	-0.06 (-0.24, 0.12)	-0.05 (-0.23, 0.13)
Term (> 37 weeks)	3926	*Reference*	*Reference*	*Reference*	*Reference*
P for trend		*<0.01*	*<0.01*	*0.87*	*0.72*
Birth weight					
Low (<2499 g)	152	-0.26 (-0.44, -0.08)*	-0.26 (-0.43, -0.08)*	-0.05 (-0.23, 0.13)	-0.05 (-0.22, 0.13)
Normal (2500 – 3999 g)	3856	*Reference*	*Reference*	*Reference*	*Reference*
High (> 4000 g)	107	0.08 (-0.13, 0.28)	0.03 (-0.17, 0.23)	0.08 (-0.13, 0.28)	0.07 (-0.14, 0.27)
P for trend		*<0.01*	*<0.01*	*0.67*	*0.81*
Gestational age- and sex-adjusted birth weight					
Small for gestational age (< -1.36)	371	0.04 (-0.08, 0.15)	0.04 (-0.08, 0.16)	-0.02 (-0.13, 0.10)	-0.01 (-0.13, 0.10)
Appropriate for gestational age (-1.36 -1.21)	3325	*Reference*	*Reference*	*Reference*	*Reference*
Large for gestational age (> 1.21)	415	0.10 (-0.01, 0.21)	0.08 (-0.03, 0.19)	0.09 (-0.02, 0.20)	0.09 (-0.02, 0.19)
P for trend		*0.06*	*0.06*	*0.54*	*0.60*

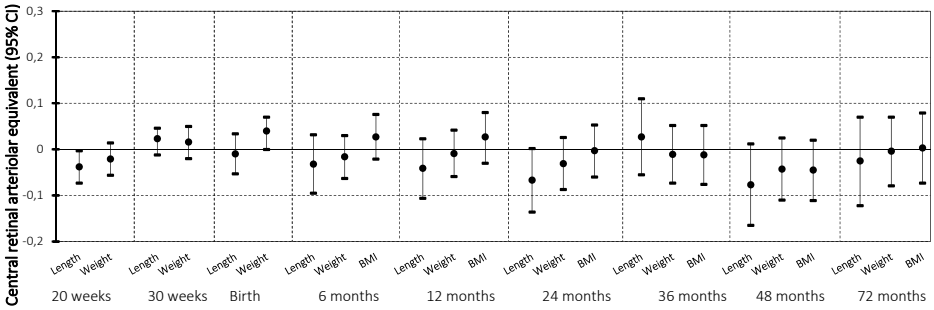
Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal vessel calibers. Basic model is adjusted for child's age at visit, sex and grader. Confounder model is adjusted for image grader, and age and sex of the child, and lifestyle socio-demographic confounders (maternal age, education, pre-pregnancy body mass index, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, pregnancy complications, plus child's ethnicity, breastfeeding and TV watching). Mediator model is additionally adjusted for childhood body mass index and blood pressure. Lower birth weight was defined as weight at birth lower than 2499 g. Small for gestational age was defined as age- and sex-adjusted birth weight < 10%. Large for gestational age was defined as age- and sex-adjusted birth weight > 10%. * P < 0.01. Models adjusted for image grader, and age and sex of the child only are given in the Supplemental Table S2.4.2.

Fetal and childhood growth patterns and retinal microvasculature

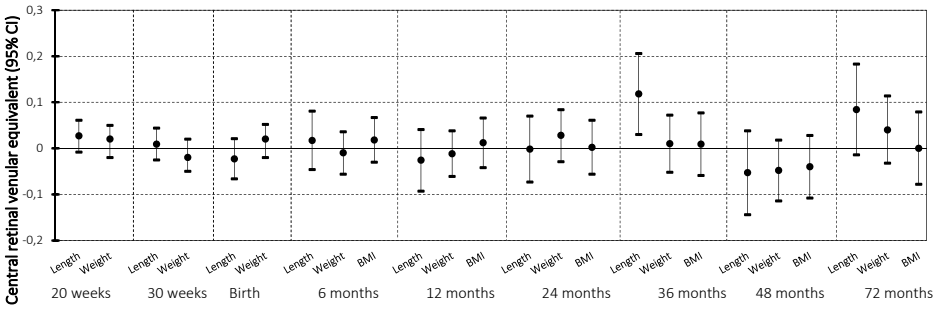
Figure 2.4.1 gives the associations of growth characteristics at each age point with childhood retinal vessel calibers at age 6 years, independent from growth measures at other ages. We observed that length and body mass index at any age were not independent associated with retinal arteriolar caliber. Independent from growth in weight in fetal life, higher weight at birth was associated with wider retinal arteriolar caliber (p-values<0.05) (**Figure 2.4.1A**). No consistent associations were present for length, weight and body mass index growth with retinal venular at the age of 6 years (**Figure 2.4.1B)**. Models adjusted for image grader, and age and sex of the child only are given in Supplementary **Figure S2.4.2**. Similar results were present after imputation of the missing growth variables (data not shown). Supplemental **Table S2.4.3** gives the associations of fetal and infant anthropometrics with childhood retinal vessel calibers using normal multiple linear regression models and shows no associations of growth measurements with retinal arteriolar and venular calibers among different models. Supplemental **Table S2.4.4** shows that first trimester crown to rump length was not associated with retinal arteriolar and venular calibers.

Figure 2.4.1. Associations of fetal and infant growth measures conditional on prior measures with retinal vessel calibers at the age of 6 years (N=4, 122)

A. Central retinal arteriolar equivalent



B. Central retinal venular equivalent



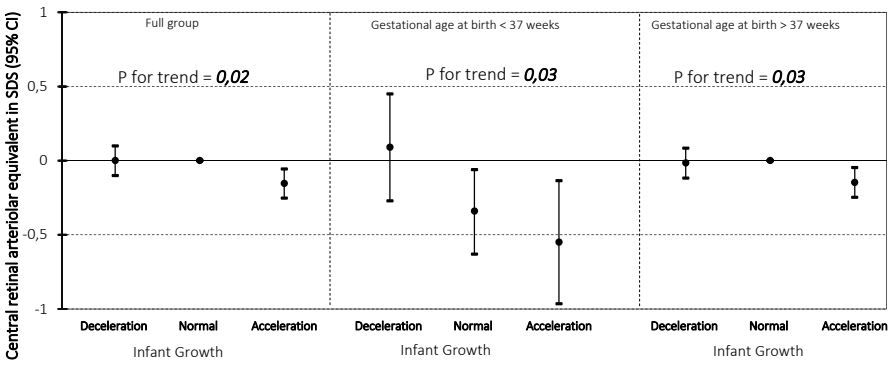
Values are standardized regression coefficients (95% CI) obtained from conditional analyses. The estimates represent differences in retinal vessels diameter per standardized residual change of fetal and infant growth measures. Analyses with length and weight gain variables considered as starting point growth measures at 20 weeks of gestational age, whereas analysis with body mass index considered as starting point body mass index at the age of 6 months. Models are adjusted for image grader, and age and sex of the child, and maternal age, education, pre-pregnancy body mass index, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching. Models adjusted for image grader, and age and sex of the child only are given in the Supplemental Figure S2.4.2.

Gestational age at birth, infant growth and childhood retinal microvasculature

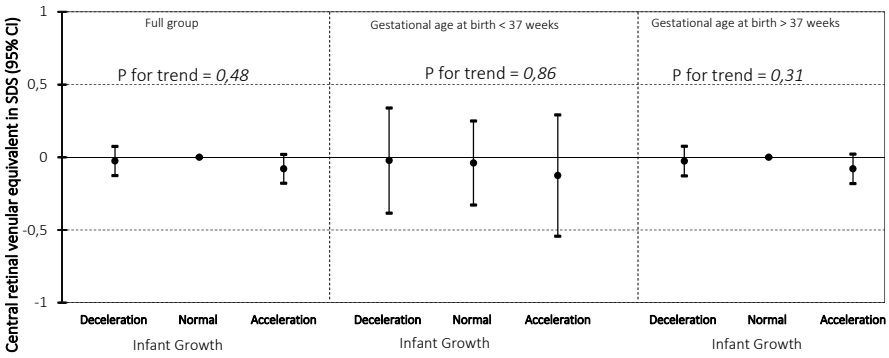
For the total group, children with an accelerated infant growth had narrower retinal arteriolar caliber then children with a normal infant growth (-0.15 SDS (95% CI -0.25, -0.06), for accelerated infant growth) (**Figure 2.4.2**). No differences in retinal venular caliber were present among children with normal and accelerated infant growth. As compared to children born term and with a normal infant growth, preterm born children with a normal or accelerated infant growth had smaller retinal arteriolar caliber (-0.34 SDS (95% CI -0.63, -0.06), and -0.55 SDS (95% CI -0.96, -0.13)), for normal and accelerated infant growth, respectively). Children born term with infant growth acceleration had smaller retinal arteriolar caliber (P for trend = 0.03), than children born term and with a normal infant growth. However, the statistical interaction term between gestational age at birth and infant growth patterns was not significant. Additional adjustment for childhood body mass index and blood pressure only slightly influenced the observed associations (Supplemental **Table S2.4.5**). No associations of gestational age at birth and infant growth patterns with retinal venular caliber were present.

Figure 2.4.2. Associations of gestational age at birth and infant growth with retinal vessel calibers at the age of 6 years

A. Central retinal arteriolar equivalent



B. Central retinal venular equivalent



Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal vessels compared to children born on term with a normal infant growth. Models are adjusted for image grader, and age and sex of the child, and lifestyle socio-demographic confounders (maternal age, education, pre-pregnancy body mass index, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching). P-values for interaction between gestational age at birth with infant growth patterns are 0.45 and 0.40, for arteriolar and venular vessels, respectively.

Discussion

In this population-based prospective cohort study we observed that children born preterm or with accelerated infant growth have a narrower retinal arteriolar caliber in childhood. These associations were largely independent of maternal and infant sociodemographic and lifestyle-related characteristics and childhood body mass index and blood pressure. Birth parameters and infant growth patterns did not influence retinal venular caliber.

Methodological considerations

We used a population-based prospective cohort design including a large number of subjects whom we studied from early fetal life onwards. The large number of repeated fetal and infant anthropometric measurements enabled us to explore potential critical periods in early life for the development of an adverse cardiovascular profile. Selection bias in follow-up studies mainly arises from loss to follow-up rather than from non-response at baseline.[28] Of all children with information about gestational age at birth and birth weight, 81% did participate in the follow-up measurements at the age of 6 years, of whom at least 64% participated in the retinal vessel measurements. Loss to follow-up would lead to selection bias if the associations of fetal and infant anthropometrics with retinal vessel calibers would be different between children included and not included in the final analyses, but we consider this unlikely. Information on anthropometrics at the age of 24 months was missing in 37% of the study population. These missing values are mainly due to the design of the study, which included growth data collected from birth until the age of 4 years in only a subgroup of the total study population.[12] We used validated techniques to measure retinal vessel calibers. We did not take into account other ocular factors that might affect retinal vessel measurements, such as axial length and refractive error.[29, 30] However, it has been previously shown that these factors have only a small impact on the measurement of retinal vessel calibers.[31] Although, we used a population based prospective cohort of healthy children and we performed an extensive adjustment for a large number of potential confounders, residual confounding in the observed associations might still occur, as in any observational study.

Comparison of main findings with other studies

Fetal life and early childhood have been recognized as critical periods for the development of cardiovascular diseases in later life.[1] Previous studies have shown that growth in weight in early childhood is associated with higher blood pressure in childhood and adulthood.[2, 3] Early life structural and functional microvasculature adaptations may be part of the mechanisms leading to cardiovascular diseases.[32] In humans, the peripheral microvasculature can non-invasively be assessed by using retinal vascular imaging.[33] Several longitudinal studies among adults and children have shown that retinal vessel narrowing reflects generalized peripheral resistance and is influenced by various factors in early life, and can therefore be used as an early marker of cardiovascular disease risk in adulthood.[21, 34] In the current study, we explored whether various fetal and childhood growth patterns are related to microvasculature alterations in childhood.

Preterm born individuals are at increased risk for developing hypertension in later life.[35] A study among 47 women aged 23-30 years showed that women born preterm had a higher length index for arterioles and a decreased number of vascular branching points compared with women born on term.[36] A study among 50 children, showed that extreme preterm born children, with a median gestational age of 26 weeks, had an abnormal retinal vessel structure, characterized by a higher tortuosity index for venules and a decreased number of vascular branching points at the age of 7 years, independent of retinopathy of prematurity.[37] In the current large population based cohort study, we observed narrower retinal arteriolar caliber among preterm born children as compared to children born a term. These associations persisted after adjustment for maternal and childhood factors. These findings demonstrate the adverse effect of preterm birth on the development of microvasculature structures.

Studies in adults showed that low birth weight is associated with retinal arteriolar narrowing [10] and increased risks of cardiovascular diseases in adulthood.[38] A study in the US among 3800 adults aged 51-72 years showed that lower birth weight was associated with retinal arteriolar narrowing, but not with venular narrowing, even after adjustment for potential confounders and blood pressure at baseline.[10] Another study among 2,353 children in Australia found that children with lower birth weight were more likely to have narrower retinal arteriolar vessels. These associations were independent of prematurity or childhood body mass index.[39] Similarly, a study among 1,369 children aged 6 years reported narrower retinal arteriolar caliber among children born with a lower birth weight, even after adjustment for prematurity.[9] We observed that the associations of lower birth weight with narrower retinal arteriolar caliber were fully explained by gestational age at birth, suggesting that not birth weight, but maturity is a critical factor in the pathogenesis of the microvasculature adaptations.

Preterm birth and low birth weight are the results of various fetal growth patterns and the starting point of different infant growth patterns. Previously, we have suggested that fetal life and infancy might be critical periods for the development of an adverse fat distribution in childhood.[40] A study among 6,621 children showed that only growth during infancy was associated with a higher childhood blood pressure, independent from growth in weight in previous ages [41] Another study among 217 healthy subjects showed that rapid weight gain in the early postnatal period is associated with higher blood pressure and higher fat mass percentage at the age 18 to 24 years.[42] Accelerated infant growth has been suggested as a possible mechanisms linking the associations of preterm birth with the development of an adverse cardiovascular profile in later life.[2, 42] Another study among 104 newborn children showed that accelerated growth, defined as Z score for weight, height and weight for height, was associated with endothelium dysfunction [43], which is shown to be a sensitive marker of higher blood pressure.[44] To the best of our knowledge, the current study assessed for the first time the associations of longitudinal fetal and infant growth patterns with retinal vessel caliber in childhood. We did not find strong associations of length, weight and body mass index in different fetal and childhood periods with retinal vessel calibers. Among preterm born infants, accelerated infant growth tended to be associations with narrower retinal arteriolar caliber. Thus, our findings suggest that especially preterm birth is an important risk factor for microvasculature adaptations.

The mechanisms underlying the observed associations remain unclear. Fetal hypoxia and suboptimal nutrition might play an important role in early vascular maladaptations. Chronic hypoxia during fetal life is shown to stimulate structural vascular changes and exaggerated neovascularization.[45] Previously, we have shown that increased feto-placental vascular resistance, which is related to suboptimal fetal nutrition and oxygen supply, is associated with fetal growth restriction and increased systolic blood pressure in childhood.[46] Next to adverse fetal influences, early childhood environmental influences might be important for the development of micro-vessel abnormalities. A previous study has suggested that microvasculature changes among preterm children are not present at birth, but develop in early life.[47] A premature child has to adapt to a higher extra-uterine oxygen tension, which down-regulates the endothelial growth factor release and subsequently inhibits normal growth of the vessels and neo-angiogenesis.[37, 48] Therefore, immaturity of a preterm child may be partly responsible for the vaso-obliteration and alterations on micro-vessels morphology. Rapid growth during early life might also have an adverse effect on the microvasculature. It has been suggested that overfeeding of preterm and low birth weight children and a rapid growth in infancy might stimulate hypothalamus-pituitary-adrenal axis and growth hormone insulin like axis, which may lead to increased blood pressure in later life.[49] Another mechanism might be that micro-vessels are unable to adapt to the immediate rise of blood volume accompanying fast growth, therefore it can lead to structural and functional alterations.[43] Further studies are needed focused on potential mechanisms underlying the observed associations.

Although the observed effect estimates for the associations of gestational age at birth with retinal vessel caliber are small, these finding are important from an etiological perspective and provide further insight into the developmental origins of cardiovascular disease. To the best of our knowledge, no previous studies have examined tracking of microvasculature alterations from childhood to adulthood. However, other cardiovascular risk factors, including blood pressure are shown to track from childhood into adulthood and are related to cardiovascular disease in later life.[1, 50] Thus, these studies suggest that even subclinical differences in risk factors for cardiovascular disease in childhood are related to the development of cardiovascular disease in later life. Further studies are needed to examine the long-term consequences of the observed differences in microvasculature throughout the life course.

Conclusions

Low birth weight, preterm birth, and childhood growth acceleration are associated with higher blood pressure in later life. Early microvasculature adaptations might be part of the underlying mechanisms in the development of cardiovascular diseases. Previous studies showed that children born preterm and with a lower birth weight have narrower retinal vasculature in later life. This is the first study that assessed the associations of longitudinal fetal and infant growth patterns with retinal vessel caliber in childhood. We did not find significant effects of detailed growth patterns on microvasculature. Preterm born children had narrower retinal arteriolar caliber, but not venular caliber in childhood, as compared to a term born children. Children with infant growth acceleration had also narrower retinal arteriolar caliber. These associations tended to be stronger

among preterm born infants. Thus, our results suggest that prematurity and accelerated growth in infancy may have an adverse impact on microvasculature structure and function. Whether these differences in microvasculature structure lead to cardiovascular disease in later life should be further studied.

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Supplemental Material

Method S2.4.1. Conditional analyses

To take account for the correlation between fetal and early childhood growth characteristics we used conditional regression analyses to examine associations of these growth characteristics with retinal vessel calibers at the age of 6 years. We constructed length, weight and body mass index variables, which are statistically independent from each other by using standardized residuals obtained from linear regression models in which length, weight and body mass index were regressed on all prior corresponding growth measurements[1]. This allows simultaneous inclusion of all growth measures in one regression model, and thereby identification of independent periods of fetal and childhood growth associated with retinal vessel calibers.[1]

References

1. Cole T. A new index of child weight-for-height based on weight and height Z scores. *Ann Hum Biol.* 1994; 21:96.

Figure S2.4.1. Flow chart of participants in study

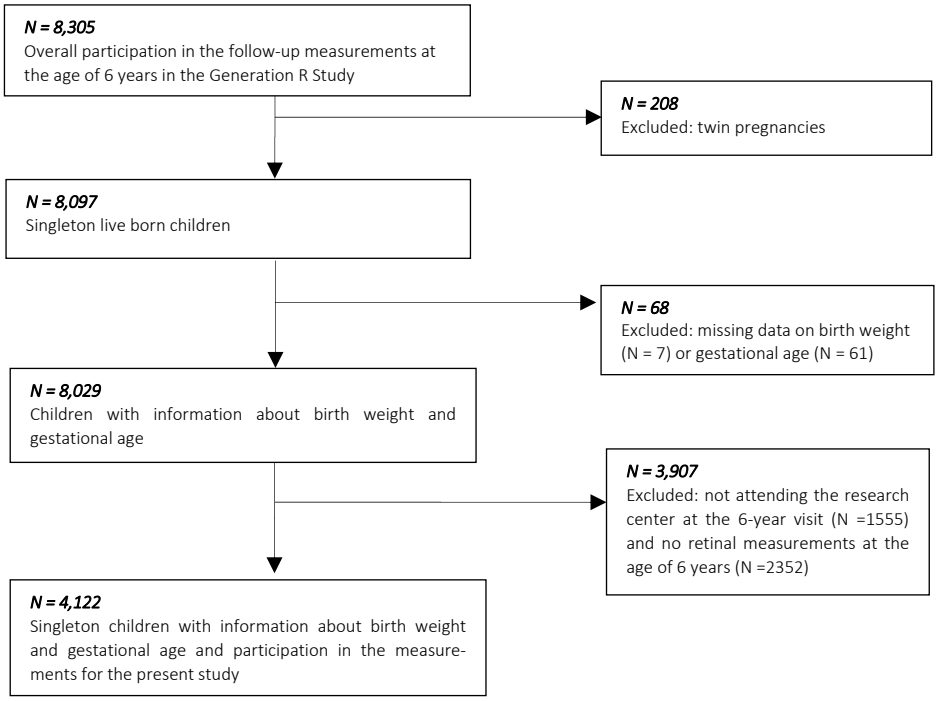
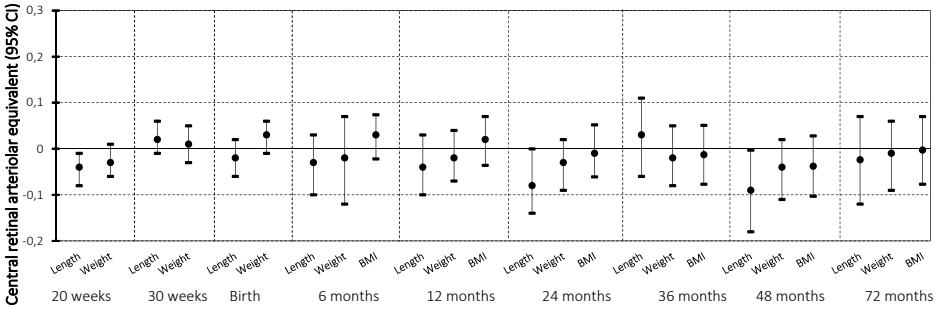
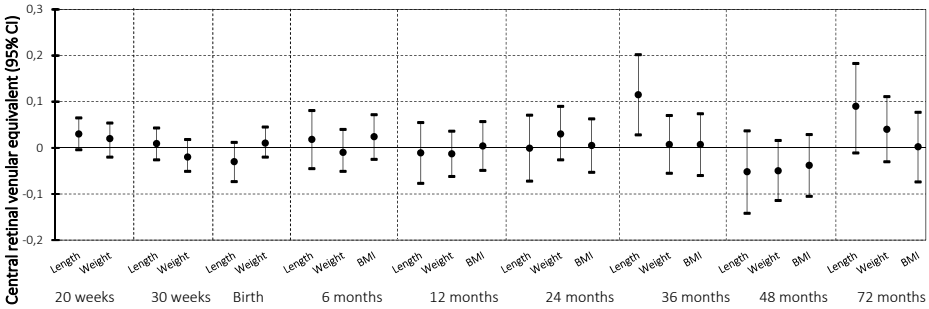


Figure S2.4.2. Associations of fetal and infant growth measures conditional on prior measures with retinal vessel calibers at the age of 6 years (N=4, 122)

A. Central retinal arteriolar equivalent



B. Central retinal venular equivalent



Values are standardized regression coefficients (95% CI) obtained from conditional analyses. The estimates represent differences in retinal vessel calibers per standardized residual change of fetal and infant growth measures. Analyses with length and weight gain variables considered as starting point growth measures at 20 weeks of gestational age, whereas analysis with body mass index considered as starting point body mass index at the age of 6 months. Models are adjusted for image grader, and age and sex of the child.

Table S2.4.1. Comparison of subject characteristics between children included and not included in the analyses

	Participation	No participation	P value
Maternal characteristics			
Age, median (95% range), y	31.0 (19.7, 39.9)	30.6 (19.3, 39.6)	*<0.01*
Gestational age at intake, median (95% range), weeks	13.9 (10.8, 22.5)	13.9 (9.9, 24.4)	*0.37*
Height, mean (SD), cm	168.0 (7.4)	168.0 (7.3)	*0.78*
Pre-pregnancy weight, mean (SD), kg	64.0 (12.8)	64.0 (12.3)	*0.02*
Pre-pregnancy body mass index, median (95% range), kg/m^2	23.8 (19.4, 33.2)	23.8 (18.7, 36.3)	*0.09*
Systolic blood pressure, mean (SD), mm/Hg	115.3 (12.0)	115.6 (12.4)	*0.40*
Diastolic blood pressure, mean (SD), mm/Hg	67.9 (9.5)	67.0 (9.4)	*0.67*
Parity, nulliparous (%)	55.9	55.2	*0.28*
Education, higher (%)	46.1	43.9	*0.16*
Folic acid use, never (%)	26.1	27.5	*0.42*
Smoked during pregnancy, Yes (%)	26.6	26.9	*0.39*
Gestational diabetes, Yes, (%)	1.0	1.2	*0.31*
Gestational hypertension, Yes, (%)	4.3	3.6	*0.07*
Preeclampsia, Yes, (%)	1.8	2.2	*0.14*
Birth and infant characteristics			
Gestational age at birth, median (full range), weeks	39.9 (26.3, 43.6)	40.1 (25.2, 43.4)	*0.02*
Birth weight, mean (SD), grams	3439 (542)	3403 (576)	*0.01*
Early preterm birth (< 34 weeks) (%)	1.1	1.4	
Late preterm (34-37 weeks) (%)	3.6	4.4	*<0.01*
Low birth weight (<2500 g) (%)	2.7	5.7	*<0.01*
Small for gestational age (%)	10.0	11.3	*0.13*
Ethnicity, European (%)	60.1	61.1	*0.19*
Ever breastfeeding (%)	92.6	91.1	*0.02*
TV watching >= 2 hours per day (%)	19.2	19.4	*0.48*

Values are means (SD), percentages (%), or medians (95% range). Small for gestational age was defined as the lowest 10th percentiles of gestational age- and sex-adjusted birth weight.

Table S2.4.2. Associations of birth outcomes with retinal vessel calibers at the age of 6 years (N = 4,122)

Birth characteristics	Difference in retinal vessel calibers in SDS (95% Confidence Interval)		
		Arteriolar caliber	Venular caliber
	N	Basic Model	Basic Model
Gestational age at birth			
<34 weeks	44	-0.49 (-0.80, -0.18)*	-0.06 (-0.37, 0.25)
34-37 weeks	145	-0.36 (-0.42, -0.05)*	-0.06 (-0.24, 0.13)
> 37 weeks	3861	*Reference*	*Reference*
P for trend		*<0.01*	*0.97*
Birth weight			
Low (<2499 g)	152	-0.27 (-0.44, -0.09)*	0.06 (-0.24, 0.11)
Normal (2500 – 3999 g)	3856	*Reference*	*Reference*
High (> 4000 g)	107	0.04 (-0.16, 0.24)	0.06 (-0.14, 0.27)
P for trend		*<0.01*	*0.70*
Gestational age- and sex- adjusted birth weight			
Small for gestational age (< -1.36)	371	0.03 (-0.08, 0.15)	-0.02 (-0.13, 0.10)
Appropriate for gestational age (-1.36 -1.21)	3325	*Reference*	*Reference*
Large for gestational age (> 1.21)	415	0.07 (-0.03, 0.18)	0.07 (-0.03, 0.18)
P for trend		*0.14*	*0.65*

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal vessel calibers. Model is adjusted for image grader, and age and sex of the child. Lower birth weight was defined as weight at birth lower than 2500 g. Preterm birth was defined as a gestational age of <37 weeks. Small for gestational age was defined as age- and sex-adjusted birth weight < 10%. Large for gestational age was defined as age- and sex-adjusted birth weight > 10%.
* P < 0.01.

Table S2.4.3. Associations of fetal and infant anthropometrics with retinal vessel calibers (N = 4,122)

Growth characteristics (SDS)	N	Difference in retinal vessel calibers SDS (95% Confidence Interval)					
		Arteriolar caliber			Venular caliber		
		Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
2nd trimester							
Length	3539	-0.04 (-0.08, -0.01)*	-0.04 (-0.07, 0.00)*	-0.03 (-0.07, 0.00)*	0.03 (0.00, 0.07)	0.03 (-0.01, 0.06)	0.03 (-0.01, 0.06)
Weight	3526	-0.03 (-0.06, 0.01)	-0.02 (-0.06, 0.01)	-0.02 (-0.05, 0.02)	0.02 (-0.02, 0.05)	0.02 (-0.02, 0.05)	0.02 (-0.01, 0.06)
Abdominal circumference	3537	-0.00 (-0.04, 0.03)	0.00 (-0.03, 0.04)	0.01 (-0.03, 0.04)	0.01 (-0.03, 0.04)	0.01 (-0.02, 0.05)	0.01 (-0.02, 0.05)
3rd trimester							
Length	3646	0.00 (-0.03, 0.04)	0.01 (-0.03, 0.04)	0.00 (-0.03, 0.04)	0.03 (-0.01, 0.06)	0.02 (-0.01, 0.06)	0.02 (-0.01, 0.06)
Weight	3635	-0.00 (-0.04, 0.03)	0.01 (-0.03, 0.04)	0.01 (-0.03, 0.04)	-0.01 (-0.04, 0.03)	-0.01 (-0.04, 0.03)	-0.01 (-0.04, 0.03)
Abdominal circumference	3641	0.00 (-0.04, 0.03)	0.00 (-0.03, 0.04)	0.01 (-0.03, 0.04)	-0.02 (-0.06, 0.01)	-0.02 (-0.06, -0.01)*	-0.02 (-0.06, 0.01)
Birth							
Length	2606	-0.01 (-0.05, 0.02)	0.00 (-0.04, 0.03)	-0.01 (-0.04, 0.03)	-0.01 (-0.04, 0.03)	0.00 (-0.04, 0.04)	0.00 (-0.04, 0.03)
Weight	4115	0.02 (-0.01, 0.06)	0.04 (0.00, 0.07)*	0.03 (0.00, 0.07)*	0.01 (-0.03, 0.04)	0.01 (-0.02, 0.05)	0.01 (-0.02, 0.05)
6 months							
Height	2734	0.00 (-0.05, 0.04)	0.00 (-0.04, 0.04)	0.01 (-0.04, 0.05)	-0.02 (-0.07, 0.02)	-0.02 (-0.07, 0.02)	-0.02 (-0.07, 0.02)
Weight	3047	0.00 (-0.04, 0.04)	-0.01 (-0.05, 0.04)	0.01 (-0.03, 0.05)	-0.01 (-0.05, 0.03)	-0.02 (-0.06, 0.02)	-0.02 (-0.06, 0.02)
Body mass index	2716	0.00 (-0.04, 0.04)	-0.01 (-0.05, 0.03)	0.00 (-0.04, 0.05)	0.01 (-0.03, 0.05)	0.00 (-0.04, 0.04)	-0.01 (-0.05, 0.04)
12 months							
Height	2790	0.00 (-0.05, 0.04)	0.00 (-0.04, 0.04)	0.02 (-0.03, 0.06)	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.04)
Weight	2794	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)	0.02 (-0.02, 0.07)	0.00 (-0.04, 0.04)	-0.01 (-0.05, 0.04)	-0.01 (-0.05, 0.04)
Body mass index	2774	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)	0.01 (-0.03, 0.06)	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)
24 months							
Height	2563	-0.05 (-0.09, -0.01)*	-0.04 (-0.08, -0.00)*	-0.02 (-0.06, 0.02)	-0.02 (-0.06, 0.02)	-0.02 (-0.06, 0.02)	-0.01 (-0.05, 0.03)
Weight	2597	-0.03 (-0.07, 0.02)	-0.03 (-0.07, 0.01)	0.01 (-0.03, 0.06)	-0.01 (-0.05, 0.03)	-0.02 (-0.06, 0.03)	-0.01 (-0.06, 0.04)
Body mass index	2556	0.01 (-0.03, 0.05)	0.01 (-0.03, 0.05)	0.04 (-0.01, 0.09)	0.00 (-0.14, 0.02)	0.00 (-0.04, 0.04)	-0.01 (-0.05, 0.04)

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal vessel calibers. Model 1 is adjusted for child's age at visit, sex and grader. Model 2 is additionally adjusted for lifestyle socio-demographic confounders (maternal age, education, pre-pregnancy body mass index, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching). Model 3 is additionally adjusted for body mass index and systolic blood pressure at the age of 6 years. * P value < 0.05

Table S2.4.4. Associations of crown to rump length at first trimester of pregnancy with retinal vessel calibers (N = 757)

Growth characteristics (SDS)	N	Difference in retinal vessel calibers SDS (95% Confidence Interval)					
		Arteriolar caliber			Venular caliber		
		Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
1st trimester							
Crown to rump length		-0.01 (-0.08, 0.06)	0.00 (-0.08, 0.07)	-0.01 (-0.08, 0.07)	-0.02 (-0.06, 0.09)	0.02 (-0.05, 0.10)	0.03 (-0.05, 0.10)

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal vessel calibers. Model 1 is adjusted for child's age at visit, sex and grader. Model 2 is additionally adjusted for lifestyle socio-demographic confounders (maternal age, education, pre-pregnancy body mass index, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching). Model 3 is additionally adjusted for body mass index and systolic blood pressure at the age of 6 years.* P < 0.01.

Table S2.4.5. Associations of gestational age at birth and infant growth with retinal vessel calibers at the age of 6 years (N = 2,595)

		Difference in retinal vessel calibers in SDS (95% Confidence Interval)				
		Arteriolar caliber			Venular caliber	
Gestational age at birth	Infant growth	Nr.	Basic Model	Mediator Model	Basic Model	Mediator Model
All children	Deceleration	657	-0.01 (-0.11, 0.09)	0.01 (-0.09, 0.10)	-0.04 (-0.14, 0.06)	-0.02 (-0.12, 0.08)
	Normal	1246	*Reference*	*Reference*	*Reference*	*Reference*
	Acceleration	692	-0.15 (-0.24, -0.05)*	-0.15 (-0.25, -0.05)*	-0.07 (-0.16, 0.03)	-0.08 (-0.18, 0.02)
P for trend			*0.05*	*0.06*	*0.89*	*0.77*
<37 weeks	Deceleration	25	0.04 (-0.32, 0.40)	0.09 (-0.27, 0.45)	-0.06 (-0.43, 0.30)	-0.02 (-0.38, 0.34)
	Normal	53	-0.37 (-0.66, -0.08)*	-0.34 (-0.63, -0.06)*	-0.07 (-0.36, 0.22)	-0.04 (-0.33, 0.25)
	Acceleration	31	-0.54 (-0.95, -0.12)*	-0.55 (-0.96, -0.13)*	-0.13 (-0.54, 0.29)	-0.13 (-0.54, 0.29)
P for trend			*<0.01*	*0.06*	*0.82*	*0.91*
>37 weeks	Deceleration	624	-0.03 (-0.13, 0.07)	-0.02 (-0.12, 0.09)	-0.04 (-0.14, 0.06)	-0.03 (-0.13, 0.08)
	Normal	1215	*Reference*	*Reference*	*Reference*	*Reference*
	Acceleration	680	-0.14 (-0.24, -0.04)*	-0.15 (-0.25, 0.05)	-0.07 (-0.17, 0.03)	-0.08 (-0.18, 0.02)
P for trend			*0.06*	*0. 44*	*0.58*	*0.53*

Values are standardized regression coefficients (95% confidence interval) and reflect the difference for retinal vessel calibers compared to children born on term with a normal infant growth. Basic model is adjusted for child's age at visit, sex, grader and gestational age at birth (all children only). Mediator model is additionally adjusted for lifestyle socio-demographic confounders (maternal age, education, pre-pregnancy body mass index, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, plus child's ethnicity, breastfeeding and TV watching) and for body mass index and systolic blood pressure at the age of 6 years.* P < 0.01.

Chapter 3 | Childhood factors

Chapter 3.1

Infant diet and metabolic outcomes in childhood

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Abstract

Background: Breastfeeding is associated with the risks of cardio-metabolic diseases in adulthood. We examined the associations of infant feeding patterns with metabolic outcomes in children, and whether any association was explained by family-based socio-demographic factors, maternal life style related factors, or childhood factors.

Methods and Results: We performed a population-based prospective cohort study in 3,417 children to examine the associations of breastfeeding duration and exclusivity and age at introduction of solid foods with blood levels of lipids, insulin and c-peptide, and risk of clustering of cardio-metabolic risk factors at the median age of 6.0 years (90% range 5.7 – 6.8). We observed that in the models only adjusted for child's age and sex, ever breastfeeding was not associated with childhood blood levels of lipids, but was associated with higher insulin and c-peptide concentrations (p-value<0.05). Breastfeeding duration and exclusivity were not consistently associated with metabolic outcomes. Early introduction of solid foods was associated with higher levels of total-cholesterol (p-value<0.05), but not with HDL- and LDL-cholesterol, triglycerides and insulin levels. Shorter breastfeeding duration and exclusive breastfeeding were associated with increased risks of clustering of cardio-metabolic risk factors. After additional adjustment for family, maternal and childhood factors, none of these associations remained significant.

Conclusions: In conclusion, we found no consistent associations of infant feeding patterns with metabolic outcomes at school-age, after taking account for family-based socio-demographic factors, maternal life style related factors, or childhood factors. Whether infant diet composition influence metabolic outcomes in later life should be further studied

Introduction

Breastfeeding during infancy has been suggested to have a protective effect on the development of cardio-metabolic diseases and type 2 diabetes and their risk factors in later life.[1-6] Also, recent studies suggested that longer breastfeeding duration is related to a better cardio-metabolic profile in childhood.[2, 7-9] The mechanisms underlying these associations are not known, but may include breast milk composition. Human breast milk contains a variety of enzymes, hormones, growth factors and long chain polyunsaturated fatty acids, which may beneficially influence infant growth and metabolic development.[10]

Studies in children are important, because of the limited influences of other life style related behaviors on metabolic outcomes. Thus far, results focused on the associations of breastfeeding with cardio-metabolic outcomes in childhood are inconsistent, which may be due to differences at ages at outcome measurements, different breastfeeding definitions with respect to exclusive breastfeeding and breastfeeding duration, or differences in adjustments for confounders.[2, 4, 11] The associations of infant breastfeeding with blood pressure in childhood seems to be fully explained by parental socio-demographic or lifestyle related factors, such as maternal educational level, pre-pregnancy BMI, and smoking during pregnancy.[12] Thus, when considering the beneficial associations of breastfeeding with later metabolic development, confounding might be an important issue.[11, 13-15] Family-based socio-demographic factors and maternal lifestyle related factors need to be considered before suggesting a causal effect of breastfeeding on lipid levels and insulin concentrations.[13, 16]

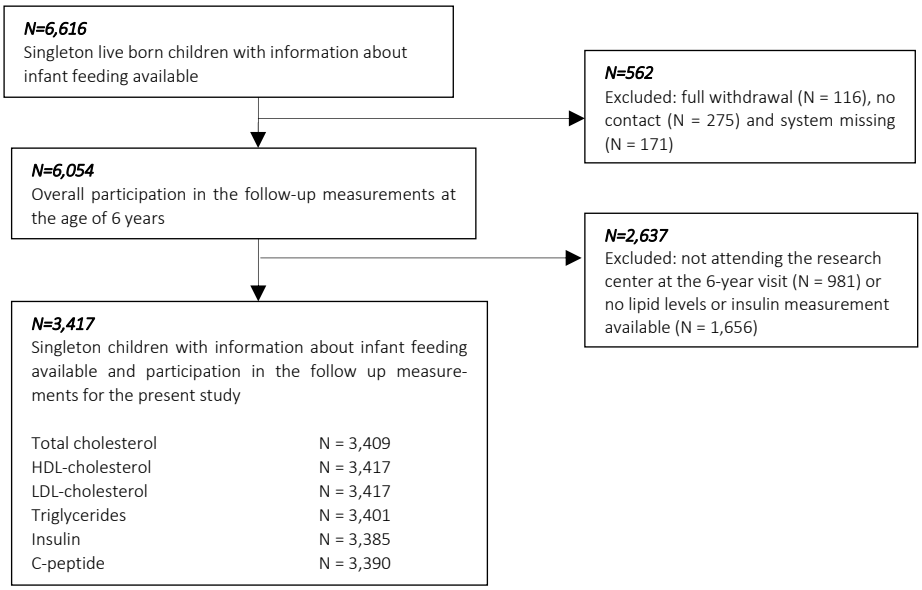
Therefore, we examined in a population-based cohort study among 3,417 school age children, the associations of breastfeeding duration and exclusiveness, and the age at introduction of solid foods with cholesterol, triglycerides, and insulin levels and the risk of a clustering of cardio-metabolic risk factors. We further explored whether any association was explained by family-based socio-demographic factors, maternal lifestyle related factors, or childhood factors.

Methods

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands. [17] Enrolment in the study was aimed in first trimester but was allowed until the birth of the child. The local Medical Ethical Committee approved the study. Written consent was obtained from parents. All children were born between 2002 and 2006. Response rate at birth was 61%.[17] Information on breastfeeding was available in 6,616 singleton live born children. Of these children, 6,054 participated in the follow-up measurements until the median age of 6.0 years (90% range 5.7 – 6.8), of whom 3,417 participated in the blood sample measurements for the current study (**Figure 3.1.1**). This lower number is mainly due to non-consent for venous puncture or crying of the child.

Figure 3.1.1. Flow chart of participants in study



Infant feeding assessments

Information on breastfeeding initiation and continuation was obtained from delivery reports and postal questionnaires at the ages of 2, 6 and 12 months after birth, as previously described.[12, 17, 18] Briefly, mothers were asked whether they ever breastfed their child (yes, no) and at what age they quitted breastfeeding. Duration of exclusive breastfeeding was defined by using information about at what age other types of milk and/or solids were introduced in the first 6 months of life, according to a short food frequency questionnaire. Among breastfed children, breastfeeding duration was categorized into four groups: 1) 0-1.9 months; 2) 2-3.9 months; 3) 4-5.9 months and 4) >= 6 months. The information on exclusiveness of breastfeeding was categorized into the following two categories: 1) non-exclusive until 4 months; and 2) exclusive until 4 months. 'Non-exclusive until 4 months' indicates infants who received both breastfeeding and formula feeding or solids during the first 4 months. 'Exclusive until 4 months' indicates infants who have been breastfed, without any other milk, solids or fluids during the first 4 months. The analyses focused on breastfeeding duration and exclusivity were performed after exclusion of never breastfed children. Information on introduction of solid foods included fruit and vegetable snacks and was obtained from the same food frequency questionnaire. Age at introduction of solid foods was categorized as: 1) <3.9 months; 2) 4-4.9 months; and 3) >5 months. These categories were based on our questionnaires and used in previous published studies from the same cohort study.[12, 19, 20]

Childhood metabolic outcomes

Thirty-minutes fasting blood samples were collected to measure total-, HDL-, and LDL-cholesterol, triglycerides, insulin and C-peptide concentrations, using a Cobas 8000 analyser (Roche, Almere, The Netherlands). Quality control samples demonstrated intra

and inter-assay coefficients of variation ranging from 0.77-1.17%, and 0.87-1.69%, respectively. Blood samples were available in 69% of the children. In line with previous definitions used among paediatric populations to define childhood metabolic syndrome phenotype [21], we defined clustering of cardio-metabolic risk factors as having any of the 3 or more following components: android fat mass % =>75th percentile; systolic or diastolic blood pressure =>75th percentile; HDL-cholesterol <=25th percentile or triglycerides => 75th percentile; and insulin level =>75th percentile. We used android fat mass as a percentage of total body fat mass, and as proxy for waist circumference since waist circumference was not available. We measured total body and regional fat mass with a Dual-energy X-ray absorptiometry (DXA) scanner (iDXA, GE-Lunar, 2008, Madison, WI, USA) and analyzed with the enCORE software v.12.6.[22] Systolic and diastolic blood pressure was measured at the right brachial artery, four times with one-minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus TM (Paramus, NJ, USA) and we used the mean of the last three blood pressure measurements for the analyses.[23]

Covariates

Information on maternal age, pre-pregnancy BMI, educational level, ethnicity and parity was obtained from the first questionnaire at enrolment in the study. Ethnicity and educational level were defined according to the classification of Statistics Netherlands.[24, 25] Information on maternal smoking during pregnancy was retrieved from questionnaires during pregnancy. Gestational age at birth, birth weight and sex were obtained from midwife and hospital registries at birth. The average TV watching time at the age of 6 years was assessed by questionnaire.[26.] According to the American Academy of Pediatrics[26] the children's media time should be limited to 1 to 2 hours/day. Therefore, we dichotomized television viewing time in ≥ 2hours/day and <2 hours/day. Anthropometrics of the child were measured at the age of 6 years in a dedicated research center by a well-trained staff. Height was measured to the nearest 0.1 cm and weight to the nearest grams. Body mass index (kg/m^2) was calculated.

Statistical analysis

First, we compared subject characteristics between the different infant feeding categories using One-Way ANOVA tests for continued variables, and Chi-square tests for categorical variables. Second, we explored the associations of breastfeeding duration and exclusivity and the age at introduction of solid foods with lipids, insulin and c-peptide levels at the age of 6 years by using multiple linear regression models. These models were first adjusted for child's age at visit and sex (crude models) and subsequently additionally for potential confounders. We tested the role of: (1) family-based sociodemographic factors (maternal age, ethnicity, education); (2) maternal lifestyle related factors (pre-pregnancy BMI, parity, smoking during pregnancy); and (3) childhood factors (gestational age at birth, birth weight, TV watching and body mass index at visit). Analyses focused on the age at introduction of solid foods were additionally adjusted for breastfeeding duration. Covariates were included in the models based on their associations with cardio-metabolic risk factors in previous studies, an association with outcomes, or a change in effect estimates of >10%. As triglycerides and insulin measures

had skewed distributions, we applied natural log transformation. To enable comparison of effect estimates, results are presented in outcome SDS ((observed value – mean) / SD). We did not create age adjusted SDS since all outcome measurements were performed in a small age range. Tests for trends were performed by treating the categorized variable as a continuous term. Finally, we used logistic regression models to examine the associations of breastfeeding and age at introduction of solid foods with the risk of clustering of cardio-metabolic risk factors in childhood using similar models. A sensitivity analysis performed in European mothers only showed similar results as for the total group (data not shown). We did not observe significant interaction terms between the breastfeeding and solid food category strata and child's sex, birth weight, gestational age at birth and maternal education level (P-value for interaction > 0.05) in relation to outcomes. In order to reduce potential bias associated with missing data, we performed multiple imputations of missing covariates by generating 5 independent datasets using the Markov Chain Monte Carlo (MCMC) method after which the pooled effect estimates were calculated.[27] Imputations were based on the relationships between covariates, determinants and outcomes. Statistical analyses were performed using the Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Table 3.1.1 shows that of all children, 92.5% (N = 3,161) had ever been breastfed. Mothers who ever breastfed their children had a lower body mass index and were higher educated as compared to mothers who never breastfed their children (P-values < 0.01). Tables S3.1.1- S3.1.3 show the distributions of maternal and childhood characteristics according to breastfeeding duration and exclusiveness and age at introduction of first solid food categories. Non-response analyses are given in the Supplemental Table S3.1.4 and S3.1.5 show that mothers and children without information about breastfeeding available and without blood levels measurements at the age of 6 years were respectively less educated and had lower birth weight and lower gestational age at birth. Children who did not participate in the follow-up measurements at the age of 6 years were less frequently breastfed and breastfed for a shorter period (Table S3.1.6)

Infant feeding and metabolic outcomes in childhood

Table 3.1.2 shows results from the crude models. Never breastfeeding was not associated with lipid levels, but was associated with higher insulin and c-peptide levels (p-value<0,05) in childhood. As compared to duration of breastfeeding longer than 6 months, duration of breastfeeding from 2 to 3.9 months was associated with higher blood levels of HDL-cholesterol. Breastfeeding duration and exclusivity were not consistently associated with blood levels of total- and LDL-cholesterol, triglycerides, insulin and c-peptide in childhood. Introduction of solid foods before 4 months of age was positively associated with higher blood levels of total-cholesterol. Table 3.1.3 shows results from the fully adjusted models. After adjustment for family-based socio-demographic factors, maternal life style related and childhood factors, infant feeding patterns were not consistently associated with metabolic outcomes at the age of 6

years. Similarly, infant feeding habits were not associated with C-peptide. We observed no dose-response associations. Models adjusted for family-based socio-demographic factors, maternal lifestyle related factors and childhood factors separately are given in Supplemental **Tables S3.1.7-S3.1.9.** Maternal educational level explained the largest amount of these associations.

Table 3.1.1. Subject characteristics according to category of breastfeeding

Characteristics	Total group N = 3,417	Never breastfed N = 256	Ever breastfed N = 3,161	P value
Maternal characteristics				
Age (years)	31.7 (30.5 – 39.9)	30.8(21.2 – 39.6)	31.7 (20.4 – 40.0)	*0.16*
Height (cm)	168.3 (7.4)	168.1 (7.0)	168.2 (7.5)	*0.95*
Weight (kg)	65.0 (49.0, 98.0)	65.0 (49.5 – 107.7)	65.0 (49.0 – 97.0)	*<0.01*
Pre-pregnancy body mass index (kg/m^2)	22.6 (18.2 – 34.3)	23.5 (18.0 – 38.8)	22.6 (18.2 – 33.7)	*<0.01*
Education (%)				
Lower	6.9	6.4	6.9	
Middle	39.5	62.3	37.7	*<0.01*
Higher	53.6	31.4	55.4	
Ethnicity (%)				
Dutch or European	66.9	78.3	65.9	*<0.01*
Non-European	33.1	21.7	34.1	
Smoking during pregnancy (%)				
Ever	24	40.8	22.6	*0.01*
Never	76	59.2	77.4	
Parity (%)				
0	55.7	48.0	56.4	*<0.01*
>= 1	44.3	52.0	43.6	
Birth characteristics				
Boys (%)	50.9	52.0	50.8	*0.73*
Gestational age (weeks)	40.1 (36.0 – 42.3)	39.9 (35.9 – 42.1)	40.1 (36.0 – 42.3)	*<0.01*
Preterm birth (%)	4.3	3.5	4.3	*0.53*
Weight (grams)	3 462 (540)	3 423 (565)	3 465 (539)	*0.23*
Low birth weight (%)	3.5	4.7	3.4	*0.09*
Small size for gestational age (%)	9.4	11.4	9.3	*0.27*
Introduction of solid foods (%)				
< 3.9 months	8.8	17.2	8.2	
4 – 4.9 months	60.5	69.4	59.9	*<0.01*
> 5 months	30.7	13.4	31.9	
Childhood characteristics				
Age at visit (years)	6.0 (5.6 – 7.4)	6.0 (5.7 – 7.2)	6.0 (5.6 – 7.5)	*0.55*
Height (cm)	118.8 (5.6)	119.0 (5.8)	119.2 (5.6)	*0.13*
Weight (kg)	23.0 (3.9)	23.1 (4.1)	23.0 (3.8)	*0.68*
Body mass index (kg/m^2)	15.8 (13.7 – 20.5)	15.8 (13.6 – 22.2)	15.8 (13.7 – 20.5)	*0.30*
Systolic blood pressure (mmHg)	102.3 (7.9)	102.7 (8.1)	102.3 (7.9)	*0.461*
Diastolic blood pressure (mmHg)	60.4 (6.7)	60.7 (6.9)	60.4 (6.7)	*0.43*
Total cholesterol (mmol/l)	4.2 (0.6)	4.1 (0.6)	4.2 (0.6)	*0.31*
HDL-cholesterol (mmol/l)	1.3 (0.3)	1.4 (0.3)	1.3 (0.3)	*0.55*
LDL-cholesterol (mmol/l)	2.4 (0.6)	2.3 (0.5)	2.4 (0.6)	*0.57*
Triglycerides (mmol/l)	0.95 (0.39, 2.34)	0.96 (0.38, 2.15)	0.95 (0.40, 2.36)	*0.50*
Insulin (U/l)	114.0 (17.0, 394.5)	112.3 (18.0, 404.8)	114.1 (17.0, 394.4)	*0.07*
C-peptide (ng/ml)	0.96 (0.30, 2.13)	0.99 (0.33, 2.19)	0.96 (0.30, 2.13)	*0.07*
Clustering of cardio-metabolic risk factors (%)	10.5	12	10.4	*0.23*

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the never breastfed group) were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables. Preterm birth was defined as gestational age < 37 weeks. Low birth weight was defined as birth weight < 2500 g. Small size for gestational age was defined as gestational age- and sex-adjusted birth weight < 10%. Subject characteristics according to breastfeeding duration and exclusivity and to age at introduction of solid groups are given in the Supplemental Tables S3.1.1-S3.1.3.

Table 3.1.2. Associations of infant feeding with lipid levels and insulin at the age of 6 years adjusted for age and sex

	Difference in outcome measure SDS (95% Confidence Interval)					
	Total- cholesterol N = 3,409	**HDL- cholesterol N = 3,417**	**LDL- cholesterol N = 3,417**	**Triglycerides N = 3,401**	**Insulin N = 3,385**	**C-peptide N=3,390**
Breastfeeding						
Never (N = 256)	-0.06 (-0.19, 0.06)	0.04 (-0.09, 0.16)	-0.03 (-0.16, 0.09)	-0.01 (-0.14, 0.12)	0.11 (0.00, 0.22)*	0.10 (0.04, 0.17)*
Ever (N = 3,161)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Duration						
0 – 1.9 months (N = 655)	0.05 (-0.05, 0.15)	0.08 (-0.02, 0.18)	-0.01 (-0.12, 0.09)	0.01 (-0.10, 0.11)	0.02 (-0.08, 0.12)	0.01 (-0.10, 0.12)
2 – 3.9 months (N = 567)	0.07 (-0.04, 0.17)	0.10 (0.00, 0.20)*	0.05 (-0.06, 0.15)	0.02 (-0.09, 0.13)	0.04 (-0.07, 0.14)	0.08 (-0.03, 0.19)
4 -5.9 months (N = 338)	0.10 (-0.02, 0.22)	0.04 (-0.09, 0.16)	0.07 (-0.05, 0.20)	0.05 (-0.18, 0.17)	0.04 (-0.18, 0.17)	0.05 (-0.01, 0.12)
≥ 6 months (N = 859)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.31*	*0.08*	*0.88*	*0.83*	*0.63*	*0.59*
Exclusive for 4 months						
No (N = 1,799)	0.01 (-0.07, 0.10)	0.02 (-0.07, 0.10)	0.01 (-0.08, 0.09)	-0.01 (-0.10, 0.08)	0.03 (-0.05, 0.12)	0.04 (-0.01, 0.08)
Yes (N = 712)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
First solid foods						
< 3.9 months (N = 201)	0.16 (0.00, 0.31)*	0.05 (-0.11, 0.21)	0.13 (-0.04, 0.28)	0.00 (-0.16, 0.16)	-0.04 (-0.20, 0.13)	-0.06 (-0.22, 0.10)
4-4.9 months (N = 1,375)	-0.02 (-0.12, 0.07)	-0.02 (-0.12, 0.07)	-0.02 (-0.12, 0.07)	0.00 (-0.10, 0.09)	-0.02 (-0.12, 0.06)	0.01(-0.09, 0.10)
> 5 months (N = 696)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.34*	*0.85*	*0.41*	*0.98*	*0.65*	*0.63*

Values are linear regression coefficients (95% confidence interval). Models are adjusted for child's age at visit and sex. Analyses with solid foods were additionally adjusted for breastfeeding duration. Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter. * P < 0.05

Table 3.1.3. Associations of infant feeding with lipid levels and insulin at the age of 6 years adjusted for age, sex, family-based socio-demographic, maternal life style related and childhood factors

	Difference in outcome measure SDS (95% Confidence Interval)					
	Total- cholesterol N = 3,409	**HDL- cholesterol N = 3,417**	**LDL- cholesterol N = 3,417**	**Triglycerides N = 3,401**	**Insulin N = 3,385**	**C-peptide N=3,390**
Breastfeeding						
Never (N = 256)	-0.06 (-0.19, 0.07)	0.07 (-0.06, 0.19)	-0.05 (-0.18, 0.08)	-0.01 (-0.14, 0.11)	0.10 (-0.03, 0.24)	0.07 (-0.06, 0.20)
Ever (N = 3,161)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Duration						
0 – 1.9 months (N = 655)	0.03 (-0.07, 0.14)	0.10 (-0.01, 0.20)	-0.05 (-0.15, 0.05)	0.00 (-0.10, 0.11)	0.05 (-0.05, 0.16)	0.06 (-0.05, 0.16)
2 – 3.9 months (N = 567)	-0.06 (-0.05, 0.16)	0.10 (-0.01, 0.20)	0.03 (-0.07, 0.14)	0.01 (-0.10, 0.12)	0.05 (-0.05, 0.15)	0.09 (-0.01, 0.20)
4 -5.9 months (N = 338)	0.10 (-0.02, 0.22)	0.06 (-0.07, 0.18)	0.07 (-0.05, 0.20)	0.03 (-0.09, 0.16)	0.03 (-0.10, 0.14)	0.02 (-0.10, 0.15)
≥ 6 months (N = 859)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.64*	*0.03*	*0.29*	*0.95*	*0.29*	*0.17*
Exclusive for 4 months						
No (N = 1,799)	-0.01 (-0.09, 0.08)	0.01 (-0.08, 0.09)	-0.02 (-0.10, 0.07)	-0.01 (-0.10, 0.08)	0.06 (-0.03, 0.15)	0.08 (-0.01, 0.17)
Yes (N = 712)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
First solid foods						
< 3.9 months (N = 201)	0.10 (-0.06, 0.27)	0.08 (-0.09, 0.24)	0.05 (-0.12, 0.21)	-0.03 (-0.20, 0.14)	-0.04 (-0.20, 0.13)	-0.02 (-0.18, 0.15)
4-4.9 months (N = 1,375)	-0.05 (-0.15, 0.04)	-0.05 (-0.15, 0.04)	-0.06 (-0.15, 0.04)	-0.01 (-0.11, 0.09)	0.02 (-0.11, 0.08)	0.03 (-0.06, 0.12)
> 5 months (N = 696)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.24*	*0.75*	*0.87*	*0.74*	*0.64*	*0.87*

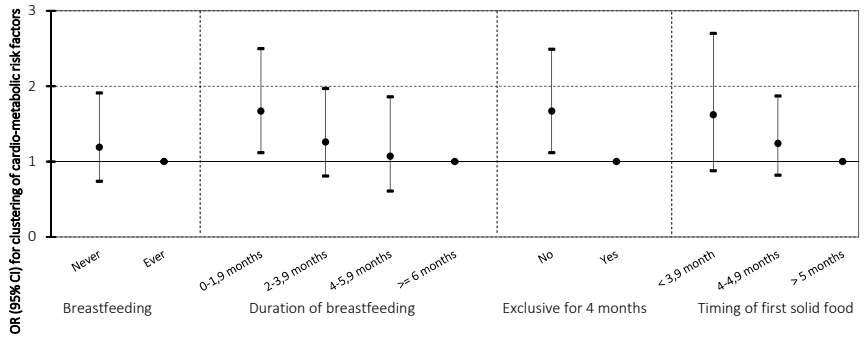
Values are linear regression coefficients (95% confidence interval). Models are adjusted for child's age at visit, sex, plus family-based socio-demographic confounders (maternal age, ethnicity, education), maternal life style related factors (pre-pregnancy BMI parity, smoking during pregnancy) and childhood factors (gestational age at birth, birth weight and TV watching, body mass index). Results from the models adjusted for family-based socio-demographic factors, maternal life style related factors, and child factors separately are given in the supplemental Tables S4-S6. Analyses with solid foods were additionally adjusted for breastfeeding duration. Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter. * P < 0.05

Infant feeding and clustering of cardio-metabolic risk factors in childhood

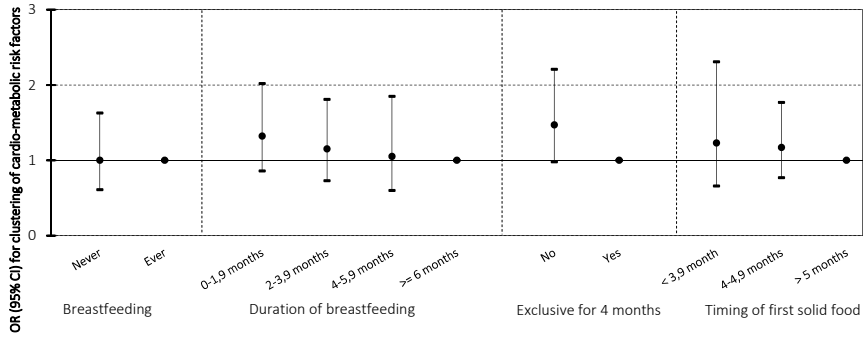
Figure 3.1.2 shows that in models adjusted only for child´s age and sex, duration of breastfeeding less than 2 months of age and non-exclusive breastfeeding were associated with higher risks of childhood clustering of cardio-metabolic risk factors. After adjustment for maternal and infant factors none of the associations remained significant. Models adjusted for family-based socio-demographic, maternal life style related and childhood factors separately are given in Supplemental Figures S3.1.1, S3.1.2 and S3.1.3.

Figure 3.1.2. Associations of infant feeding with the risks of metabolic syndrome phenotype

A. Basic models



B. Fully adjusted models



Values are odds ratios (95% confidence interval) using multiple logistic regression models. Basic models are adjusted for child's age at visit and sex. Analyses with solid foods were additionally adjusted for breastfeeding duration. Fully adjusted models are additionally adjusted for family-based socio-demographic factors (maternal age, ethnicity, education), maternal life style related factors (pre-pregnancy BMI parity, smoking during pregnancy) and childhood factors (gestational age at birth, birth weight and TV watching). Results from the models adjusted for family-based socio-demographic confounders, maternal life style related factors, and infant factors separately are given in the supplementary materials (Figures S3.1.1-S3.1.3). Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter.

Discussion

Results from this population-based prospective study do not support the hypothesis that ever breastfeeding, breastfeeding duration and exclusivity are consistently associated with childhood levels of cholesterol, triglycerides or insulin. The observed associa-

tions of breastfeeding and age at introduction of solid foods with metabolic outcomes in childhood were not-consistent and explained by family-based socio-demographic factors, maternal life style related factors and childhood factors.

Methodological considerations

We used a population-based prospective cohort design including a large number of subjects whom we studied from early fetal life onwards. Of the total group of singleton live born children, information on breastfeeding was available in 70%. We do not expect that this non-response at baseline led to biased effect estimates because biased estimates in large cohort studies mainly arise from loss to follow-up rather than from non-response at baseline.[28] Of all children with information on breastfeeding, <48% did not participate in the follow-up measurements at the age of 6 years. This loss to follow-up would lead to selection bias if the associations of infant feeding with lipid levels and insulin would differ between those included and not included in the final analyses. We observed that children who did not participate in the follow-up measurements at the age of 6 years were less frequently breastfed and breastfed for a shorter period. This might have led to an underestimation of the effect estimates. Although assessing breastfeeding initiation and duration and introduction of solid foods by questionnaires may lead to measurement error, this method seems to be valid in population-based samples, especially when recall spans over a short period (<3 years), which is the case in our study.[29, 30] Also, we used three questionnaires to assess infant feeding during the first 12 months. We did not assess the use of water during exclusive breastfeeding period, which is not in the line with the strict criteria used by the WHO.[31] However, we did ask for the most commonly introduced solids and fluids. Furthermore, in The Netherlands, it is not common that children receive breast-feeding in combination with water to prevent dehydration. Therefore, we think that our measurement of exclusive breast-feeding is a good proxy for exclusive breast-feeding according to the WHO criteria. We used 30-minutes fasting blood samples, which may have led to underestimation of the observed associations. However, it has been shown in adults that non-fasting lipid levels can accurately predict increased risks of cardiovascular events in later life [32]. We used a previously proposed definition of clustering of metabolic risk factors in children. The criteria used in pediatric studies have been adapted from adult standards with the use of gender- and age-dependent normal values.[21] However, since there is a lack of hard clinical end points in the pediatric setting, the relationship between the individual risk factors and their clustering on the atherosclerosis disease process remains difficult to define. We could not adjust for detailed measures of childhood diet, as this information was available only in a small subgroup of the study population. Finally, although we performed adjustment for a large number of potential maternal and infant confounders, residual confounding might still occur, as in any observational study.

Interpretation of main findings

Several studies in the last decades have evaluated whether breastfeeding has an effect on development of cardio-metabolic outcomes in later life, and explored underlying mechanisms for these associations. Previous studies have reported that cholesterol levels in adulthood are influenced by infant feeding habits.[33] Breastfed infants had high-

er total cholesterol levels in infancy than those formula-fed, but these associations were not consistent in later childhood.[9, 34] The presence of an effect in infancy only might be due to higher cholesterol concentrations in breastfed infants during the period of breastfeeding only and not after ceasing breastfeeding. Compared to formula milk, human breast milk is rich in long-chain polyunsaturated fatty acids (LC-PUFAs), which seem to have a protective effect on the development of obesity and diabetes in later life.[35] A deficiency in the LC-PUFA in formula feed children during first year of life may increase the risk of insulin resistance.[35] It has also been suggested that breastfeeding has a protective effect on development of obesity [7, 36], which may subsequently lead to lower levels of cholesterol, triglycerides and insulin resistance in later life. However, the previously suggested associations may also be explained by family related socio-demographic characteristics and maternal life style related factors.

In this study, we observed that early introduction of solid foods was positively associated with total-cholesterol in the basic models. After full adjustment for maternal and infant factors, infant feeding was not significantly associated with lipid levels in childhood. In agreement with our results, another longitudinal study among 489 participants showed no significant effects of breastfeeding on total- and LDL-cholesterol levels after adjustment for maternal and child confounders.[37] Demmers et al.[38] reported from a prospective clinical trial among 47 infant that there were not significant differences in LDL-cholesterol and triglycerides levels among breastfed infant as compared to those formula-fed at the age of 4 months. This study showed that at the age of 4 months total- and HDL-cholesterol were higher among breastfed infant, but these differences did not persist at the age of 18 months.[38] Also, a meta-analyses did not provide strong evidence for long term benefits of breastfeeding on blood cholesterol levels in adulthood.[36]

A meta-analysis among 24 studies reported lower serum glucose and marginally lower serum insulin levels in breastfed infants compared to formula fed infants.[2] However, these results might be influenced by publication bias and uncontrolled or residual confounding. Another study among 679 young adults found no evidence of associations between formula milk intake in infancy and insulin sensitivity or insulin secretion, after fully adjustment for potential confounders.[4] Also, no consistent evidence has been shown for a protective and a causal effect of breastfeeding on overweight and obesity and subsequent development of diabetes in later life.[18, 33, 34, 39] Results from a randomized control trial showed that longer breastfeeding duration and exclusive breastfeeding among healthy term infants, did not influence glucose and insulin levels at the age of 11.5 years.[40] We also did not observe associations of infant feeding with insulin and c-peptide levels in the fully adjusted model, suggesting that previously reported beneficial effects of breastfeeding may be the result of confounding. However, we cannot exclude the possibility that the associations of infant feeding with insulin resistance and diabetes may arise in later adulthood.

Studies focused on the associations of breastfeeding duration, exclusive breastfeeding and age at introduction of solid food with the clustering of cardio-metabolic risk factors also showed inconsistent results.[11, 41] We found a significant association of shorter and exclusivity breastfeeding with the risk of clustering of cardio-metabolic risk factors at the age of 6 years, but these associations attenuated into non-significant after

adjustment for maternal and childhood confounders. Similarly, a systematic review among 10, 912 young adults from five prospective birth-cohort studied showed no evidence that ever breastfeed, longer breastfeeding duration and late introduction of complementary food were associated with risk of adult hypertension, diabetes and overweight.[34]

Results from our study suggest that any association of infant feeding with lipid levels and insulin in childhood are explained by family-based socio-demographic, maternal life-style and child factors. Maternal education level was the strongest confounder in these associations. However other explanations are possible. We did not have information about breast milk composition. Differences in breast milk composition, such as long chain polyunsaturated fatty acids content across populations and over time, might complicate direct comparisons between studies.[42] Also, the associations of breastfeeding with later cardio-metabolic outcomes might appear at older ages, or be confined to specific populations such as preterm infants or children born underweight.

Conclusions

In this population-based prospective cohort study, we observed that the associations of breastfeeding and age at introduction of solid foods with lipid levels and insulin in children were explained by family-based socio-demographic, maternal life-style and childhood related characteristics. Further studies are needed to study whether breast-milk composition or infant feeding habits may have long-term benefits for metabolic health.

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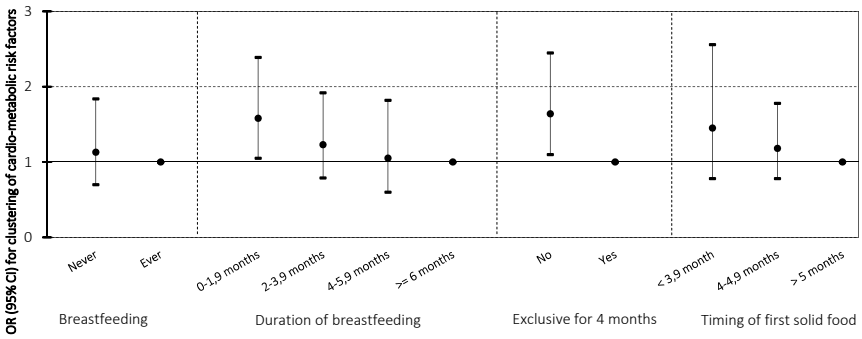
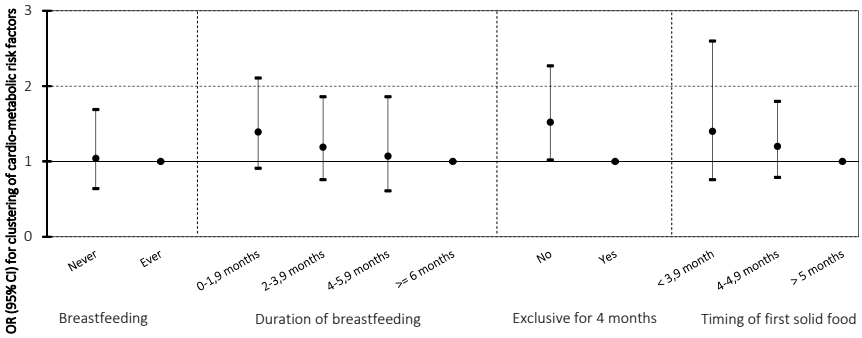
Supplemental Material

Figure S3.1.1. Associations of infant feeding with the risk of childhood clustering of cardio-metabolic risk factors adjusted for age, sex, and family-based socio-demographic



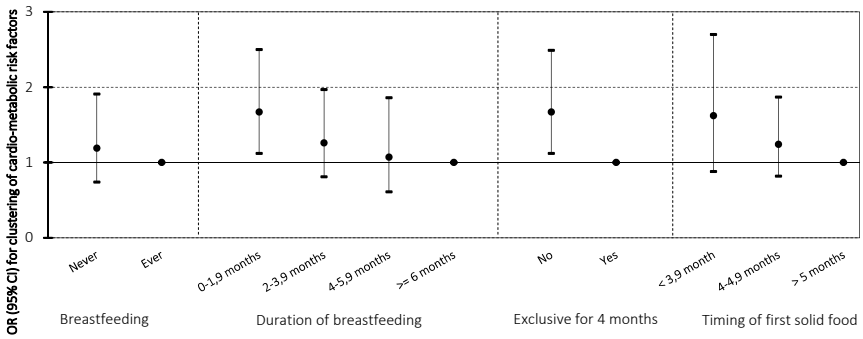
Values are odds ratios (95% confidence interval) using multiple logistic regression models. Models are adjusted for child's age at visit and sex, plus family-based socio-demographic confounders (maternal age, ethnicity, education). Analyses with solid foods were additionally adjusted for breastfeeding duration. Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter.

Figure S3.1.2. Associations of infant feeding with the risk of childhood clustering of cardio-metabolic risk factors adjusted for age, sex, and maternal life style related factors



Values are odds ratios (95% confidence interval) using multiple logistic regression models. Models are adjusted for child's age at visit and sex, maternal life style related factors (pre-pregnancy BMI parity, smoking during pregnancy). Analyses with solid foods were additionally adjusted for breastfeeding duration. Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter.

Figure S3.1.3. Associations of infant feeding with the risk of childhood clustering of cardio-metabolic risk factors adjusted for age, sex, and childhood factors



Values are odds ratios (95% confidence interval) using multiple logistic regression models. Models are adjusted for child's age at visit and sex and childhood factors (gestational age at birth, birth weight and TV watching and body mass index). Analyses with solid foods were additionally adjusted for breastfeeding duration. Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter.

Table S3.1.1. Subject characteristics according to category of breastfeeding duration

	0 – 1.9 months	2 -3.9 months	4 – 5.9 months	>= 6 months	P value
Maternal characteristics					
Age (years)	30.6 (19.6, 39.1)	31.5 (20.3, 39.6)	32.1 (21.5, 39.4)	32.6 (22.2, 40.4)	*< 0.01*
Height (cm)	167.6 (7.4)	168.9 (7.6)	169.6 (6.9)	168.9 (7.4)	*< 0.01*
Weight (kg)	66.0 (48.0, 103.0)	64.0 (49.0, 96.9)	64.0 (50.0, 94.0)	64.0 (50.0, 92.0)	*< 0.01*
Pre-pregnancy body mass index (kg/m^2)	23.3 (18.0, 39.1)	22.1 (18.4, 33.2)	22.2 (18.1, 31.8)	22.3 (18.4, 32.2)	*< 0.01*
Education (%)					
Lower	8.6	4.2	1.8	5.0	*< 0.01*
Middle	51.7	39.9	27.6	28.5	
Higher	39.7	55.9	70.6	66.6	
Ethnicity (%)					
Dutch or European	64.6	68.6	76.3	71.7	*< 0.01*
Non-European	35.4	31.2	23.7	28.3	
Smoking during pregnancy (%)					
Ever	66.1	23.4	19.6	15.1	*< 0.01*
Never	33.9	76.6	80.4	84.9	
Parity (%)					
0	67.2	62.8	58.2	52.9	*< 0.01*
>= 1	37.3	37.2	41.8	47.1	
Birth characteristics					
Boys (%)	49.3	51.9	52.1	50.3	*0.78*
Gestational age (weeks)	40.1 (35.7, 42.2)	40.1 (35.6, 42.3)	40.2 (35.7, 42.4)	40.3 (36.7, 42.4)	*0.01*
Preterm birth (%)	4.7	5.1	5.0	2.9	*0.13*
Weight (grams)	3450 (534)	3450 (554)	3465 (569)	3532 (513)	*< 0.01*
Low birth weight (%)	4.6	4.6	3.0	2.2	*0.03*
Small size for gestational age(%)	9.5	10.3	8.6	8.1	*0.47*
Introduction of solid foods (%)					
< 3.9 months	15.0	9.6	6.5	3.7	*< 0.01*
4 – 4.9 months	66.3	70.7	63.9	45.6	
> 5 months	18.7	19.7	29.6	50.7	
TV watching >= 2 hours/day (%)	22.7	17.3	7.7	14.5	*<0.01*
Childhood outcomes					
Age at visit (years)	6.0 (5.6, 7.5)	5.9 (5.6, 7.4)	5.9 (5.5, 6.8)	5.9 (5.6, 7.0)	*0.01*
Height (cm)	119 (5.9)	119 (5.5)	119 (4.8)	119 (5.4)	*0.30*
Weight (kg)	23.1 (4.0)	22.9 (3.4)	22.3 (3.5)	22.7 (3.6)	*0.31*
Body mass index (kg/m^2)	15.8	15.9	15.8	15.8	*0.32*
Systolic blood pressure (mmHg)	102.4 (8.3)	102.5 (7.9)	101.9 (7.7)	101.8 (7.9)	*0.32*
Diastolic blood pressure (mmHg)	60.7 (6.8)	60.2 (6.8)	60.0 (6.7)	60.2 (6.6)	*0.26*
Total cholesterol (mmol/l)	4.2 (0.6)	4.2 (0.7)	4.2 (0.7)	4.2 (0.6)	*0.40*
HDL-cholesterol (mmol/l)	1.35 (0.3)	1.35 (0.3)	1.33 (0.3)	1.32 (0.3)	*0.21*
LDL-cholesterol (mmol/l)	2.35 (0.6)	2.38 (0.6)	2.40 (0.6)	2.36 (0.5)	*0.58*
Triglycerides (mmol/l)	0.94 (0.40, 2.56)	0.98 (0.41, 2.37)	0.95 (0.41, 2.63)	0.94 (0.39, 2.15)	*0.79*
Insulin (U/l)	117.4 (16.1, 405.7)	115.6 (15.8, 421.3)	122.2 (18.1, 391.0)	114.0 (17.1, 391.5)	*0.65*
C-peptide (ng/ml)	0.95 (0.30, 2.27)	0.99 (0.30, 2.11)	0.99 (0.31, 2.16)	0.96 (0.28, 2.10)	*0.31*
Clustering of cardio-metabolic risk factors (%)	11.5	10.6	9.9	8.8	*0.38*

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the never breastfed group) were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables. Non-European: Indonesian, Moroccan, Antillean, Surinamese, CapeVerdean, African, American, Asian and Australian. Preterm birth was defined as gestational age < 37 weeks. Low birth weight was defined as birth weight < 2500 g. Small size for gestational age was defined as gestational age- and sex-adjusted birth weight < 10%.

Table S3.1.2. Subject characteristics according to category of breastfeeding exclusivity

	No N = 1,799	Yes N = 712	P value
Maternal characteristics			
Age (years)	31.5 (20.2, 40.0)	32.5 (23.0, 40.4)	*< 0.01*
Height (cm)	168.0 (7.6)	169.6 (7.1)	*< 0.01*
Weight (kg)	65.0 (48.5, 98.0)	64.0 (50.0, 93.3)	*0.18*
Pre-pregnancy body mass index (kg/m^2)	22.7 (18.3, 34.1)	22.1 (18.4, 32.3)	*< 0.01*
Education (%)			
Lower	7.2	3.2	
Middle	41.6	26.4	*< 0.01*
Higher	51.2	70.4	
Ethnicity (%)			
Dutch or European	64.9	75.7	*< 0.01*
Non-European	35.1	24.3	
Smoking during pregnancy (%)			
Ever	25.7	15.0	*< 0.01*
Never	74.3	85.0	
Parity (%)			
0	59.9	54.2	*<0.01*
>= 1	40.1	45.8	
Birth characteristics			
Boys (%)	50.4	50.4	*0.99*
Gestational age (weeks)	40.1 (35.9, 42.3)	40.3 (36.6, 42.4)	*0.19*
Preterm birth (%)	4.4	4.1	*0.69*
Weight (grams)	3450 (539)	3560 (529)	*< 0.01*
Low birth weight (%)	4.0	2.0	*0.01*
Small size for gestational age(%)	10.4	5.9	*< 0.01*
Introduction of solid foods (%)			
< 3.9 months	12.4	0.1	
4 – 4.9 months	65.6	48.8	*< 0.01*
> 5 months	22.0	50.8	
TV watching >= 2 hours/day (%)	19.7	9.3	*<0.01*
Childhood outcomes			
Age at visit (years)	6.0 (5.6, 7.3)	5.9 (5.7, 6.8)	*<0.01*
Height (cm)	119.1 (5.7)	118.7 (5.2)	*0.09*
Weight (kg)	13.0 (3.7)	22.6 (3.3)	*0.02*
Body mass index (kg/m^2)	15.8 (13.6, 20.3)	15.7 (13.7, 19.4)	*0.04*
Systolic blood pressure (mmHg)	102.5 (8.0)	101.4 (7.6)	*0.00*
Diastolic blood pressure (mmHg)	60.5 (6.7)	59.8 (6.6)	*0.03*
Total cholesterol (mmol/l)	4.2 (0.6)	4.2 (0.6)	*0.73*
HDL-cholesterol (mmol/l)	1.3 (0.3)	1.3 (0.3)	*0.55*
LDL-cholesterol (mmol/l)	2.4 (0.6)	2.4 (0.6)	*0.88*
Triglycerides (mmol/l)	0.96 (0.40, 2.37)	0.95 (0.40, 2.34)	*0.81*
Insulin (U/l)	114.7 (15.7, 401.7)	114.0 (17.8, 391.0)	*0.25*
C-peptide (ng/ml)	0.96 (0.29, 2.15)	0.95 (0.29, 2.11)	*0.14*
Clustering of cardio-metabolic risk factors (%)	11.3	7.2	*0.02*

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the never breastfed group) were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables. Non-European: Indonesian, Moroccan, Antillean, Surinamese, Cape Verdean, African, American, Asian and Australian. Preterm birth was defined as gestational age < 37 weeks. Low birth weight was defined as birth weight < 2500 g. Small size for gestational age was defined as gestational age- and sex-adjusted birth weight < 10%.

Table S3.1.3. Subject characteristics according to category of age at first solid food

	< 3.9 months	4 – 4.9 months	>5 months	P value
Maternal characteristics				
Age (years)	29.1 (18.6, 38.8)	31.7 (22.1, 40.1)	32.5 (22.4, 40.1)	*< 0.01*
Height (cm)	167.6 (7.7)	168.3 (7.3)	169.8 (7.3)	*< 0.01*
Weight (kg)	68.0 (49.0, 105.5)	65.0 (49.0, 97.0)	65.0 (50.0, 93.0)	*< 0.01*
Pre-pregnancy body mass index (kg/m^2)	23.9 (17.7, 39.5)	22.6 (18.3, 34.1)	22.2 (18.3, 32.2)	*< 0.01*
Education (%)				
Lower	9.0	6.0	3.0	
Middle	61.2	40.2	24.7	*< 0.01*
Higher	29.8	53.8	72.3	
Ethnicity (%)				
Dutch or European	63.1	67.6	78.3	*< 0.01*
Non-European	36.9	32.4	21.7	
Smoking during pregnancy (%)				
Ever	35.7	23.4	17.0	*< 0.01*
Never	63.3	76.6	83.0	
Parity (%)				
0	64.3	59.0	57.3	*0.21*
>= 1	35.7	41.0	42.7	
Birth characteristics				
Boys (%)	48.8	50.8	51.0	*0.84*
Gestational age (weeks)	40.1 (34.8, 42.4)	40.1 (36.3, 42.3)	40.3 (35.4, 42.4)	*0.91*
Preterm birth (%)	4.0	3.9	4.3	*0.89*
Weight (grams)	3415 (509)	3490 (519)	3530 (574)	*<0.01*
Low birth weight (%)	4.5	3.0	4.3	*0.22*
Small size for gestational age(%)	15.4	9.2	8.2	*0.01*
TV watching >= 2 hours/day (%)	27.0	16.7	10.2	*<0.01*
Childhood outcomes				
Age at visit (years)	5.9 (5.6, 7.8)	5.9 (5.7, 6.8)	5.9 (5.6, 6.7)	*0.01*
Height (cm)	118.4 (5.8)	118.7 (5.3)	118.8 (5.3)	*0.70*
Weight (kg)	22.9 (4.0)	22.7 (3.6)	22.5 (3.1)	*0.16*
Body mass index (kg/m^2)	15.9 (13.4, 22.6)	15.8 (13.8, 20.0)	15.7 (13.6, 19.1)	*< 0.01*
Systolic blood pressure (mmHg)	102.6 (8.1)	102.3 (8.1)	101.3 (7.5)	*0.02*
Diastolic blood pressure (mmHg)	60.8 (6.6)	60.5 (9)	59.5 (6.3)	*0.00*
Total cholesterol (mmol/l)	4.3 (0.6)	4.2 (0.6)	4.2 (0.6)	*0.04*
HDL-cholesterol (mmol/l)	1.4 (0.3)	1.3 (0.3)	1.3 (0.3)	*0.48*
LDL-cholesterol (mmol/l)	2.5 (0.5)	2.4 (0.6)	2.4 (0.6)	*0.10*
Triglycerides (mmol/l)	0.99 (0.46, 2.22)	0.96 (0.39, 2.43)	0.97 (0.42, 2.35)	*0.61*
Insulin (U/l)	110.7 (19.5, 336.3)	114 (18.4, 413.6)	116.3 (15.3, 390.5)	*0.96*
C-peptide (ng/ml)	0.94 (0.28, 1.98)	0.97 (0.31, 2.17)	0.95 (0.29, 2.08)	*0.44*
Clustering of cardio-metabolic risk factors (%)	14.4	10.1	8.5	*0.05*

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the never breastfed group) were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables. Preterm birth was defined as gestational age < 37 weeks. Low birth weight was defined as birth weight < 2500 g. Small size for gestational age was defined as gestational age- and sex-adjusted birth weight < 10%.

Table S3.1.4. Characteristics of the children without information of breastfeeding available

	No information about breastfeeding N=538	Breastfeeding information available N=5,073	P value
Maternal characteristics			
Age (years)	29.3 (19.2, 41.1)	31.5 (20.3, 39.7)	*< 0.01*
Height (cm)	164.4 (7.0)	167.7 (7.2)	*< 0.01*
Weight (kg)	60.0 (48.1, 93.9)	64.0 (49.0, 98.0)	*< 0.01*
Pre-pregnancy body mass index (kg/m^2)	22.7 (17.6, 35.9)	22.6 (18.2, 34.2)	*0.60*
Education (%)			
Lower	22.6	7.3	
Middle	56.1	40.2	
Higher	21.3	52.5	*< 0.01*
Ethnicity (%)			
Dutch or European	62.4	66.5	
Non-European	37.6	33.5	*< 0.01*
Smoking during pregnancy (%)			
Ever	33.4	24.3	
Never	66.6	75.7	*< 0.01*
Parity (%)			
0	54.0	57.4	
>= 1	46.0	42.6	*< 0.01*
Birth characteristics			
Boys (%)	53.5	49.8	*0.10*
Gestational age (weeks)	39.8 (33.2, 42.1)	40.1 (36.0, 42.3)	*< 0.01*
Preterm birth (%)	9.5	4.3	*< 0.01*
Weight (grams)	3480 (539)	3440 (554)	*0.01*
Low birth weight (%)	8.8	4.1	*< 0.01*
Small size for gestational age(%)	14.3	10.0	*< 0.01*
TV watching >= 2 hours/day (%)	26.9	17.0	*< 0.01*
Childhood outcomes			
Age at visit (years)	6.1 (5.7, 8.1)	6.0 (5.6, 7.4)	*< 0.01*
Height (cm)	120.2 (6.4)	118.9 (5.7)	*< 0.01*
Weight (kg)	24.0 (4.9)	22.9 (3.9)	*< 0.01*
Body mass index (kg/m^2)	16.0 (13.6, 22.0)	15.8 (13.6, 20.6)	*0.90*
Systolic blood pressure (mmHg)	103.5 (8.5)	102.6 (8.1)	*0.01*
Diastolic blood pressure (mmHg)	61.3 (7.0)	60.6 (6.7)	*0.04*
Total cholesterol (mmol/l)	4.2 (0.6)	4.2 (0.6)	*0.36*
HDL-cholesterol (mmol/l)	1.4 (0.3)	1.3 (0.3)	*0.01*
LDL-cholesterol (mmol/l)	2.3 (0.5)	2.4 (0.6)	*0.05*
Triglycerides (mmol/l)	0.9 (0.4, 2.3)	1.0 (0.4, 2.3)	*0.16*
Insulin (U/l)	105.3 (21.2, 386.9)	113.9 (17.0, 394.5)	*0.93*
C-peptide (ng/ml)	0.90 (0.3, 2.1)	1.0 (0.3, 2.1)	*0.43*
Clustering of cardio-metabolic risk factors (%)	16.6	10.5	*< 0.01*

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the never breastfed group) were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables. Preterm birth was defined as gestational age < 37 weeks. Low birth weight was defined as birth weight < 2500 g. Small size for gestational age was defined as gestational age- and sex-adjusted birth weight < 10%.

Table S3.1.5. Characteristics of the children without blood levels measurements at the age of 6 years

	Blood measures available N=3,417	No blood measures available N=1,656	P value
Maternal characteristics			
Age (years)	31.2 (20.0, 39.7)	31.7 (20.5, 39.9)	*< 0.01*
Height (cm)	167.7 (7.2)	168.3 (7.4)	*0.01*
Weight (kg)	64.0 (49.0, 98.0)	65.0 (49.0, 98.0)	*0.40*
Pre-pregnancy body mass index (kg/m^2)	22.5(18.1, 34.2)	22.6 (18.2, 34.2)	*0.97*
Education (%)			
Lower	8.1	6.9	*0.02*
Middle	41.5	39.5	
Higher	50.4	53.6	
Ethnicity (%)			
Dutch or European	68.8	66.9	*0.45*
Non-European	34.2	33.1	
Smoking during pregnancy (%)			
Ever	25.0	24.0	*0.47*
Never	75.0	76.0	
Parity (%)			
0	60.9	55.7	*< 0.01*
>= 1	39.1	44.3	
Birth characteristics			
Boys (%)	47.3	50.9	*0.02*
Gestational age (weeks)	40.1 (36.3, 42.3)	40.1 (36.0, 42.3)	*0.89*
Preterm birth (%)	4.3	4.3	*0.90*
Weight (grams)	3440 (554)	3480 (539)	*0.03*
Low birth weight (%)	4.1	3.5	*0.31*
Small size for gestational age(%)	11.3	9.4	*0.04*
Breastfeeding			
Ever	92.0	92.5	*0.65*
Never	8.0	7.5	
Breastfeeding duration			
0 – 1.9 months	30.0	27.1	
2 – 3.9 months	23.4	23.4	*0.12*
4 – 5.9 months	12.2	14.0	
≥ 6 months	34.5	35.5	
Introduction of solid foods (%)			
< 3.9 months	6.1	8.8	*0.27*
4 – 4.9 months	61.7	60.5	
> 5 months	31.2	30.6	
TV watching >= 2 hours/day (%)	16.3	17.4	*< 0.01*
Childhood outcomes			
Age at visit (years)	6.0 (5.6, 7.2)	6.0 (5.4, 7.4)	*0.10*
Height (cm)	118.3 (5.7)	119.2 (5.6)	*< 0.01*
Weight (kg)	22.7 (3.9)	23.0 (3.8)	*0.01*
Body mass index (kg/m^2)	15.8 (13.4, 20.8)	15.8 (13.7, 20.5)	*0.90*

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the never breastfed group) were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables. Preterm birth was defined as gestational age < 37 weeks. Low birth weight was defined as birth weight < 2500 g. Small size for gestational age was defined as gestational age- and sex-adjusted birth weight < 10%.

Table S3.1.6. Characteristics of the children who did not participate at the follow-up measurements at the age of 6 years

	No participation at the follow-up N= 5,611	Participation at the follow-up N=2,085	P value
Maternal characteristics			
Age (years)	29.5 (18.9, 39.0)	31.4 (20.1, 40.0)	*< 0.01*
Height (cm)	166.6 (7.5)	167.8 (7.4)	*< 0.01*
Weight (kg)	63.0 (47.0, 98.2)	64.0 (49.0, 98.0)	*0.02*
Pre-pregnancy body mass index (kg/m^2)	22.4 (17.7, 35.5)	22.6 (18.1, 34.3)	*0.94*
Education (%)			
Lower	14.1	8.4	
Middle	47.0	41.3	*< 0.01*
Higher	31.9	50.2	
Ethnicity (%)			
Dutch or European	60.6	63.9	*< 0.01*
Non-European	39.4	36.1	
Smoking during pregnancy (%)			
Ever	28.6	24.9	*< 0.01*
Never	71.4	75.1	
Parity (%)			
0	52.1	56.5	*< 0.01*
>= 1	47.9	43.5	
Birth characteristics			
Boys (%)	51.6	50.1	*0.23*
Gestational age (weeks)	40.0 (35.6, 42.3)	40.1 (35.9, 42.3)	*0.18*
Preterm birth (%)	4.3	4.8	*0.55*
Weight (grams)	3400 (564)	3436 (550)	*0.01*
Low birth weight (%)	4.8	4.3	*0.01*
Small size for gestational age(%)	11.9	10.4	*0.06*
Breastfeeding			
Ever	90.5	92.2	*0.02*
Never	9.5	7.6	
Breastfeeding duration			
0 – 1.9 months	39.1	20.0	*< 0.01*
2 – 3.9 months	23.9	23.4	
4 – 5.9 months	10.6	13.4	
≥ 6 months	26.4	35.2	
Introduction of solid foods (%)			
< 3.9 months	10.1	8.3	*0.03*
4 – 4.9 months	62.2	60.9	
> 5 months	27.7	30.8	
TV watching >= 2 hours/day (%)	15.8	17.7	*< 0.01*

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the never breastfed group) were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables. Preterm birth was defined as gestational age < 37 weeks. Low birth weight was defined as birth weight < 2500 g. Small size for gestational age was defined as gestational age- and sex-adjusted birth weight < 10%.

Table S3.1.7. Associations of infant feeding with cardio-metabolic outcomes at the age of 6 years adjusted for age, sex and family-based socio-demographic factors

	Difference in outcome measure SDS (95% Confidence Interval)					
	Total- cholesterol N = 3,409	**HDL- cholesterol N = 3,417**	**LDL- cholesterol N = 3,417**	**Triglycerides N = 3,401**	**Insulin N = 3,385**	**C-peptide N=3,390**
Breastfeeding	-0.06 (-0.19, 0.07)	0.06 (-0.06, 0.19)	-0.05 (-0.18, 0.08)	-0.01 (-0.14, 0.12)	0.12 (-0.01, 0.25)	0.09 (-0.04, 0.22)
Never (N = 256)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Ever (N = 3,161)						
Duration	0.03 (-0.07, 0.13)	0.10 (-0.00, 0.20)	-0.05 (-0.15, 0.05)	0.00 (-0.10, 0.11)	0.05 (-0.06, 0.15)	0.05 (-0.05, 0.16)
0 – 1.9 months (N = 655)	0.06 (-0.05, 0.16)	0.10 (-0.00, 0.21)	0.03 (-0.08, 0.13)	0.01 (-0.10, 0.12)	0.05 (-0.06, 0.15)	0.09 (-0.02, 0.20)
2 – 3.9 months (N = 567)	0.10 (-0.02, 0.23)	0.05 (-0.07, 0.17)	0.08 (-0.05, 0.20)	0.04 (0.09, 0.16)	0.04 (-0.09, 0.16)	0.04 (-0.09, 0.17)
4 -5.9 months (N = 338)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
≥ 6 months (N = 859)	*0.55*	*0.03*	*0.40*	*0.99*	*0.34*	*0.21*
P for trend						
Exclusive for 4 months	-0.01 (-0.09, 0.08)	0.01 (-0.08, 0.09)	-0.02 (-0.11, 0.07)	-0.01 (-0.10, 0.08)	0.06 (-0.03, 0.15)	0.08 (-0.01, 0.17)
No (N = 1,799)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Yes (N = 712)						
First solid foods	0.12 (-0.05, 0.28)	0.06 (-0.10, 0.23)	0.07 (-0.10, 0.23)	-0.02 (-0.19, 0.14)	-0.00 (-0.16, 0.16)	0.00 (-0.16, 0.17)
< 3.9 months (N = 201)	-0.05 (-0.14, 0.05)	-0.04 (-0.13, 0.06)	-0.05 (-0.14, 0.05)	-0.00 (-0.10, 0.09)	-0.00 (-0.10, 0.09)	0.04 (-0.06, 0.13)
4-4.9 months (N = 1,375)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
> 5 months (N = 696)	*0.61*	*0.88*	*0.93*	*0.85*	*0.95*	*0.68*
P for trend	-0.06 (-0.19, 0.07)	0.06 (-0.06, 0.19)	-0.05 (-0.18, 0.08)	-0.01 (-0.14, 0.12)	0.12 (-0.01, 0.25)	0.09 (-0.04, 0.22)

Values are linear regression coefficients (95% confidence interval). Models are adjusted for child's age at visit, sex, plus family-based socio-demographic confounders (maternal age, ethnicity, education). Analyses with solid foods were additionally adjusted for breastfeeding duration. Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter.
* P < 0.05

Table S3.1.8. Associations of infant feeding with cardio-metabolic outcomes at the age of 6 years adjusted for age, sex and maternal life style related factors

	Difference in outcome measure SDS (95% Confidence Interval)					
	Total- cholesterol N = 3,409	**HDL- cholesterol N = 3,417**	**LDL- cholesterol N = 3,417**	**Triglycerides N = 3,401**	**Insulin N = 3,385**	**C-peptide N=3,390**
Breastfeeding	-0.06 (-0.19, 0.07)	0.06 (-0.07, 0.18)	-0.05 (-0.18, 0.08)	-0.01 (-0.14, 0.12)	0.10 (-0.03, 0.23)	0.08 (-0.05, 0.21)
Never (N = 256)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Ever (N = 3,161)						
Duration	0.02 (-0.08, 0.12)	0.11 (0.01, 0.22)*	-0.07 (-0.17, 0.04)	0.01 (-0.11, 0.10)	0.03 (-0.08, 0.14)	0.05 (-0.05, 0.16)
0 – 1.9 months (N = 655)	0.05 (-0.06, 0.15)	0.11 (-0.00, 0.21)	0.02 (-0.09, 0.13)	0.00 (-0.10, 0.12)	0.04 (-0.07, 0.15)	0.09 (-0.01, 0.20)
2 – 3.9 months (N = 567)	0.10 (-0.02, 0.23)	0.05 (-0.07, 0.17)	0.07 (-0.05, 0.20)	0.04 (-0.09, 0.16)	0.03 (-0.09, 0.16)	0.03 (-0.10, 0.15)
4 -5.9 months (N = 338)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
≥ 6 months (N = 859)	*0.68*	*0.02*	*0.28*	*0.85*	*0.53*	*0.30*
P for trend						
Exclusive for 4 months	-0.01 (-0.10, 0.07)	0.01 (-0.07, 0.10)	-0.03 (-0.12, 0.06)	-0.02 (-0.10, 0.07)	0.05 (-0.04, 0.14)	0.08 (-0.01, 0.17)
No (N = 1,799)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Yes (N = 712)						
First solid foods	0.11 (-0.05, 0.28)	0.07 (-0.09, 0.24)	0.06 (-0.10, 0.23)	-0.03 (-0.19, 0.14)	-0.02 (-0.19, 0.14)	-0.06 (-0.21, 0.11)
< 3.9 months (N = 201)	0.05 (-0.14, 0.05)	-0.04 (-0.13, 0.06)	-0.05 (-0.14, 0.05)	-0.00 (-0.10, 0.09)	-0.01 (-0.10, 0.09)	0.00 (-0.09, 0.10)
4-4.9 months (N = 1,375)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
> 5 months (N = 696)	*0.63*	*0.81*	*0.98*	*0.80*	*0.83*	*0.66*
P for trend	-0.06 (-0.19, 0.07)	0.06 (-0.07, 0.18)	-0.05 (-0.18, 0.08)	-0.01 (-0.14, 0.12)	0.10 (-0.03, 0.23)	0.08 (-0.05, 0.21)

Values are linear regression coefficients (95% confidence interval). Models are adjusted for child's age at visit, sex and height, life style related factors (pre-pregnancy BMI parity, smoking during pregnancy). Analyses with solid foods were additionally adjusted for breastfeeding duration. Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter.
* $P < 0.05$

Table S3.1.9. Associations of infant feeding with cardio-metabolic outcomes at the age of 6 years adjusted for age, sex, and childhood factors

	Difference in outcome measure SDS (95% Confidence Interval)					
	Total- cholesterol N = 3,409	**HDL- cholesterol N = 3,417**	**LDL- cholesterol N = 3,417**	**Triglycerides N = 3,401**	**Insulin N = 3,385**	**C-peptide N=3,390**
Breastfeeding						
Never (N = 256)	-0.06 (-0.20, 0.05)	0.03 (-0.09, 0.16)	-0.04 (-0.16, 0.08)	-0.01 (-0.14, 0.12)	0.11 (-0.02, 0.23)	0.10 (-0.02, 0.23)
Ever (N = 3,161)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Duration						
0 – 1.9 months (N = 655)	0.04 (-0.07, 0.14)	0.08 (-0.02, 0.18)	-0.03 (-0.13, 0.07)	0.01 (-0.09, 0.11)	0.02 (-0.08, 0.12)	0.02 (-0.08, 0.13)
2 – 3.9 months (N = 567)	0.06 (-0.05, 0.16)	0.09 (-0.02, 0.19)	0.04 (-0.07, 0.14)	0.02 (-0.09, 0.13)	0.04 (-0.07, 0.15)	0.09 (-0.02, 0.19)
4 -5.9 months (N = 338)	0.10 (-0.03, 0.22)	0.04 (-0.08, 0.17)	0.07 (-0.05, 0.20)	0.04 (0.09, 0.17)	0.03 (-0.10, 0.15)	0.04 (-0.09, 0.17)
≥ 6 months (N = 859)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.48*	*0.08*	*0.61*	*0.88*	*0.61*	*0.44*
Exclusive for 4 months						
No (N = 1,799)	-0.01 (-0.09, 0.08)	0.01 (-0.08, 0.10)	-0.01 (-0.10, 0.07)	-0.02 (-0.11, 0.07)	0.03 (-0.05, 0.12)	0.05 (-0.04, 0.14)
Yes (N = 712)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
First solid foods						
< 3.9 months (N = 201)	0.09 (-0.04, 0.28)	0.06 (-0.10, 0.22)	0.08 (-0.08, 0.25)	-0.02 (-0.18, 0.14)	-0.08 (-0.24, 0.08)	-0.06 (-0.22, 0.10)
4-4.9 months (N = 1,375)	-0.04 (-0.13, 0.06)	-0.02 (-0.11, 0.07)	-0.04 (-0.13, 0.05)	-0.01 (-0.10, 0.09)	-0.03 (-0.13, 0.06)	0.01 (-0.09, 0.10)
> 5 months (N = 696)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.51*	*0.78*	*0.75*	*0.81*	*0.31*	*0.67*

Values are linear regression coefficients (95% confidence interval). Models are adjusted for child's age at visit, sex, and childhood factors (gestational age at birth, birth weight and TV watching and body mass index). Analyses with solid foods were additionally adjusted for breastfeeding duration. Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter. * $P < 0.05$

Chapter 3.2

Influence of breastfeeding on retinal vessel calibers in school-age children.

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Abstract

Background: A shorter breastfeeding duration is associated with an increased risk of cardiovascular disease in adulthood. Early microvasculature structure adaptations may be part of the underlying mechanism. We examined the associations of infant feeding patterns with microvasculature in children.

Methods and Results: In this population-based prospective cohort study in 3,220 children, information on breastfeeding duration and exclusivity and age at introduction of solid foods was obtained from postal questionnaires at the ages of 2, 6 and 12 months after birth. At the median age of 6.0 years (90% range 5.7 – 6.8), we measured retinal arteriolar and venular calibers from digitized retinal photographs. We observed that in the models only adjusted for child's age, sex and ethnicity, children who were never breastfed had narrower retinal arteriolar and venular calibers in childhood, as compared to children who were breastfed (all p-value < 0.05). After additional adjustment for maternal and childhood socio-demographic and lifestyle-related characteristics, never breastfeeding was only associated with narrower retinal venular caliber (difference: -0.15 SDS (95% CI -0.29, -0.02)). We did not observe associations of breastfeeding duration and exclusivity or age at introduction of solid foods with retinal vessel calibers.

Conclusions: Children who were never breastfed tended to have narrower retinal venular calibers. We did not observe associations of breastfeeding duration with retinal vessel calibers. These results should be considered as hypothesis generating for further observational and experimental studies.

Introduction

A shorter breastfeeding duration and non-exclusivity in infancy have been associated with higher risks of hypertension in adulthood.[1-4] Studies in children also suggested that shorter duration of breastfeeding and early age at introduction of solid foods are related to higher blood pressure in childhood.[5-7] Moreover, recent recommendations from the National Health, Lung and Blood Institute and World Health Organization have suggested that breastfeeding for the first 6 months of life have a strong beneficial effect on cardiovascular health. The mechanisms underlying the associations of breastfeeding patterns and cardiovascular disease are not known, but might involve effects of specific ingredients and their combination within breast milk (including enzymes, hormones, growth factors and long chain polyunsaturated fatty acids) on early microvascular development.[8] Microvasculature can non-invasively be assessed by advances in retinal photography.[9] Previous studies among children and adults have shown that retinal arteriolar narrowing is associated with higher blood pressure and increased risks of hypertension.[10, 11] Studies in children are important, in order to understand the early structural processes and because of the limited influences of lifestyle and cardiovascular risk factors on micro-vessels structure. Thus far, no previous study has examined the associations of infant breastfeeding with microvasculature adaptations in later life.

Therefore, we examined in a population-based cohort study among 3,220 school-age children, the associations of breastfeeding duration and exclusiveness, and age at introduction of solid foods with retinal vessel calibers. We further explored whether any association was explained by maternal or childhood socio-demographic and lifestyle-related factors.

Methods

Study design

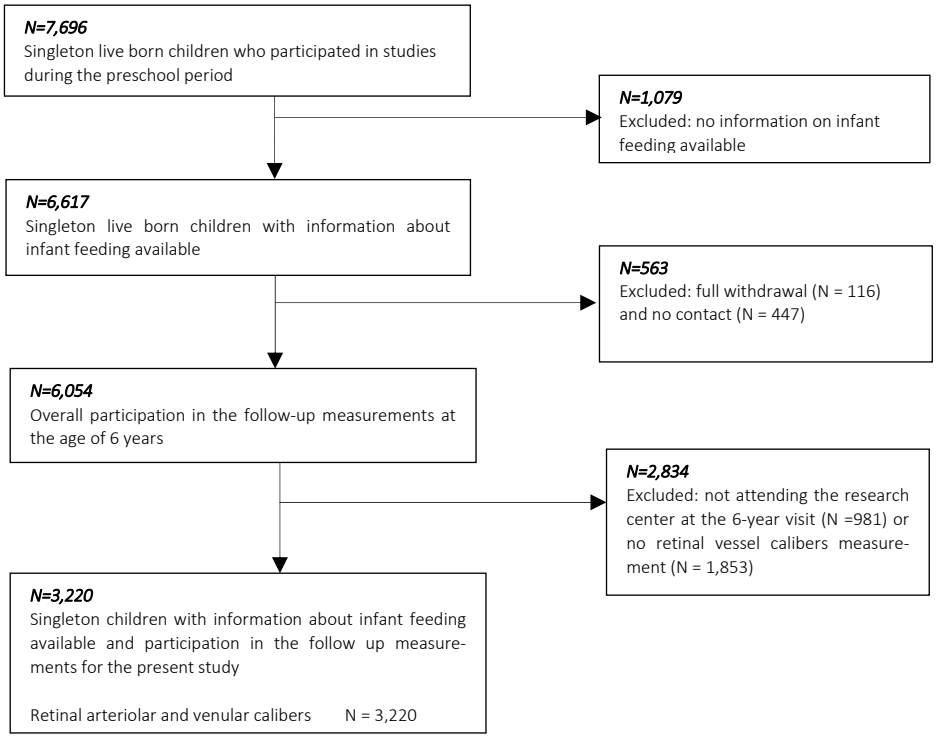
This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[12, 13] Enrolment in the study was aimed in first trimester but was allowed until the birth of the child. The local Medical Ethical Committee approved the study. Written consent was obtained from parents. All children were born between 2002 and 2006. Response rate at baseline was 61%.[12] Information on breastfeeding was available in 6,616 singleton live born children. Of these children, 6,054 participated in the follow-up measurements until the median age of 6.0 years (90% range 5.7 – 6.8), of whom 3,220 participated in the retinal vessel measurements for the current study (**Figure 3.2.1**). Missing retinal vessel measurements were mainly due to the start of these measurements subsequent to the children participating in the respective follow-up visit.

Infant feeding assessments

Information on breastfeeding initiation and continuation was obtained from delivery reports and postal questionnaires at the ages of 2, 6 and 12 months after birth, as previously described.[5, 12, 14] Briefly, mothers were asked whether they ever breastfed their child (yes, no) and at what age they quitted breastfeeding. Duration of exclusive breastfeeding was defined by using information about at what age other types of milk and/or

solids were introduced in the first 6 months of life, according to a short food frequency questionnaire. Among breastfed children, breastfeeding duration was categorized into four groups: 1) >0-1.9 months; 2) 2-3.9 months; 3) 4-5.9 months and 4) >= 6 months. The information on exclusiveness of breastfeeding was categorized into the following two categories: 1) non-exclusive until 4 months; and 2) exclusive until 4 months. 'Non-exclusive until 4 months' indicates infants who received both breastfeeding and formula feeding or solids at any stage during the first 4 months. 'Exclusive until 4 months' indicates infants who have been breastfed, without any other milk, solids or fluids during the first 4 months. The analyses focused on breastfeeding duration and exclusivity were performed after exclusion of never breastfed children. Information on introduction of solid foods included fruit and vegetable snacks, and was obtained from the same food frequency questionnaire. Age at introduction of solid foods was categorized as: 1) <3.9 months; 2) 4-4.9 months; and 3) >5 months.

Figure 3.2.1. Flow chart of participants in study



Retinal microvasculature assessment

At the age of 6 years retinal photographs were taken by a well-trained staff in a dedicated research center. Digital retinal photographs centered on the optic disc were taken in one eye with images resolution 4096 and 3072 pixels, using Topcon digital retinal camera (model TRC, NW300). We use the same methods and protocols to measure retinal vascular caliber from the digitized retinal photographs, as described in previous

studies among adults and children.[15, 16] Briefly, a semi-automatic computer imaging program was used to measure the caliber of the 6 largest retinal arterioles and venules located one half to 1 disc diameter from the optic disc margin.[17] Using the revised Knudtson-Parr-Hubbard formula, absolute arteriolar and venular diameters were estimated in micrometers and subsequently were summarized as central retinal arteriolar and venular equivalents, representing the average arteriolar and venular calibers of that eye, respectively.[18] Graders, masked to birth parameters and other participant characteristics, operated the computer program and monitored all retinal measurements. We constructed grader specific standard deviation scores for both central retinal and arteriolar equivalents. Interclass correlation coefficients between graders were 0.79 for retinal arteriolar caliber and 0.89 for retinal venular caliber, which suggest adequate reproducibility.

Covariates

We collected information about maternal age, parity, educational level, pre-pregnancy body mass index, smoking during pregnancy and folic acid supplement use by questionnaires. Maternal blood pressure was assessed at enrollment and pregnancy complications (hypertensive disorders, gestational diabetes) were obtained from medical records. Child's ethnicity (European, Non-European) was classified by the countries of birth of the parents.[19] Information on average TV watching time was assessed by questionnaire. At the age of 6 years, we measured height by a Harpenden stadiometer (Holtain Limited, Dyfed, UK), and weight with a mechanical personal scale (SECA, Almere, The Netherlands), and we calculated BMI. Systolic and diastolic blood pressure was measured at the right brachial artery, four times with one-minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus TM (Paramus, NJ, USA) and we used the mean of the last three blood pressure measurements for the analyses.[20]

Statistical analysis

First, we compared subject characteristics between ever breastfeeding categories using One-Way ANOVA tests for continued variables, and Chi-square tests for categorical variables. We also compared the subject characteristics between children included and not included in the analyses. Second, we explored the associations of breastfeeding duration and exclusivity and the age at introduction of solid foods with retinal vessel calibers at the age of 6 years by using multiple linear regression models. These models were first adjusted for child's age at visit, sex and ethnicity (crude models) and subsequently additionally for potential confounders selected based on previous publication, an association with outcomes, or a change in effect estimates of >10%. We tested the role of: (1) maternal socio-demographic and lifestyle-related characteristics (maternal age, ethnicity, education, pre-pregnancy body mass index, parity, blood pressure at intake, smoking and folic acid supplement use during pregnancy, and pregnancy complications); and (2) childhood factors (gestational age at birth, birth weight, TV watching and BMI and blood pressure at visit). Tests for trends were performed by treating the categorized breastfeeding variable as a continuous term. A sensitivity analysis performed in European mothers only showed similar results as for the total group (data not shown). We did not observe significant interaction terms between the breastfeeding

and solid food category strata and child's sex, gestational age and weight at birth, and maternal education level (P-value for interaction > 0.05) in relation to outcomes. We performed multiple imputations for missing values of covariates (<25% missing values). Five datasets were created and analyzed together.[21] Statistical analyses were performed using the Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Table 3.2.1 shows that of all children, 92.6% (N = 2,982) had ever been breastfed of whom 23.7% had been exclusively breastfeed until 4 months. As compared to mothers who never breastfed their children, mothers who ever breastfed their children were higher educated, had a lower systolic blood pressure, and smoked less during pregnancy (P-values < 0.01). Non-response analyses showed that children who did not participate in the follow-up measurements at the age of 6 years were less frequently breastfed and breastfed for a shorter period, compared to children included in this study (Supplemental Table S3.2.1).

Infant feeding and microvasculature in childhood

Table 3.2.2 shows that in the models adjusted for child's age, sex and ethnicity, never breastfeeding was associated with narrower retinal arteriolar and venular calibers in childhood (differences in retinal arteriolar and venular calibers, respectively: -0.16 SDS (95% CI -0.29, -0.03) and -0.18 SDS (95% CI -0.32, -0.04)). As compared to duration of breastfeeding longer than 6 months, duration of breastfeeding from 2 to 3.9 months was associated with narrower retinal arteriolar and venular calibers (p-values<0.05). However, non-significant trend estimates were present for these associations. Exclusive breastfeeding was not associated with childhood retinal vessel calibers. As compared to age of introduction of solid foods after 5 months of age, earlier introduction of solid food was not associated with childhood retinal vessel calibers. After adjustment for maternal socio-demographic and lifestyle-related characteristics and childhood factors, children who were never breastfed had narrower retinal venular caliber (difference: -0.15 SDS (95% CI -0.29, -0.02)), but not different retinal arteriolar caliber.

No associations of breastfeeding duration and exclusivity, and age at introduction of solid foods with childhood retinal vessel calibers were present in the fully adjusted models. Models adjusted for maternal socio-demographic and lifestyle-related characteristics and childhood factors separately are given in Supplemental Tables S3.2.2 and S3.2.3. Introduction of solid food after 6 months was also not associated with retinal vessel calibers in childhood (data not shown).

Table 3.2.1. Subject characteristics according to category of breastfeeding (N = 3,220)

Characteristics	Total group N = 3,220	Never breastfed N = 238	Ever breastfed N = 2,982	P value
Maternal characteristics				
Age, median (95% range), y	31.5 (20.2 – 40.0)	31.5 (20.2 – 40.3)	31.4 (20.2, 39.9)	*0.70*
Gestational age at intake, median (95% range), weeks	13.6 (9.8, 25.8)	13.6 (9.8, 30.1)	13.6 (9.8, 25.4)	*0.64*
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.6 (18.2, 34.7)	22.8 (18.3, 36.9)	22.6 (18.2, 34.6)	*0.47*
Systolic blood pressure, mean (SD), mmHg	115.5 (12.0)	117.9 (13.2)	115.3 (11.9)	*<0.01*
Diastolic blood pressure, mean (SD), mmHg	68.1 (9.5)	69.1 (10.6)	68.1 (9.4)	*0.17*
Parity, nulliparous (%)	56.6	47.8	57.3	*<0.01*
Education, higher (%)	52.2	32.4	53.7	*<0.01*
Folic acid use, never (%)	21.1	19.8	21.2	*0.36*
Smoked during pregnancy, Yes (%)	24.0	34.6	23.1	*<0.01*
Gestational diabetes, Yes, (%)	0.7	0.4	0.8	*0.49*
Gestational hypertension, Yes, (%)	4.3	5.6	4.3	*0.37*
Preeclampsia, Yes, (%)	1.9	2.6	1.8	*0.28*
Birth characteristics				
Boys (%)	49.5	51.7	49.6	*0.73*
Gestational age at birth, median (95% range),weeks	40.1 (36.0 – 42.3)	39.9 (35.9 – 42.2)	40.1 (36.3, 42.3)	*<0.01*
Birth weight, mean (SD), grams	3 462 (540)	3,420 (560)	3470 (533)	*0.16*
Ethnicity, European (%)	67.4	79.1	66.5	*<0.01*
TV watching >= 2 hours per day (%)	16.7	21.4	16.4	*0.05*
Introduction of solid foods (%)				
< 3.9 months	8.6	15.2	8.1	
4 – 4.9 months	60.1	67.7	59.5	*<0.01*
> 5 months	31.3	17.1	32.4	
Childhood characteristics				
Age at visit, median (95% range), years	6.0 (5.7 – 7.6)	6.0 (5.7 – 7.4)	6.0 (5.7, 7.6)	*0.79*
Body mass index, mean (SD), kg/m^2	16.1 (1.8)	16.2 (1.9)	16.1 (1.8)	*0.67*
Systolic blood pressure, mean (SD), mmHg	102.7 (8.0)	103.3 (8.5)	102.6 (8.0)	*0.20*
Diastolic blood pressure, mean (SD), mmHg	60.7 (6.7)	61.2 (6.8)	60.7 (6.7)	*0.20*
Retinal arteriolar caliber, mean (SD), µm	159.4 (14.9)	157.3 (13.9)	159.6 (15.0)	*0.02*
Retinal venial caliber, mean (SD), µm	219.2 (20.3)	216.0 (19.4)	219.5 (20.3)	*0.01*

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the never breastfed group) were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables.

Table 3.2.2. Associations of infant feeding with retinal vessel calibers at the age of 6 years adjusted for child age, sex and ethnicity (N = 3,220)

	Retinal vessel calibers in SDS (95% Confidence Interval)			
	Arteriolar caliber		Venular caliber	
	Basic model N = 3,220	Full model N = 3,220	Basic model N = 3,220	Full model N = 3,220
Breastfeeding				
Never (N = 238)	-0.16 (-0.29, -0.03)*	-0.09 (-0.22, 0.04)	-0.18 (-0.32, -0.04)*	-0.15 (-0.29, -0.02)*
Ever (N = 2,982)	*Reference*	*Reference*	*Reference*	*Reference*
Breastfeeding duration				
>0 – 1.9 months (N = 613)	-0.05 (-0.10, 0.00)	-0.03 (-0.13, 0.08)	-0.03 (-0.08, 0.03)	-0.04 (-0.15, 0.07)
2 – 3.9 months (N =549)	-0.08 (-0.14, -0.02)*	-0.08 (-0.19, 0.03)	-0.08 (-0.13, -0.02)*	-0.08 (-0.19, 0.03)
4 – 5.9 months (N = 314)	0.03 (-0.09, 0.15)	0.03 (-0.10, 0.16)	0.13 (0.00, 0.25)	0.13 (-0.01, 0.26)
≥ 6 months (N = 831)	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.21*	*0.34*	*0.27*	*0.23*
Exclusive breastfeeding for 4 months				
No (N = 1,681)	-0.07 (-0.16, 0.02)	-0.04 (-0.13, 0.05)	-0.03 (-0.11, 0.05)	-0.02 (-0.11, 0.08)
Yes (N = 708)	*Reference*	*Reference*	*Reference*	*Reference*
Age at first solid foods				
< 4 months (N = 192)	-0.03 (-0.19, 0.14)	0.05 (-0.12, 0.21)	0.05 (-0.03, 0.14)	0.09 (-0.09, 0.25)
4 - 4.9 months (N = 1,347)	-0.05 (-0.14, 0.05)	0.00 (-0.09, 0.09)	-0.01 (-0.10, 0.09)	0.01 (-0.09, 0.10)
>= 5 months (N = 700)	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.54*	*0.68*	*0.69*	*0.45*

Values are linear regression coefficients (95% confidence interval). Basic models are adjusted for child's age at visit, sex and ethnicity. Full models are additionally adjusted for maternal socio-demographic and lifestyle-related characteristics (maternal age, education, pre- BMI, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, pregnancy complications) and childhood factors (gestational age at birth and birth weight, TV watching and current BMI and blood pressure). Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter. Models adjusted for maternal socio-demographic and lifestyle-related characteristics and childhood factors separately are given in Supplemental Tables S3.2.2 and S3.2.3.

* $P < 0.05$

Discussion

Results from this population-based prospective study show that children who were never breastfed tended to have narrower retinal vessel calibers, this was particularly the case for venular calibers. Breastfeeding duration and exclusivity and age at introduction of solid foods were not associated with retinal vessel calibers.

Methodological considerations

We used a population-based prospective cohort design including a large number of subjects whom we studied from early fetal life onwards. Of the total group of singleton live born children, information on breastfeeding was available in 80%. We do not expect that this non-response at baseline led to biased effect estimates because biased estimates in large cohort studies mainly arise from loss to follow-up rather than from non-response at baseline.[22] Of all children with information on breastfeeding, 53% had retinal vessel measurements available. Missing retinal vessel measurements were mainly due to the later start of these measurements during the follow-up visits. This loss to follow-up would lead to selection bias if the associations of infant feeding with childhood retinal vessel calibers would differ between those included and not included in the final analyses. We observed that children who did not participate in the follow-up measurements at the age of 6 years were less frequently breastfed and breastfed for a shorter period. This might have led to an underestimation of the effect estimates. Assessing breastfeeding initiation and duration and introduction of solid foods by questionnaires may lead to measurement error. However, this method seems to be valid in population-based samples, especially when recall spans over a short period (<3 years), which is the case in our study. [23, 24] Also, we used three questionnaires to assess infant feeding during the first 12 months. Finally, although we performed adjustment for a large number of potential maternal and infant confounders, residual confounding might still occur, as in any observational study. For example, we could not adjust for detailed measures of childhood diet, as this information was available only in a small subgroup of the study population.

Interpretation of main findings

Several studies in the last decades have evaluated whether breastfeeding has an effect on cardiovascular development in later life.[25-28] A meta-analyses among 17,503 subjects reported a reduction in diastolic blood pressure among breastfed infants, whereas not all studies found a lower systolic blood pressure among breastfed infants.[1] This inconsistency was mainly explained by differences in adjustment for socioeconomic status or maternal antenatal factors. Previously, we have shown that never breastfeeding was associated with a higher carotid-femoral pulse wave velocity and lower left ventricular mass, whereas an early age of introduction of solid foods was associated with higher systolic and diastolic blood pressure.[5] These associations were not explained by maternal lifestyle-related characteristics or childhood birth characteristics. Another small study among 545 children also showed that early introduction of solid foods is associated with higher systolic and diastolic blood pressure at 7.2 years of age.[29] However, this

study did not adjust extensively for potential confounding factors. Early microvasculature structure alterations might be involved in the mechanisms underlying the associations of breastfeeding patterns with an adverse cardiovascular profile. It has been shown among children and adults that microvasculature alterations, measured by retinal photographs, precede the development of hypertension, and can therefore be used as an early marker of cardiovascular disease risk.[10, 15]

In this study, we found that never breastfeeding tended to be associated with narrower retinal arteriolar and venular calibers. After adjustment for maternal socio-demographic and lifestyle-related characteristics and childhood factors significant associations were present only for narrower retinal venular caliber. Our results showed no associations of breastfeeding duration and exclusivity and age of introduction of solid foods with retinal vessel calibers. To the best of our knowledge no previous study examined the associations of breastfeeding patterns with retinal vessel calibers in later life. A study among 159 children aged 11-14 years showed that endothelial function of skin microvasculature was significantly better among breastfed children, as compared to the ones fed with infant formula.[30] These associations were present independent from family-based socio-demographic confounders. Another study among 1667 young adults found that breastfed men have better brachial endothelial function compared to formula fed men.[31]

The mechanisms that could explain structural and functional microvasculature alterations due to breastfeeding remain unclear. Compared to formula milk, human breast milk is rich in long-chain polyunsaturated fatty acids (LC-PUFAs). The endothelial system uses LC-PUFAs for its structural and functional integrity.[32] Higher LC-PUFAs concentrations in children can inhibit the production of inflammatory markers such as TNF-α and C-reactive protein and enhance the synthesis of nitric oxide (NO), which can therefore inhibit endothelial dysfunction.[32] Thus, narrower retinal vessel calibers among never breastfed infant might reflect structural microvasculature changes and pathophysiological processes related to endothelial function, due to lower LC-PUFAs concentrations. However, the observed associations in our study may also be explained by residual confounding due to missing detailed information on childhood diet and further lifestyle factors. Results from a randomized control trial suggested that longer breastfeeding duration and exclusive breastfeeding among healthy term infants, did not influence cardiovascular risk factors, including blood pressure, fasting insulin, and the presence of metabolic syndrome, at the age of 11.5 years.[7] In addition, taking into account the number of tests performed, our findings may also reflect chance findings. We did not apply Bonferroni correction for multiple testing as the different studied exposure and outcome variables are related. Therefore, further studies are needed to replicate these observations and to investigate which components of breast-milk may influence retinal vessel calibers and whether the observed small differences are related to adverse cardiovascular outcomes in later life.

Conclusions

In this population-based prospective cohort study, we observed that children who were never breastfeed tended to have narrower retinal vessel calibers. Breastfeeding duration and exclusivity and age at introduction of solid foods were not associated with

retinal vessel calibers. Further studies are needed to replicate our findings and to study whether breast-milk composition or infant feeding habits may have long-term benefits for microvasculature development.

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Supplemental Material

Table S3.2.1. Comparison of subject characteristics between children included and not included in the analyses

Characteristics	Participation N=3,220	Non-participation N=2,834	P value
Maternal characteristics			
Age, median (95% range), y	31.5 (20.2 – 40.0)	31.2 (20.0 – 39.5)	*0.01*
Gestational age at intake, median (95% range), weeks	13.6 (9.8, 25.8)	13.6 (9.8, 24.2)	*0.74*
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.6 (18.2, 34.7)	22.5 (18.1, 36.7)	*<0.01*
Systolic blood pressure, mean (SD), mmHg	115.5 (12.0)	115.7 (12.1)	*0.52*
Diastolic blood pressure, mean (SD), mmHg	68.1 (9.5)	68.0 (9.3)	*0.55*
Parity, nulliparous (%)	56.6	55.8	*0.46*
Education, higher (%)	52.2	51.9	*0.53*
Folic acid use, never (%)	21.1	22.1	*0.24*
Smoked during pregnancy, Yes (%)	24.0	23.6	*0.38*
Gestational diabetes, Yes, (%)	0.7	0.9	*0.23*
Gestational hypertension, Yes, (%)	4.3	3.6	*0.11*
Preeclampsia, Yes, (%)	1.9	2.1	*0.29*
Birth characteristics			
Boys (%)	49.5	50.1	*0.18*
Gestational age at birth, median (95% range),weeks	40.1 (36.0 – 42.3)	40.1 (35.9 – 42.3)	*0.08*
Birth weight, mean (SD), grams	3 462 (540)	3,435 (555)	*0.02*
Ethnicity, European (%)	67.4	70.5	*0.01*
Breastfeeding, yes (%)			
TV watching >= 2 hours per day (%)	16.7	16.9	*0.46*
Introduction of solid foods (%)			
< 3.9 months	8.6	8.1	*0.50*
4 – 4.9 months	60.1	61.9	
> 5 months	31.3	30.0	

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics were evaluated using ANOVA for continuous variables, and Chi-squared tests for categorical variables.

Table S3.2.2. Associations of infant feeding with retinal vessel calibers at the age of 6 years adjusted for child age, sex, ethnicity and maternal factors (N = 3,220)

	Retinal vessel calibers in SDS (95% Confidence Interval)	
	Arteriolar caliber N = 3,220	**Venular Caliber N = 3,220**
Breastfeeding		
Never (N = 238)	-0.11 (-0.25, 0.02)	-0.15 (-0.29, -0.02)*
Ever (N = 2,982)	*Reference*	*Reference*
Breastfeeding duration		
>0 – 1.9 months (N = 613)	-0.03 (-0.14, 0.08)	-0.03 (-0.14, 0.08)
2 – 3.9 months (N =549)	-0.08 (-0.19, 0.03)	-0.08 (-0.19, 0.03)
4 – 5.9 months (N = 314)	0.03 (-0.10, 0.16)	0.13 (-0.01, 0.26)
≥ 6 months (N = 831)	*Reference*	*Reference*
P for trend	*0.37*	*0.26*
Exclusive breastfeeding for 4 months		
No (N = 1,681)	-0.06 (-0.15, 0.04)	-0.03 (-0.17, 0.06)
Yes (N = 708)	*Reference*	*Reference*
Age at first solid foods		
< 4 months (N = 192)	0.04 (-0.1, 0.20)	0.07 (-0.10, 0.24)
4 - 4.9 months (N = 1,347)	-0.02 (-0.12, 0.07)	-0.01 (-0.10, 0.09)
>= 5 months (N = 700)	*Reference*	*Reference*
P for trend	*0.94*	*0.51*

Values are linear regression coefficients (95% confidence interval). Models are adjusted for child's age at visit, sex and ethnicity, and additionally for maternal socio-demographic and lifestyle-related characteristics (maternal age, education, pre-BMI, parity, blood pressure at intake, folic acid use, maternal smoking during pregnancy, pregnancy complications). Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter. * P < 0.05

Table S3.2.3. Associations of infant feeding with retinal vessel calibers at the age of 6 years adjusted for child age, sex, ethnicity and childhood factors (N = 3,220)

	Retinal vessel calibers in SDS (95% Confidence Interval)	
	Arteriolar caliber N = 3,220	**Venular Caliber N = 3,220**
Breastfeeding		
Never (N = 238)	-0.11 (-0.24, 0.01)	-0.18 (-0.31, -0.05)*
Ever (N = 2,982)	*Reference*	*Reference*
Breastfeeding duration		
>0 – 1.9 months (N = 613)	-0.02 (-0.13, 0.08)	-0.03 (-0.13, 0.08)
2 – 3.9 months (N =549)	-0.07 (-0.18, 0.03)	-0.08 (-0.18, 0.03)
4 – 5.9 months (N = 314)	0.03 (-0.09, 0.16)	0.13 (0.01, 0.25)*
≥ 6 months (N = 831)	*Reference*	*Reference*
P for trend	*0.38*	*0.27*
Exclusive breastfeeding for 4 months		
No (N = 1,681)	-0.04 (-0.13, 0.05)	-0.02 (-0.11, 0.07)
Yes (N = 708)	*Reference*	*Reference*
Age at first solid foods		
< 4 months (N = 192)	0.02 (-0.14, 0.18)	0.07 (-0.09, 0.24)
4 - 4.9 months (N = 1,347)	-0.01 (-0.10, 0.08)	0.01 (-0.10, 0.09)
>= 5 months (N = 700)	*Reference*	*Reference*
P for trend	*0.91*	*0.56*

Values are linear regression coefficients (95% confidence interval). Models are adjusted for child's age at visit, sex and ethnicity and additionally for childhood factors (gestational age at birth and birth weight, TV watching and childhood BMI and blood pressure). Non-exclusive breastfeeding until 4 months includes partial until 4 months, partial thereafter; and partial until 4 months, not thereafter. Exclusive breastfeeding until 4 months includes exclusive until 6 months; exclusive until 4 months, partial thereafter; exclusive until 4 months, not thereafter. * P < 0.05

Chapter 3.3

Body fat distribution and cardiovascular risk factors in childhood

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Abstract

Background: More specific total body and abdominal fat mass measures might be stronger associated with cardiovascular risk factors in childhood, than body mass index. We examined the independent associations of total and abdominal fat measures with cardiovascular risk factors in school age children.

Methods and Results: We performed a population-based cohort study among 6,523 children. At the age of 6 years we measured childhood body mass index, and general and abdominal fat mass, using Dual-energy X-ray Absorptiometry and ultrasound and cardiovascular risk factors. Conditional on body mass index, higher fat mass percentage and abdominal fat mass were associated with higher blood pressure, total- and LDL-cholesterol, insulin and c-peptide levels, but with lower left ventricular mass and HDL-cholesterol (p-values<0,05). These associations differed between underweight, normal weight, overweight and obese children. Higher childhood adiposity measures were associated with increased odds of cardiovascular risk factors clustering, with the strongest effect for fat mass percentage (OR: 3.01 (95% CI 2.67, 3.9).

Conclusions: Our results suggest that general and abdominal fat measures are associated with cardiovascular risk factors in childhood, independent from body mass index. These measures may provide additional information for identification of children with an adverse cardiovascular profile.

Introduction

Childhood obesity is a major public health problem.[1] It seems that not only overweight and obesity, but also higher body mass across the full range is associated with risk factors for cardiovascular and metabolic diseases in childhood and adulthood.[2-5] Body mass index (BMI) does not distinguish lean mass from fat mass.[6] Among adults and children, total body fat mass assessed by Dual-energy X-ray Absorptiometry (DXA) seems to be, independent from body mass index, associated with cardiovascular risk factors.[3, 7] Also, waist circumference, as proxy for abdominal fat mass, was independent of body mass index related to the risk of mortality in adults, suggesting that central or abdominal adiposity is more strongly associated with adverse health outcomes.[4, 8] Abdominal fat mass is an accumulation of both subcutaneous and visceral adipose tissues. In adults, visceral adipose tissue accumulation is stronger related with an adverse glucose and lipid profile than subcutaneous adipose tissue accumulation.[8] Thus far, population-based studies focused on the associations of different detailed total body and abdominal fat mass measures with cardiovascular risk factors in children show inconsistent results. Abdominal fat mass in children has been identified as a stronger predictor of cardiovascular risk factors, as compared to body mass index, though results are not consistent.[2, 3, 9] These inconsistent findings may be explained by different measures of fat mass and variation of children's age included in these studies.

Therefore, we examined in a population-based cohort study among 6,523 school-age children, the independent associations of body mass index and total body and abdominal fat mass measures with risk factors for cardiovascular disease.

Methods

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards in Rotterdam, the Netherlands.[10] The study was conducted according to the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam (MEC 198.782/2001/31). In total, 9,778 mothers with a delivery date from April 2002 until January 2006 were enrolled in the study. Response rate at baseline was 61%. Written consent was obtained from parents. 8,305 children participated in follow-up studies at the age of 6 years (median 6.0 years (95% range 5.6, 7.9 years)), of whom 6,523 (78%) children participated in detailed cardiovascular follow-up measurements and 4,294 (66%) had blood samples available. This lower number for blood samples is mainly due to non-consent for venous puncture. Children who did not participate in the follow-up measures at 6 years had a lower gestational age at birth and lower birth weight (Supplemental **Table S3.3.6**, (online)).

Childhood assessments

At the age of 6 years we measured height and weight without shoes and heavy clothing. Weight was measured to the nearest gram using an electronic scale (SECA 888, Almere,

The Netherlands). Height was measured to the nearest 0.1 cm by a stadiometer (Holtain Limited, Crosswell, Crymych, UK). Body mass index (kg/m^2) was calculated. Childhood underweight, normal weight, overweight and obesity were defined by the International Obesity Task Force cut offs.[11]

Total body and regional fat mass percentages were measured using DXA (iDXA, GE-Lunar, 2008, Madison, WI), and analysed with the enCORE software v.12.6.[7] iDXA can accurately detect whole-body fat mass within less than 0.25% coefficient of variation. Children were placed without shoes, heavy clothing and metal objects in supine position on the DXA table. Fat mass percentage (%) was calculated as percentage of total body weight (kg) measured by DXA. We calculated the ratio of android and gynoid fat mass. The android/gynoid fat ratio reflects the central body fat distribution in the abdomen (android) and hip (gynoid) regions.[7]

Abdominal examinations were performed with ultrasound, as described in detail before [12]. Briefly, preperitoneal and subcutaneous fat thicknesses were measured with a linear (L12-5 MHz) transducer[13], which was placed perpendicular to the skin surface on the median upper abdomen. We scanned longitudinally just below the xiphoid process to the navel along the midline (linea alba). All measurements were performed off-line. Subcutaneous fat mass distance (SC-distance) was measured as distance of the inner surface of subcutaneous tissue to the linea alba. Preperitoneal fat mass distance (PP-distance) was measured as distance of the linea alba to the peritoneum on top of the liver. Subcutaneous and preperitoneal fat mass areas were measured as areas of 2 cm length along the midline starting from the maximum preperitoneal distance in direction of the navel (SC-area, PP-area). We measured three times the areas of 2 cm length along midline, and we used the mean value of these measures. The intra-observer reproducibility and the intra-class correlation coefficients ranged from 0.93 to 0.97.

Blood pressure was measured at the right brachial artery four times with one-minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus TM (Paramus, NJ).[14] We calculated the mean value for systolic and diastolic blood pressure using the last three blood pressure measurement of each participant. Echocardiography measurements were performed using methods recommended by the American Society of Echocardiography, and used to calculate the left ventricular mass.[15, 16]

Thirty-minutes fasting blood samples were collected to measure total-, HDL-, and LDL-cholesterol, triglycerides, insulin and C-peptide concentrations, using Cobas 8000 analyser (Roche, Almere, The Netherlands). Quality control samples demonstrated intra and inter assay coefficients of variation ranging from 0.77-1.39%, and 0.87-2.40%, respectively.

We defined hypertension as systolic and diastolic blood pressure above the 95th percentile, using age- and height-specific cut-points.[17] Recommendations from National Cholesterol Education Program for children age 2-9 years were used to define adverse levels of cholesterol; high cholesterol (>5.1 mmol/l).[18] For defining children with clustering of cardiovascular risk factors, we used the previously described definition of childhood metabolic syndrome phenotype [19], which means having three or more of the following components: android fat mass % =>75th percentile; systolic or diastolic blood pressure =>75th percentile; HDL-cholesterol <=25th percentile or triglycerides => 75th

percentile; and insulin level =>75th percentile. We used android fat mass as percentage of total body fat mass, as proxy for waist circumference.

Statistical analysis

First, we compared childhood characteristics between different childhood obesity categories using One-Way ANOVA tests. We examined the correlations between all childhood adiposity and cardiovascular outcomes using Pearson or Spearman rank correlation coefficients. Second, we assessed the associations of childhood fat measures with cardiovascular risk factors using different linear regression models. Model 1 was adjusted for child's age at measurements, sex, ethnicity and height. Model 2 was additionally adjusted for child's current body mass index. For these analyses, we log-transformed not normally distributed abdominal fat mass measures and cardiovascular risk factors. We constructed standard deviation scores ((observed value- mean)/SD) for all variables to enable comparison in effect size of different outcome measures. We explored whether adding specific fat mass measures to the model with body mass index explained more of the variance for each outcome. To take account for the correlation between different adiposity measures, we also examined the associations of detailed childhood fat mass measures with cardiovascular risk factors, independent from body mass index by performing linear regression analyses to assess the associations of fat mass measures conditional on body mass index.[20] We constructed fat mass variables, which are statistically independent of body mass index, allowing simultaneous inclusion in multiple regression models. Details of these models are given in the Supplemental **Methods** (online). Third, we tested potential interactions between childhood body mass index categories and childhood adiposity measures. Subsequently, we performed linear regression analyses to examine the associations of childhood adiposity measures with cardiovascular risk factors in different body mass index categories. Finally, we used logistic regression models to examine the associations of childhood body mass index, fat mass percentage and abdominal fat mass measures with the risks of hypertension, hypercholesterolemia and clustering of cardiovascular risk factors. These models were adjusted for child's age at measurements, sex and ethnicity. All analyses were performed using the Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc, Chicago, IL).

Results

Subject characteristics

Table 3.3.1 presents children characteristics. The correlation coefficients between childhood adiposity measures and cardiovascular risk factors are given in the Supplemental **Tables S3.3.1** and **S3.3.2** (online).

Table 3.3.1. Subject characteristics according to body mass index categories

Characteristics	Underweight N=333	Normal weight N=5027	Overweight N=858	Obese N=294	P value
Childhood Characteristics					
Age (years)	6.1 (0.5)	6.2 (0.5)	6.3 (0.6)	6.4 (0.7)	*<0.001*
Sex, No. (%)					
Boys	179 (53.8)	2608 (51.9)	372 (43.4)	110 (37.4)	*<0.001*
Girls	154 (46.2)	2419 (48.1)	486 (56.6)	184 (62.6)	
Ethnicity, No. (%)					
European	210 (64)	334 (68.2)	421 (50.5)	184 (65.2)	*<0.001*
Non-European	118 (36)	1560 (31.8)	412 (49.5)	98 (34.8)	
Gestational age at birth (weeks)	39.9 (32.8, 42.1)	40.1 (35.7, 42.3)	40.1 (36.4, 40.4)	39.9 (36, 42.1)	*<0.001*
Birth weight (g)	3132 (558)	3428 (548)	3495 (546)	3468 (560)	*<0.001*
Anthropometrics					
Height (m)	1.2 (0.1)	1.2 (0.1)	1.2 (0.1)	1.2 (0.1)	*<0.001*
Weight (kg)	18.8 (2.0)	22.2 (2.7)	27.4 (3.7)	34.1 (5.7)	*<0.001*
Body mass index (kg/m2)	13.5 (0.4)	15.7 (0.9)	18.5 (0.8)	21.9 (1.9)	*<0.001*
Body fat distribution					
Fat mass percentage (%)	20.6 (3.4)	23.4 (4.1)	30.7 (4.6)	38.3 (4.6)	*<0.001*
Android/ gynoid fat mass ratio	0.2 (0.0)	0.2 (0.1)	0.3 (0.1)	0.4 (0.1)	*<0.001*
Abdominal fat mass distribution					
Subcutaneous area (cm^2)	0.3 (0.1, 0.8)	0.4 (0.2, 1.1)	0.9 (0.4, 2.1)	1.7 (0.8, 3.2)	*<0.001*
Preperitoneal area (cm^2)	0.3 (0.1, 0.6)	0.4 (0.2, 0.8)	0.6 (0.2, 1.4)	1.0 (0.3, 1.5)	*<0.001*
Cardiovascular risk factors					
Systolic blood pressure (mm/Hg)	99.8 (7.5)	102.2 (7.9)	105.4 (8.1)	108.4 (9.2)	*<0.001*
Diastolic blood pressure (mm/Hg)	59.7 (6.5)	60.5 (6.8)	61.5 (6.9)	63.3 (6.9)	*<0.001*
Left ventricular mass (g/ m^2)	46.7 (10.3)	52.5 (11.0)	58.1 (12.1)	64.3 (12.6)	*<0.001*
Total-cholesterol (mmol/l),	4.1 (0.6)	4.2 (0.6)	4.3 (0.7)	4.4 (0.6)	*<0.001*
LDL-cholesterol (mmol/l)	2.3 (0.5)	2.3 (0.6)	2.0 (0.6)	2.6 (0.6)	*<0.001*
HDL- cholesterol (mmol/l)	1.4 (0.3)	1.4 (0.3)	1.3 (0.3)	1.3 (0.30	*0.001*
Triglycerides (mmol/l)	0.9 (0.4, 2.5)	0.9 (0.4, 2.3)	1.0 (0.4, 2.4)	1.1 (0.4, 3.1)	*<0.001*
Insulin U/l	116 (14, 391)	108 (16, 372)	139 (23, 448)	148 (29, 537)	*<0.001*
C-Peptide ng/ml	0.97 (0.28, 2.25)	0.94 (0.29, 2.07)	1.02 (0.38, 2.33)	1.08 (0.39, 2.60)	*<0.01*
Clustering of cardiovascular risk factors(%)	2.1 (0.1)	6.7 (0.3)	26.2 (0.4)	42.2 (0.5)	*<0.01*

Abbreviations: N; number; SD; standard deviation. Values are means (standard deviation), percentages or median (95% range). P-value was estimated by using One-Way ANOVA test for continuous variables and Chi-square tests for categorical variables

Total and abdominal adiposity measures and cardiovascular risk factors

Higher childhood body mass index was associated with higher blood pressure, higher left ventricular mass, and higher blood levels of total- and LDL-cholesterol, triglycerides, insulin and c-peptide, and with lower blood levels of HDL-cholesterol levels (**Table 3.3.2,** all p-vales< 0.05). As compared to associations of body mass index, the associations of fat mass percentage and abdominal fat measures with systolic blood pressure, left ventricular mass and blood levels of insulin and c-peptide were weaker, whereas those with blood levels of lipids were stronger. No differences in the strength of these associations were present between subcutaneous and preperitoneal abdominal fat mass measures. Additionally adjusting these associations for body mass index attenuated the associations of fat mass percentage and abdominal fat mass measures with blood pressure and blood levels of insulin and c-peptide into non-significant (Supplemental **Table S3.3.3** (online)). Supplemental **Table S3.3.4** (online) shows that adding fat mass percentage and abdominal fat mass measures to the models slightly increased the portion of variance already explained by body mass index.

Table 3.3.2. Associations of childhood total body and abdominal fat mass measures with cardiovascular risk factors

Cardiovascular risk factors	General adiposity		Body fat distribution			Abdominal fat mass distribution		
	N	Body mass index (SDS)	N	Fat mass percentage (SDS)	Android/Gynoid fat mass (SDS)	N	Subcutaneous fat mass area (SDS)	Preperitoneal fat mass area (SDS)
Systolic blood pressure (SDS)	6069	0.22 (0.19, 0.24)**	5958	0.16 (0.14, 0.19)**	0.11 (0.09, 0.14)**	5020	0.16 (0.13, 0.18)**	0.09 (0.06, 0.12)**
Diastolic blood pressure (SDS)	6069	0.07 (0.04, 0.09)**	5958	0.08 (0.06, 0.11)**	0.05 (0.03, 0.08)**	4971	0.06 (0.03, 0.09)**	0.04 (0.01, 0.07)*
Left ventricular mass (SDS)	5966	0.35 (0.33, 0.37)**	5825	0.09 (0.06, 0.11)*	0.10 (0.08, 0.12)**	5072	0.04 (0.01, 0.07)*	-0.01 (-0.04, 0.01)
Total-cholesterol (SDS)	4321	0.08 (0.05,0.11)**	4140	0.13 (0.10, 0.17)**	0.09 (0.06, 0.12)**	3445	0.12 (0.09, 0.16)**	0.14 (0.10, 0.18)**
LDL- cholesterol (SDS)	4216	0.08 (0.05, 0.12)**	4143	0.15 (0.11, 0.18)**	0.10 (0.06, 0.13)**	3448	0.14 (0.10, 0.17)**	0.12 (0.09, 0.16)**
HDL- cholesterol (SDS)	4217	-0.08 (-0.11, -0.05)**	4144	-0.10 (-0.13, -0.06)**	-0.12 (-0.15, -0.09)**	4352	-0.07 (-0.11, -0.03)**	-0.04 (-0.06, -0.00)†
Triglycerides (SDS)	4202	0.11 (0.07, 0.14)**	4130	0.13 (0.09, 0.16)**	0.18 (0.15, 0.21)**	3438	0.11 (0.07, 0.15)**	0.13 (0.09, 0.16)**
Insulin (SDS)	4173	0.17 (0.14, 0.20)**	4093	0.13 (0.09, 0.17)**	0.11 (0.07, 0.14)**	3410	0.10 (0.06, 0.14)**	0.09 (0.06, 0.13)**
C-peptide (SDS)	4182	0.14 (0.11, 0.17)**	4102	0.11 (0.07, 0.14)**	0.11 (0.08, 0.14)**	3419	0.07 (0.03, 0.11)**	0.07 (0.04, 0.11)**

Abbreviations: N; number; SDS; standard deviation scores; Values are linear regression coefficients (95% confidence interval). The estimates represent differences in cardiovascular risk factors (outcomes) per SDS of childhood anthropometrics, total body and abdominal fat mass measures (determinants). Models are adjusted for age, sex and ethnicity. Models with body fat and abdominal fat mass measures are additionally adjusted for height. Models adjusted for body mass index are given in are given in Supplemental Table S3.3.3. † < 0.05 * P-value<0.01 ** P-value<0.001

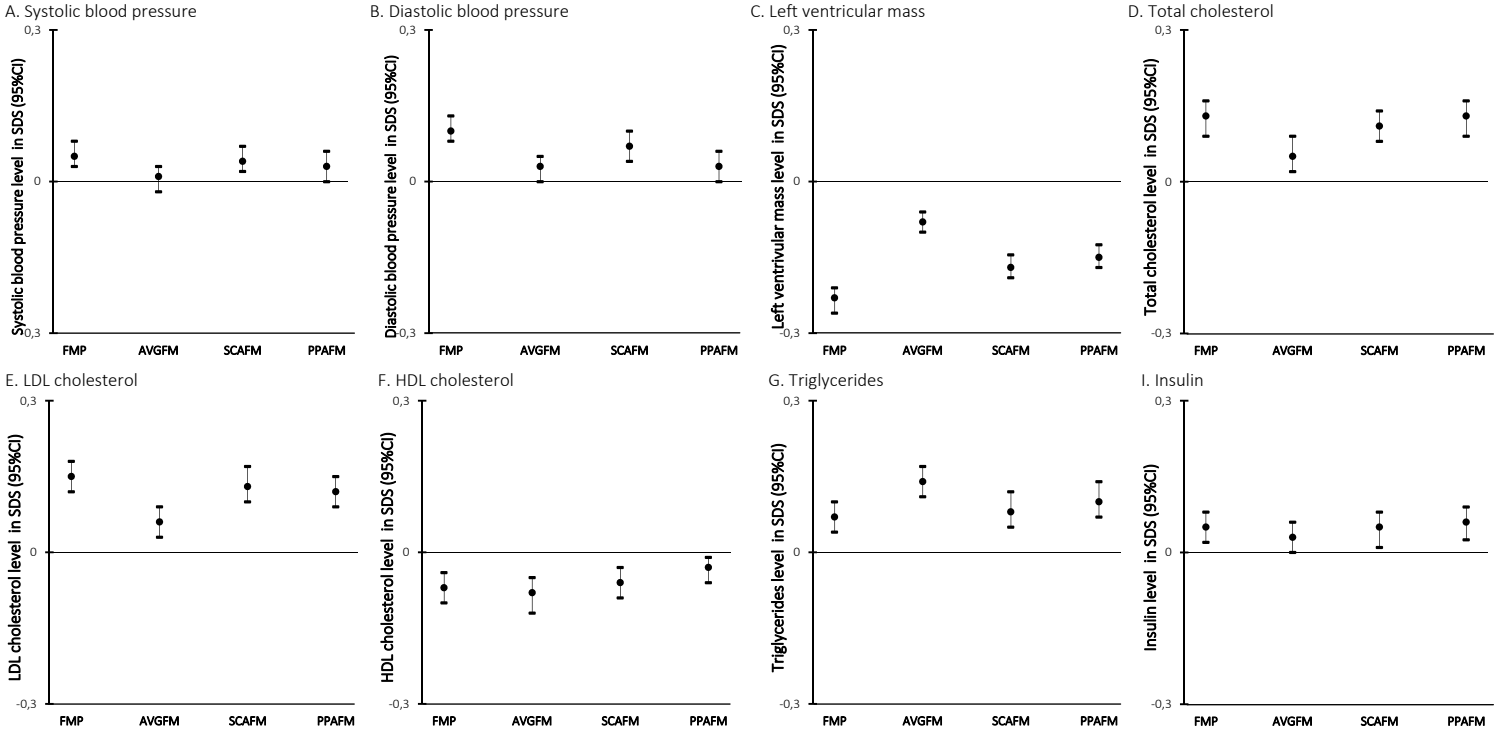
Figure 3.3.1 shows that conditional on BMI, fat mass percentage and subcutaneous fat mass area, but not android/gynoid fat mass ratio and preperitoneal abdominal fat mass area, were positively associated with blood pressure, and inversely with left ventricular mass. Higher fat mass percentage and abdominal fat mass measures were associated with higher total-cholesterol, LDL-cholesterol, triglycerides, insulin and c-peptide blood levels and lower HDL-cholesterol blood levels. For blood pressure, left ventricular mass, and total and LDL-cholesterol levels, the strongest effect estimates were observed for fat mass percentage, whereas for triglycerides the strongest effect estimates were observed for android/gynoid fat mass ratio.

Table 3.3.3 shows that among normal weight and overweight children, higher fat mass percentage was associated with higher blood pressure, whereas among obese children higher fat mass percentage was stronger associated with HDL-cholesterol and insulin. Higher android/gynoid fat mass ratio was most strongly associated with higher blood pressure among obese children, but not consistently with other cardiovascular risk factors. We observed the strongest associations of higher fat mass percentage and abdominal fat mass measures with lower left ventricular mass among underweight children. The associations of abdominal fat mass measures with cardiovascular risk factors were higher among obese children. Interaction terms were constructed by using body mass index in four categories and adiposity measures as continuous variables. The interaction terms between body mass index categories and adiposity measures for total-cholesterol were not significant. Associations of childhood total body and abdominal fat mass measures with c-peptide levels among different obesity categories are shown in Supplemental Table S3.3.5 (online). For all analyses, sensitivity analyses were performed among boys and girls separately and no consistent sex differences were present (results not shown).

Risks of hypertension, hypercholesterolemia and clustering of cardiovascular risk factors

Figure 3.3.2 shows that higher body mass index and fat mass percentage and abdominal fat mass tended to be associated with higher risks of hypertension, hypercholesterolemia, and clustering of cardiovascular risk factors, with similar effect estimates for the associations of the different fat measures with the risks of hypertension and hypercholesterolemia. For clustering of cardiovascular risk factors, we observed the strongest effect estimate for fat mass percentage (Odds ratio 3.01 (95 % Confidence Interval (CI) 2.67, 3.39) per SDS total body fat mass. After excluding the android fat mass percentage as a component from the definition of clustering of cardiovascular risk factors, similar results were observed for the associations of body fat distribution measurements with clustering of cardiovascular risk factors(Supplemental Figure S3.3.1 (online))

Figure 3.3.1. Associations of total body and abdominal fat mass measures with cardiovascular risk factors, conditional on body mass index



Abbreviations: SDS; standard deviation score; TBFM; total body fat mass; AGRFM; android/gynoid fat mass ratio; SCAFM; subcutaneous fat mass area; PPAFM; preperitoneal fat mass area; Values are standardized regression coefficients (95% Confidence Interval (CI)) from conditional analyses. The estimates represent differences in systolic and diastolic blood pressure, left ventricular mass, different lipid levels and insulin per standardized residual change of total and abdominal fat mass measures. Models are adjusted for age, sex and ethnicity.

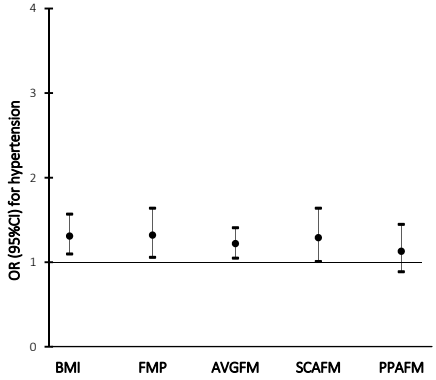
Table 3.3.3. Associations of childhood total body and abdominal fat mass measures with cardiovascular risk factors among underweight, normal weight, overweight and obese children

	Cardiovascular risk factors in SDS						
	Systolic blood pressure	Diastolic blood pressure	Left ventricular mass	LDL-cholesterol	HDL-cholesterol	Triglycerides	Insulin
Fat mass percentage (SDS)							
Underweight	-0.10 (-0.27, 0.07)	-	-0.30 (-0.46, -0.13)**	-	-0.13 (-0.36, 0.11)	-	0.07 (-0.16, 0.30)
Normal weight	0.13 (0.09, 0.17)**	-	-0.21 (-0.25, -0.18)**	-	-0.07 (-0.12, -0.02)*	-	0.05 (0.00, 0.10)†
Overweight	0.09 (0.00, 0.17)†	-	-0.27 (-0.36, -0.18)**	-	-0.13 (-0.23, -0.03)*	-	0.18 (0.06, 0.30)*
Obese	0.04 (-0.13, 0.22)	-	0.07 (-0.09, 0.24)	-	-0.24 (-0.42, -0.06)*	-	0.33 (0.12, 0.59)**
P-value for interaction	*<0.01*	*0.68*	*<0.01*	*0.07*	*0.05*	*0.10*	*<0.01*
Android/Gynoid (SDS)							
Underweight	0.03 (-0.14, 0.20)	0.00 (-0.17, 0.18)	0.12 (-0.04, 0.28)	-0.20 (-0.41, -0.02) †	-0.32 (-0.55, -0.09)*	-	0.05 (-0.17, 0.28)
Normal weight	0.04 (0.01, 0.08)†	0.02 (-0.02, 0.05)	-0.02 (-0.05, 0.02)	0.06 (0.02, 0.11) †	-0.06 (-0.11, -0.01)†	-	0.02 (-0.03, 0.07)
Overweight	0.01 (-0.06, 0.09)	0.04 (-0.04, 0.11)	-0.12 (-0.19, -0.05)**	0.14 (0.05, 0.22)*	-0.15 (-0.23, -0.07)**	-	0.14 (0.05, 0.23)*
Obese	0.13 (0.01, 0.24)†	0.15 (0.04, 0.25)*	0.09 (-0.03, 0.20)	0.09 (-0.04, 0.23)	-0.19 (-0.31, -0.08)*	-	0.10 (-0.05, 0.25)
P-value for interaction	*<0.01*	*<0.01*	*<0.01*	*<0.01*	*0.02*	*0.62*	*<0.01*
Subcutaneous area (SDS)							
Underweight	-0.08 (-0.24, 0.07)	-	-0.12 (-0.26, 0.00)	-	-0.08 (-0.27, 0.12)	0.12 (-0.09, 0.33)	0.00 (-0.20, 0.20)
Normal weight	0.11 (0.07, 0.15)**	-	-0.10 (-0.14, -0.06)**	-	-0.03 (-0.08, 0.02)	0.06 (0.01, 0.11)†	0.05 (0.01, 0.10)†
Overweight	0.09 (-0.02, 0.19)	-	-0.16 (-0.26, -0.05)*	-	-0.12 (-0.24, 0.01)	0.16 (0.03, 0.29)*	0.09 (-0.06, 0.24)
Obese	0.24 (0.02, 0.47)†	-	0.05 (-0.18, 0.27)	-	-0.23 (-0.48, 0.02)	0.36 (0.10, 0.62)*	0.21 (-0.08, 0.50)
P-value for interaction	*<0.01*	*0.10*	*<0.01*	*0.52*	*<0.01*	*<0.01*	*<0.01*
Preperitoneal area (SDS)							
Underweight	-0.08 (-0.23, 0.08)	-0.13(-0.28, 0.03)	-0.17 (-0.30, -0.04)*	0.01 (-0.18, 0.19)	0.05 (-0.15, 0.24)	0.01 (-0.20, 0.21)	-0.03 (-0.22, 0.16)
Normal weight	0.06 (0.02, 0.09)*	0.04 (-0.00, 0.07)	-0.13 (-0.16, -0.10)**	0.13 (0.08, 0.17)**	0.02 (-0.02, 0.07)	0.08 (0.03, 0.12)*	0.07 (0.03, 0.12)*
Overweight	0.04 (-0.04, 0.13)	0.01 (-0.08, 0.10)	-0.15 (-0.23, -0.06)*	0.20 (0.10, 0.30)**	-0.05 (-0.15, 0.05)	0.18 (0.08, 0.29)*	0.06 (-0.06, 0.18)
Obese	0.12 (-0.02, 0.25)	0.08 (-0.04, 0.21)	0.03 (-0.11, 0.16)	0.14 (-0.02, 0.30)	-0.14 (-0.30, 0.01)	0.23 (0.07, 0.40)*	0.08 (-0.10, 0.25)
P-value for interaction	*<0.01*	*<0.01*	*<0.01*	*0.04*	*<0.01*	*<0.01*	*<0.01*

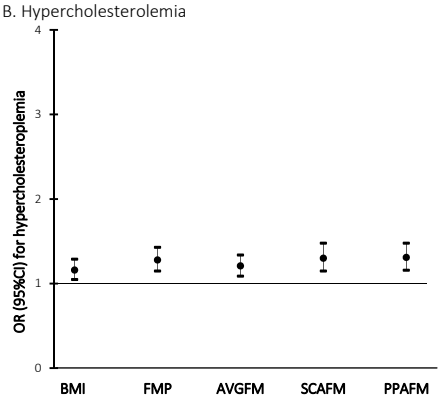
Abbreviations: SDS; standard deviation scores; Values are linear regression coefficients (95% confidence interval). The estimates represent differences in cardiovascular risk factors per SDS change of total body and abdominal fat mass measures among different obesity categories. Analyses are adjusted for age, sex and ethnicity. Results for c-peptide are similar as insulin, and are given in Supplemental Table S3.3.5. Interaction terms were constructed using body mass index in four categories and adiposity measures as continuous variables. † < 0.05 * P-value<0.01 ** P-value<0.001

Figure 3.3.2. Associations of total body and abdominal fat mass measures with the risk of hypertension, hypercholesterolemia and clustering of cardiovascular risk factors in children

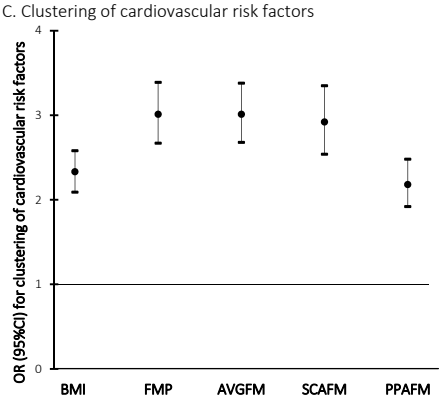
A. Hypertension



B. Hypercholesterolemia



C. Clustering of cardiovascular risk factors



Abbreviations: OR; odds ratios; CI; confidence interval; BMI; body mass index; TBFM; total body fat mass; AVGFM; android/gynoid fat mass ratio; SCAFM; subcutaneous fat mass area; PPAFM; preperitoneal fat mass area; Values are odds ratios (95% Confidence Interval (CI)) that reflect the risk of hypertension, hypercholesterolemia and clustering cardiovascular risk factors per SD change in body mass index, total body and abdominal fat mass measures in children. Models are adjusted for age, sex and ethnicity.

Discussion

This large-scale population-based study among school-age children showed that both fat mass percentage and abdominal fat mass measures were associated with cardiovascular risk factors, independent from body mass index. Higher childhood body fat distribution measures were strongly associated with increased risks of childhood hypertension, hypercholesterolemia and clustering of cardiovascular risk factors.

Methodological considerations

We performed a cross-sectional study within a population-based cohort with a large number of subjects. The response rate at baseline was 61%. Of all children participating at the age of 6 years, 78% (6,523) participated in the adiposity and cardiovascular follow-up studies. The non-response could lead to biased effect estimates if the associations of different obesity measures with cardiovascular risk factors would be different between children included and not included in the analyses.[10] Assuming that children with a higher body mass index are less likely to participate in the detailed adiposity and cardiovascular follow-up studies, our estimates may be underestimated. Birth weight was lower in those who were included in the current analyses than in those who were not included. However, it is hard to speculate whether this difference would affect the observed associations materially, but we consider this unlikely. We obtained detailed measures of childhood adiposity. DXA quantifies fat content with high precision, but cannot differentiate between abdominal visceral and subcutaneous fat compartments. However, we used abdominal ultrasound, a valid method for measuring both subcutaneous abdominal fat mass and preperitoneal fat mass.[13] Both DXA and abdominal ultrasound have been validated against CT.[12, 21] Use of 30-minutes fasting blood samples may have led to underestimation of the observed associations. However, it has been shown in adults that non-fasting lipid levels can accurately predict increased risks of cardiovascular events in later life.[22] The different adiposity measures were correlated, which may explain why the associations are difficult to interpret when are included in one regression models. The main advantage of the conditional analyses is that the effect estimates are completely statistically independent when combined in one model. Due to the cross-sectional analyses, we were not able to explore directions and causality of the observed associations. Therefore, it is of interest to perform further longitudinal analyses to examine the associations of these adiposity measures with cardiovascular risk factors in adolescence and adulthood.

Interpretation of main findings

Many studies have shown associations of different adiposity measures with cardiovascular risk factors.[1-4, 8] Body mass index may be a suboptimal measure in children, as it is unable to distinguish lean mass from fat mass. Detailed total body and abdominal fat mass measures may be useful to identify children with an adverse cardiovascular profile. Thus far not much is known about these associations in school-age children. In our study, body mass index, fat mass percentage and abdominal fat measures were strongly positively correlated. These observations suggest that the correlations between body mass index, fat mass percentage and waist circumference previously shown in both

adults and older aged children are also present in school-aged children.[3, 23, 24] The relatively weaker correlation between body mass index and preperitoneal fat mass suggests that body mass index is only weakly related to visceral fat mass.

We observed that fat mass percentage, was independent from body mass index, associated with various cardiovascular risk factors. Also, the associations of body fat mass measures with lipid levels tended to be stronger than the associations for body mass index. Similarly, a study among 5,235 English children aged 9 to 12 years observed that body mass index and total fat mass in childhood were associated with cardiovascular risk factors in adolescents, with slightly stronger effect estimates for total fat mass measures.[23] Surprisingly, we observed that independent from body mass index, fat mass percentage was inversely associated with left ventricular mass. Another study among 201 children aged 6 to 17 years old reported a similar associations [25], suggesting muscle mass is the major determinant of left ventricular mass in childhood.

Both android/gynoid fat mass ratio and subcutaneous abdominal fat mass area, which reflect waist to hip ratio and waist circumference, respectively, were associated with cardiovascular risk factors, independent from body mass index. Multiple studies in both adults and older aged children have reported similar effect estimates.[26, 27] However, waist to hip ratio has not consistently been identified as a strong predictor of cardiovascular risk factors. These inconsistencies may be due to the large variations in the level of total body and abdominal fat mass, therefore both lean and obese individuals may have the same waist to hip ratio. We measured subcutaneous and preperitoneal fat mass using ultrasound, and used preperitoneal fat mass as a measure of visceral fat mass.[12, 13] In adults and adolescents, both subcutaneous abdominal fat mass and visceral abdominal fat mass are associated with cardiovascular risk factors and visceral fat mass tends to be stronger related with HDL-cholesterol, triglycerides, and insulin resistance.[8, 28, 29] As compared to associations of preperitoneal fat mass, we observed stronger associations for subcutaneous abdominal fat mass area with most cardiovascular risk factors. Thus in children, visceral fat mass may not be strongly associated with cardiovascular risk factors, which may be explained by less pathogenic and only a small accumulation of visceral adipose tissue at younger ages. In line with our findings, a study among 783 young men [30] showed that visceral abdominal fat mass is not stronger associated with cardiovascular risk factors than subcutaneous abdominal fat mass.

The effects of specific fat measures on health outcomes may differ between normal, overweight and obese children. A study among 359,387 European adults showed that the associations of waist circumference with risk of death were stronger among subjects with a lower body mass index.[4] Similarly, a study among 2,003 adolescents showed that males with a normal body mass index and elevated waist circumference were more likely to have elevated levels of cardiovascular risk factors.[31] We observed that the associations of higher abdominal fat mass measures with cardiovascular risk factors were stronger among obese children. Another study among adults from 18 to 80 years old showed that subjects with body fat percentage within the obesity range had higher levels of cardiovascular risk factors.[32] Therefore, also in school-age children, the associations of general and abdominal fat mass may differ between the body mass index groups.

Our results suggest that children with higher levels of general and abdominal fat mass have independent of their body mass index, an adverse cardiovascular risk profile. Whether and to what extent detailed fat mass percentage and abdominal fat measures should be used in clinical practice is not known yet. The additional clinical value of detailed fat measures as compared to body mass index may be only limited. The additional variance explained in cardiovascular risk factors by more direct measures of adiposity in our models was small. Also, taking into account greater comfort, feasibility and lower costs of measuring body mass index in children, body mass index alone might be an appropriate measure for clinical practice in children. Although clinical practice may not be in direct need for detailed measures of total and abdominal fat measures at this age, our findings strongly suggest that detailed body fat distribution measurements are important tools in etiological studies focused on the early origins of cardio-metabolic diseases.

Conclusions

Fat mass percentage and abdominal fat mass measures are associated with cardiovascular risk factors in school-age children, independent from body mass index. These measures may provide additional information for identification of children with an adverse cardiovascular profile, and may be important measures for etiological research focused on development of cardiovascular and metabolic diseases. Further studies are needed to examine the longitudinal associations of these specific fat mass measures with development of cardiovascular risk factors and disease in later life.

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Supplemental Material

Supplemental Methods: Conditional analysis

The standardized residuals from multiple linear regression analysis are used in conditional modelling to assess the associations of total fat mass, subcutaneous area, preperitoneal area and subcutaneous distance/preperitoneal distance ratio with cardiovascular risk factors, statistically independent from body mass index. Conditional models of total body fat mass measures and abdominal fat mass measures were built using linear regression analysis. In these analyses, body mass index is considered as starting point. The excess total body and abdominal fat mass is similar to the standardized residuals resulting from the linear regression model of total body and abdominal fat mass measures regressed on body mass index. The standard residuals obtained from this regression model are entirely uncorrelated with body mass index.[1, 2, 3] This allows inclusion of both body mass index and more detailed fat mass measures in linear regression models, without problems regarding the correlations between these measures. After construction of these new independent variables, we subsequently used linear regression analysis to estimate the change in systolic and diastolic blood pressure, left ventricular mass, lipid levels and insulin per standardized residual change of total body fat mass, android/gynoid fat mass ratio, subcutaneous and preperitoneal area fat mass, independently from body mass index.

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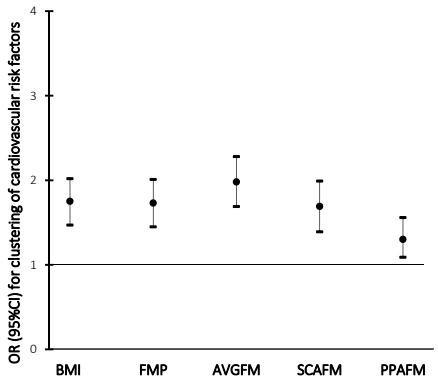
Figure S3.3.1. Associations of total body and abdominal fat mass measures with the risk of clustering of cardiovascular risk factors in children



Abbreviations: OR; odds ratios; CI; confidence interval; BMI; body mass index; FMP; fat mass percentage; AVGFM; android/gynoid fat mass ratio; SCAFM; subcutaneous fat mass area; PPAFM; preperitoneal fat mass area; Values are odds ratios (95% Confidence Interval (CI)) that reflect the risk of clustering cardiovascular risk factors per SD change in body mass index, total body and abdominal fat mass measures in children. Models are adjusted for age, sex and ethnicity.

Table S3.3.1. Correlation coefficients between childhood anthropometrics and total body and abdominal fat mass measures

	Height	Weight	BMI	FMP	AVGFM	SCAFM	PPAFM
Height	1						
Weight	0.76**	1					
BMI	0.30**	0.84**	1				
FMP	0.19**	0.58**	0.70**	1			
AVGFM	0.13**	0.48**	0.61**	0.65**	1		
SCAFM	0.21**	0.51**	0.61**	0.82**	0.56**	1	
PPAFM	0.22**	0.40**	0.42**	0.56**	0.39**	0.65**	1

Abbreviations: BMI; body mass index; FMP; fat mass percentage; AVGFM; android/gynoid fat mass ratio; SCAFM; subcutaneous area fat mass; PPAFM; preperitoneal area fat mass; Values represent Pearson correlation coefficients and Spearmen rank correlation coefficients (subcutaneous area fat mass, preperitoneal area fat mass). ** P-value<0.001

Table S3.3.2. Correlation coefficients between childhood anthropometrics and total body and abdominal fat mass measures with cardiovascular risk factors

	SBP	DBP	LVM	Total-cholesterol	LDL-cholesterol	HDL-cholesterol	TG	Insulin	C-Peptide
Height	0.21**	0.09**	0.50**	0.00	-0.01	0.04*	-0.05*	0.12**	0.08**
Weight	0.29**	0.15**	0.52**	0.07**	0.05**	0.00	-0.01	0.15**	0.13**
BMI	0.25**	0.10**	0.36*	0.09**	0.08**	-0.03 †	0.05*	0.13**	0.13**
FMP	0.21**	0.14**	0.08**	0.15**	0.17**	-0.07**	0.08*	0.09**	0.11**
AVGFM	0.15**	0.07**	0.15**	0.10**	0.10**	-0.09**	0.14**	0.08**	0.12**
SCAFM	0.19**	0.11**	0.10**	0.13**	0.15**	-0.03	0.06*	0.09**	0.05*
PPAFM	0.14**	0.07**	0.03*	0.15**	0.13**	0.02	0.07**	0.09**	0.06*
SBP	1	0.62**	0.12**	0.08**	0.05*	0.06 †	-0.01	0.07**	0.04 †
DBP		1	0.02	0.05*	0.05*	0.04*	-0.02	-0.02	-0.05*
LVM			1	-0.04*	-0.04*	0.02	-0.06*	0.07**	0.06*
Total-				1	0.86**	0.29**	0.16**	-0.02	-0.04*
LDL-cholesterol					1	-0.06*	0.13**	-0.05*	-0.06*
HDL-cholesterol						1	-0.39*	-0.06*	-0.10**
TG							1	0.19**	0.20**
Insulin								1	0.88**
C-Peptide									1

Abbreviations: BMI; body mass index; FMP; fat mass percentage; AVGFM; android/gynoid fat mass; SCAFM; subcutaneous fat mass area; PPAFM; preperitoneal fat mass area; SBP; systolic blood pressure; DBP; diastolic blood pressure; LVM; left ventricular mass; LDL-cholesterol; low density lipoprotein cholesterol; HDL-cholesterol; high density lipoprotein cholesterol; TG triglycerides; Values represent Pearson correlation coefficients and Spearmen rank correlation coefficients (subcutaneous area fat mass, preperitoneal area fat mass, TG, Insulin, C-peptide). † < 0.05 * P-value<0.01 ** P-value<0.001

Table S3.3.3. Associations of childhood total body and abdominal fat mass measures with cardiovascular risk factors, adjusted for height and body mass index

Cardiovascular risk factors	General adiposity		Body fat distribution			Abdominal fat mass distribution		
	N	Body mass index (SDS)	N	Fat mass percentage (SDS)	Android/Gynoid fat mass (SDS)	N	Subcutaneous fat mass area (SDS)	Preperitoneal fat mass area (SDS)
Systolic blood pressure (SDS)	6069	0.22 (0.19, 0.24)**	5958	0.16 (0.14, 0.19)**	0.11 (0.09, 0.14)**	5020	0.16 (0.13, 0.18)**	0.09 (0.06, 0.12)**
Diastolic blood pressure (SDS)	6069	0.07 (0.04, 0.09)**	5958	0.08 (0.06, 0.11)**	0.05 (0.03, 0.08)**	4971	0.06 (0.03, 0.09)**	0.04 (0.01, 0.07)*
Left ventricular mass (SDS)	5966	0.35 (0.33, 0.37)**	5825	0.09 (0.06, 0.11)*	0.10 (0.08, 0.12)**	5072	0.04 (0.01, 0.07)*	-0.01 (-0.04, 0.01)
Total-cholesterol (SDS)	4321	0.08 (0.05,0.11)**	4140	0.13 (0.10, 0.17)**	0.09 (0.06, 0.12)**	3445	0.12 (0.09, 0.16)**	0.14 (0.10, 0.18)**
LDL- cholesterol (SDS)	4216	0.08 (0.05, 0.12)**	4143	0.15 (0.11, 0.18)**	0.10 (0.06, 0.13)**	3448	0.14 (0.10, 0.17)**	0.12 (0.09, 0.16)**
HDL- cholesterol (SDS)	4217	-0.08 (-0.11, -0.05)**	4144	-0.10 (-0.13, -0.06)**	-0.12 (-0.15, -0.09)**	4352	-0.07 (-0.11, -0.03)**	-0.04 (-0.06, -0.00)†
Triglycerides (SDS)	4202	0.11 (0.07, 0.14)**	4130	0.13 (0.09, 0.16)**	0.18 (0.15, 0.21)**	3438	0.11 (0.07, 0.15)**	0.13 (0.09, 0.16)**
Insulin (SDS)	4173	0.17 (0.14, 0.20)**	4093	0.13 (0.09, 0.17)**	0.11 (0.07, 0.14)**	3410	0.10 (0.06, 0.14)**	0.09 (0.06, 0.13)**
C-peptide (SDS)	4182	0.14 (0.11, 0.17)**	4102	0.11 (0.07, 0.14)**	0.11 (0.08, 0.14)**	3419	0.07 (0.03, 0.11)**	0.07 (0.04, 0.11)**

Abbreviations: N; number; SDS; standard deviation scores; Values are linear regression coefficients (95% confidence interval). Effect estimates represent differences in cardiovascular risk factors per standard deviation scores of childhood total body and abdominal fat mass measures. Models are adjusted for age, sex and ethnicity. Models with body fat and abdominal fat mass measures are additionally adjusted for height and body mass index. † < 0.05 * P-value<0.01 ** P-value<0.001

Table S3.3.4. Variance explained by models in the associations of childhood total body and abdominal fat mass measures with cardiovascular risk factors

	Systolic blood pressure (SDS)	Diastolic blood pressure (SDS)	Left ventricular mass (SDS)	Total-cholesterol (SDS)	LDL-cholesterol (SDS)	HDL-cholesterol (SDS)	Triglycerides (SDS)	Insulin (SDS)	C-peptide (SDS)
General adiposity	R^2	R^2	R^2	R^2	R^2	R^2	R^2	R^2	R^2
BMI (SDS)	0.07	0.03	0.22	0.02	0.03	0.02	0.02	0.03	0.03
Body fat distribution									
FMP (SDS)									
Model 1	0.08	0.03	0.29	0.03	0.04	0.03	0.02	0.03	0.03
Model 2	0.09	0.03	0.37	0.03	0.04	0.03	0.02	0.04	0.03
AVGFM (SDS)									
Model 1	0.07	0.03	0.29	0.02	0.03	0.03	0.03	0.03	0.03
Model 2	0.09	0.03	0.35	0.02	0.03	0.03	0.03	0.04	0.03
Abdominal fat mass distribution									
SCAFM (SDS)									
Model 1	0.08	0.03	0.29	0.03	0.03	0.02	0.02	0.03	0.02
Model 2	0.09	0.03	0.36	0.03	0.03	0.02	0.01	0.03	0.03
PPAFM (SDS)									
Model 1	0.07	0.03	0.29	0.03	0.03	0.02	0.02	0.03	0.02
Model 2	0.09	0.03	0.37	0.03	0.03	0.02	0.02	0.03	0.03

Abbreviations: SDS; standard deviation scores; R^2; variance explained by models; BMI; body mass index; FMP; fat mass percentage; AVGFM; android/gynoid fat mass; SCAFM; subcutaneous fat mass area; PPAFM; preperitoneal fat mass area; Model 1 is adjusted for age, sex, ethnicity and height. Model 2 is adjusted for age, sex, ethnicity, height and body mass index.

Table S3.3.5. Association of childhood total body and abdominal fat mass measures with c-peptide levels among underweight, normal weight, overweight and obese children

	C-peptide (SDS)
Fat mass percentage (SDS)	
Underweight	0.13 (-0.12, 0.38)
Normal weight	0.03 (-0.02, 0.08)
Overweight	0.12 (0.01, 0.23)†
Obese	0.33 (0.14, 0.51)*
P-value for interaction	*<0.01*
Android/Gynoid (SDS)	
Underweight	0.13 (-0.12, 0.37)
Normal weight	0.05 (-0.00, 0.10)
Overweight	0.11 (0.02, 0.19)*
Obese	0.16 (0.03, 0.29)†
P-value for interaction	*<0.01*
Subcutaneous area (SDS)	
Underweight	-0.02 (-0.22, 0.18)
Normal weight	0.04 (-0.00, 0.09)†
Overweight	0.05 (-0.05, 0.15)
Obese	0.18 (0.02, 0.35)*
P-value for interaction	*<0.01*
Preperitoneal area (SDS)	
Underweight	0.02 (-0.18, 0.23)
Normal weight	0.04 (-0.01, 0.08)
Overweight	-0.01 (-0.14, 0.12)
Obese	0.20 (-0.05, 0.47)
P-value for interaction	*<0.01*

Abbreviations: SDS; standard deviation scores; Values are linear regression coefficients (95% confidence interval). Effect estimates represent differences in c-peptide levels per SDS change of total body fat mass measures and abdominal fat mass measures among different obesity categories. Standard deviation scores for all outcome variables were used. Analyses are adjusted for age, sex and ethnicity.† < 0.05 * P-value<0.01 ** P-value<0.001

Table S3.3.6. Comparison of subject characteristics between children included and not included in the analyses

Characteristics	Participation	No participation	P value
Childhood characteristics			
Sex, No. (%)			
Boys	3275 (50.2)	917 (51.5)	*0.18*
Girls	3248 (49.8)	864 (48.5)	
Ethnicity, No. (%)			
European	4080 (62.5)	1029 (57.7)	*0.37*
Non-European	2277 (34.9)	586 (32.9)	
Gestational age at birth (weeks), median (95% range)	40.1 (35.7, 42.3)	39.9 (33.7, 42.3)	*<0.001*
Birth weight (g), mean (SD)	3306 (643)	3423 (555)	*<0.001*

Values are means (standard deviation), percentages or median (95% range). P-value was estimated by using One-Way ANOVA test and Chi-square tests.

Chapter 3.4

Body fat distribution, metabolic and inflammatory markers and childhood retinal microvasculature

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Submitted

Abstract

Background: Childhood obesity is associatied with cardiovascular diseases in later life. Microvasculature adaptations might be involved in the development of these deseases. We examined the associations of body fatness, metabolic and inflammatory markers with retinal vessel calibers among children.

Methods and Results: We performed a population-based cohort study among 4,145 school-age children. At the median age of 6.0 years (95% range 5.8, 8.0 years), we measured body mass index, total and abdominal fat mass, metabolic and inflammatory markers (blood levels of lipids, insulin and C-peptide and C-reactive protein) and retinal vascular calibers from retinal photographs. We observed that compared to normal weight children, obese children had narrower retinal arteriolar caliber (difference -0.21 SDS (95% Confidence Interval (CI) -0.35, -0.06)), but not venular caliber. Continuous analyses showed that higher body mass index and total body fat mass, but not android/gynoid fat mass ratio and pre-peritoneal fat mass, were associated with narrower retinal arteriolar caliber (p-values <0.05 for body mass index and total body fat mass), but not with retinal venular caliber. Lipid and insulin levels were not associated with retinal vessel calibers. Higher C-reactive protein was associated with only wider retinal venular caliber (difference 0.10 SDS (95% CI 0.06, 0.14) per SDS increase in C-reactive protein). This latter association was not influenced by body mass index.

Conclusions: Higher body fatness is associated with narrower retinal arteriolar caliber, whereas increased C-reactive protein levels are associated with wider retinal venular caliber. Increased fat mass and inflammation correlate with microvascular development from school-age onwards.

Introduction

Childhood obesity is associated with cardiovascular diseases in later life.[1] Previous studies in adults have suggested that childhood obesity not only led to large-artery disease such as increased carotid artery stiffness, which is a subclinical marker of atherosclerosis [2], but may also affect endothelial and microvascular function.[3] Arteriolar and venular microvasculature structures can be assessed non-invasively by retinal photography. Previous studies among adults have suggested that obesity is associated with wider retinal venular caliber.[4, 5] Only few studies among children have examined the effect of obesity on retinal vessel calibers and suggested wider retinal venular caliber among obese children.[6, 7] In addition to body mass index, adverse body fat distribution patterns may be stronger related to adverse vascular adaptations.[8] In adults, higher waist circumference was associated with increased retinal arteriolar caliber.[5] Not much is known about the effects of specific fat mass measures on microvasculature development at younger ages.[4] Also, whether the metabolic profiles related to increased adiposity, directly affect microvascular development is not known. Inflammatory responses in obese subjects might be involved in the pathophysiological mechanisms underlying the associations of obesity with microvascular adaptations.[9] Recent studies show that inflammatory markers are associated with retinal vascular measures in adults.[9, 10] However, not much is known about the associations of inflammatory markers and microvasculature in children.

Therefore, we examined among 4,145 children participating in a population-based cohort study the cross-sectional associations of different fat mass measures and metabolic and inflammatory risk factors with retinal arteriolar and venular calibers at school-age.

Methods

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[11] All children were born between 2002 and 2006. Response rate at birth was 61%.[11] The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained from all parents. In total, 8,305 children participated in the follow-up measurements at the median age of 6.0 years (90% range 5.7 – 7.4), of whom 6,523 participated in the detailed measurements for the current study. Retinal vessel calibers were available in 4,145 children. Missing retinal vessel measurements were mainly due to the delated inclusion of these measurements in the study protocol (Flowchart given in **Figure S3.4.1**). Response versus non-response analysis showed that birth weight was lower among children who were not included in the analyses, as compared to children who were included in the final analysis (Supplemental Material **Table S3.4.1**).

Childhood body fat assessments

The methods for body fat assessments have been described previously in detail.[12] At the age of 6 years we measured height and weight without shoes and heavy clothing.

Height was measured to the nearest 0.1 cm by a stadiometer (Holtain Limited, Crosswell, Crymych, UK). Weight was measured to the nearest gram using an electronic scale (SECA 888, Almere, The Netherlands). Body mass index (kg/m^2) was calculated. We defined childhood underweight, normal weight, overweight and obesity according to the International Obesity Task Force.[13]

Total body and regional fat mass percentages were measured using Dual-energy X-ray absorptiometry (DXA)(iDXA, GE-Lunar, 2008, Madison, WI, USA), and analysed with the enCORE software v.12.6.[14] DXA can accurately detect whole-body fat mass within less than 0.25% coefficient of variation. Total body fat mass (kg) was calculated as percentage of total body weight (kg) measured by DXA. The android/gynoid fat mass ratio was calculated. The android/gynoid fat ratio reflects the central body fat distribution in the abdomen (android) and hip (gynoid) regions.[15]

Abdominal ultrasound examinations were used to measure pre-peritoneal fat as measure of visceral abdominal fat.[16] Briefly, pre-peritoneal fat thickness was measured with a linear (L12-5 MHz) transducer [17], which was placed perpendicular to the skin surface on the median upper abdomen. We scanned longitudinally just below the xiphoid process to the navel along the midline (linea alba). All measurements were performed off-line. Pre-peritoneal abdominal fat mass distance (PP-distance) was measured as distance of the linea alba to the peritoneum on top of the liver. Pre-peritoneal fat mass area was measured as area of 2 cm length along the midline starting from the maximum pre-peritoneal distance in direction of the navel (PP-area).

Metabolic and inflammatory markers

Thirty-minutes fasting blood samples were collected to measure total-, HDL-, and LDL-cholesterol, triglycerides, insulin, C-peptide and C-reactive protein levels. Quality control demonstrated intra- and inter-assay coefficients of variation ranging from 0.77-1.39%, and 0.87-2.40%, respectively.

Retinal microvasculature assessment

At the age of 6 years, retinal photographs were taken by a well-trained staff in a dedicated research center. Digital retinal photographs centered on the optic disc were taken in one eye with a Topcon digital retinal camera (model TRC, NW300). We used the same methods and protocols to measure retinal vascular caliber from the digitized retinal photographs, as described in previous studies among adults and children.[9, 18] Briefly, a semi-automatic computer imaging program was used to measure the caliber of the 6 largest retinal arterioles and venules located one half to 1 disc diameter from the optic disc margin.[19] These measurements were summarized as central retinal arteriolar and venular equivalents, representing the average arteriolar and venular calibers of that eye, respectively.[20] Graders, masked to birth parameters and other participant characteristics, operated the computer program and monitored all retinal measurements. We constructed grader specific standard deviation scores for both central retinal and arteriolar equivalents. Interclass correlation coefficients between graders were 0.79 for retinal arteriolar caliber and 0.89 for retinal venular caliber, which suggest adequate reproducibility.

Covariates

We collected information about maternal and infant socio-demographic factors. Maternal age, educational level, marital status, household income and parity were assessed by questionnaires. Gestational age was established by fetal ultrasound examination during the first ultrasound visit.[11] Birth weight was obtained from medical records. The child's ethnicity (European, Non-European) was classified by the countries of birth of the parents.[21] Information on breastfeeding and average TV watching time was assessed by questionnaire.[22]

Statistical analyses

First, we examined the correlations between all fat mass measures, metabolic and inflammatory markers and retinal vessel calibers using Pearson or Spearman rank correlation coefficients. Second, we used linear regression models to explore the associations of body mass index categories (underweight, normal weight, overweight and obesity) with retinal arteriolar and venular calibers. These models were adjusted for image grader and age, sex and ethnicity of the child. Third, we used similar models to assess the associations of continuous body mass index, total fat mass, android/gynoid fat mass ratio and pre-peritoneal fat mass, and lipid, insulin, C-peptide and C-reactive protein levels with retinal arteriolar and venular calibers in childhood. We constructed standard deviation scores ((observed value- mean)/SD) for all variables in order to enable comparison in effect size of different outcome measures. All analyses were first adjusted for image grader and age, sex, ethnicity of the child and height (fat mass measures only), and additionally for maternal and infant socio-demographic factors. Analyses with metabolic and inflammatory outcomes were additionally adjusted for body mass index of the child. To explore differences in results between boys and girls, we tested for interaction terms between child's sex and fat mass measures and metabolic and inflammatory outcomes in relation to retinal vessel calibers in childhood. Since no significant interaction terms were present, no further stratified analyses were performed. In order to reduce potential bias due to missing data, we performed multiple imputation of missing covariates (<25% missing values) by generating five independent datasets using the Markov Chain Monte Carlo method, and the pooled effect estimates (95% Confidence Interval (CI)) are presented.[23] The imputation was based on the relationships between covariates, determinants and outcomes. Analyses were performed using the Statistical Package of Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Table 3.4.1 shows characteristics of the study population. The mean (SD) arteriolar and venular calibers were 159.1 µm (14.9) and 219.0 µm (20.2), respectively. **Table 3.4.2** shows that body mass index, total body fat mass and android/gynoid fat ratio were inversely correlated with retinal arteriolar caliber (range *r* -0.04, to -0.09 (p-values<0.05), respectively) but not with retinal venular caliber. C-reactive protein levels were positively correlated with retinal venular caliber (range *r* 0.14, p-values<0.05). Lipid and insulin levels were not correlated with retinal vessel calibers

Table 3.4.1. Characteristics of study population (N= 4,145)

Characteristics	Values
Maternal age (years), mean (SD)	30.4 (5.3)
Maternal education, Higher, (%)	53.9
Marital status, Married, (%)	87.5
Household income, Higher, (%)	67.2
Multiparous, Yes, (%)	44.2
Age of the child (years), median (95 % range)	6.0 (5.77, 8.0)
Sex, Boys, (%)	50
Gestational age at birth (weeks), median (95% range)	40.1 (36.0, 42.3)
Birth weight (grams), mean (SD)	3437 (542)
Ethnicity of the child, European (%)	62.9
Ever breastfeeding, (%)	92.6
TV watching >= 2 hours per day, (%)	19.3
Fat mass measures	
Height (m), mean (SD)	1.20 (0.1)
Weight (kg), mean (SD)	23.4 (4.1)
Body mass index (kg/m2), median (95% range)	15.9 (13.6, 21.4)
Total body fat mass (%), mean (SD)	25.0 (5.8)
Android/ gynoid fat ratio, mean (SD)	0.24 (0.06)
Pre-peritoneal area (cm^2), median (95% range)	0.41 (0.17, 1.27)
Metabolic risk factors	
Total-cholesterol (mmol/l), mean (SD)	4.2 (0.6)
LDL-cholesterol (mmol/l), mean (SD)	2.3 (0.5)
HDL- cholesterol (mmol/l), mean (SD)	1.4 (0.3)
Triglycerides (mmol/l), median (95% range)	0.96 (0.39, 2.37)
Insulin (U/l), median (95% range)	114.9 (17.9, 407.2)
C-peptide (ng/ml), median (95% range)	0.96 (0.30, 2.14)
C-reactive protein (mg/l), median (95% range)	0.3 (0.1, 10.7)
Retinal vessel calibers	
Retinal arteriolar caliber (µm), mean (SD)	159.1 (14.9)
Retinal venial caliber (µm), mean (SD)	219.0 (20.2)

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

Table 3.4.2. Correlation coefficients between body fat measures, metabolic and inflammatory markers and retinal vessel calibers at the age of 6 years (N = 4,145)

	Body mass index	Total body fat	Android/ Gynoid fat ratio	Pre-peritoneal fat mass	Total-cholesterol	HDL-cholesterol	LDL-cholesterol	Triglycerides	Insulin	C-peptide	C-reactive protein	Arteriolar caliber	Venular caliber
Body mass index	1												
Total body fat mass	0.68*	1											
Android/Gynoid fat ratio	0.59*	0.66*	1										
Pre-peritoneal fat mass	0.47*	0.63*	0.48*	1									
Total-cholesterol	0.03	0.13*	0.07*	0.13*	1								
HDL- cholesterol	-0.08*	-0.11*	-0.13*	-0.04	0.30*	1							
LDL- cholesterol	0.05	0.17*	0.09*	0.13*	0.85*	-0.06*	1						
Triglycerides	0.07*	0.10*	0.14*	0.08*	0.21*	-0.40*	0.07*	1					
Insulin	0.14*	0.11*	0.08*	0.10*	0.01	-0.06*	-0.06*	0.19*	1				
C-peptide	0.11*	0.07*	0.08*	0.08*	-0.01	-0.07*	-0.07*	0.17*	0.85*	1			
C-reactive protein	0.20*	0.37*	0.19*	0.21*	-0.00	-0.12*	0.00	-0.06*	-0.03	-0.03	1		
Arteriolar caliber	-0.09*	-0.04*	-0.07*	0.04	0.03	0.02	0.03	-0.02	-0.02	-0.04	0.01	1	
Venular caliber	-0.02	0.03	0.03	0.03	0.03	-0.01	0.03	-0.04	0.04	0.02	0.14*	0.48*	1

Values represent Pearson correlation coefficients and Spearmen rank correlation coefficients (pre-peritoneal area fat, and levels of triglycerides, insulin, c-peptide and c-reactive protein).
*P-value<0.05

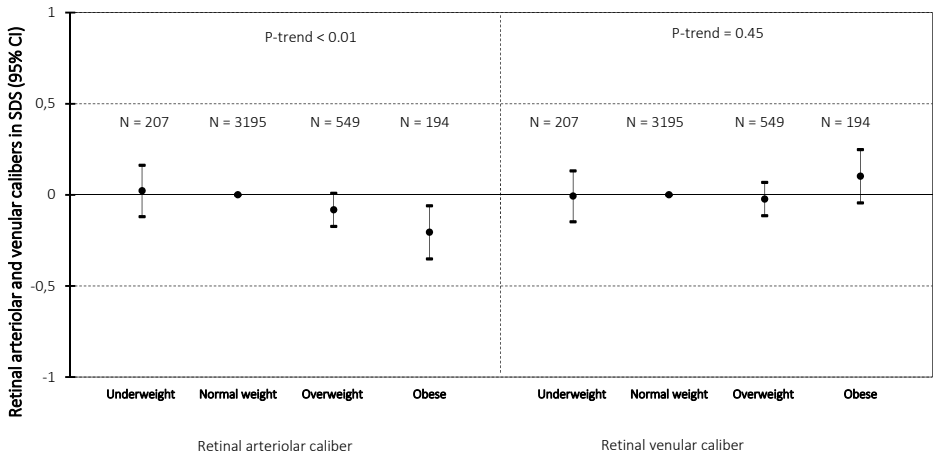
Body fat distribution, metabolic and inflammatory markers and retinal vessel calibers

Figure 3.4.1 shows that in the models adjusted for image grader, age, sex and ethnicity of the child, obese children had narrower retinal arteriolar calibers as compared to normal weight children (difference -0.21 SDS (95% CI -0.35, -0.06)). No differences in retinal venular caliber were present among children in different body mass index categories.

Table 3.4.3 presents the associations of more detailed general and abdominal fat mass measures with retinal vessel calibers in childhood. Higher body mass index and total body fat mass, but not android/gynoid fat mass ratio and pre-peritoneal fat mass, were associated with narrower retinal arteriolar caliber, with the strongest association present for body mass index (difference: -0.06 SDS (95% CI -0.09, -0.03), per SDS increase in body mass index). No associations of general and abdominal fat mass measures with retinal venular caliber in childhood were present.

Table 3.4.4 shows that in the models adjusted for image grader, age, sex and ethnicity of the child total-, LDL- and HDL-cholesterol, C-peptide and C-reactive protein levels were not associated with retinal arteriolar caliber. Total-, LDL- and HDL-cholesterol and c-peptide levels were also not associated with retinal venular caliber. Higher C-reactive protein levels were associated with wider retinal venular caliber (difference: 0.10 SDS (95% CI 0.06, 0.14), per SDS increase in C-reactive protein). This association was not explained by additional adjustment for childhood current body mass index. Adjustment for maternal and infant socio-demographic factors did not influence the observed associations (data not shown).

Figure 3.4.1. Associations of body mass index categories with retinal vessel calibers in children (N = 4,145)



Values are linear regression coefficients (95% confidence interval). The estimates represent differences in standard deviation score change of retinal vessel calibers among different obesity categories, compared with normal weight children and differences in standard deviation score change of retinal vessel calibers among children with clustering of cardiovascular risk factors. Models are adjusted for image grader, age, sex and ethnicity.

Table 3.4.3. Associations of general and abdominal fat mass measures with retinal vessel calibers in childhood (N = 4,145)

Fat mass measures	N	Arteriolar caliber (SDS)	Venular caliber (SDS)
Body mass index (SDS)	4,145	-0.06 (-0.09, -0.03)*	0.02 (-0.02, 0.05)
Total body fat mass (SDS)	4,080	-0.05 (-0.09, -0.02)*	0.01 (-0.03, 0.04)
Android/Gynoid fat ratio (SDS)	4,080	-0.04 (-0.07, 0.00)	0.00 (-0.04, 0.03)
Pre-peritoneal fat mass (SDS)	3,364	-0.03 (-0.07, 0.01)	0.02 (-0.02, 0.06)

Abbreviations: N; number; SDS; standard deviation scores; Values are linear regression coefficients (95% confidence interval). The estimates represent differences in retinal vessels caliber per standard deviation score of childhood fat mass measures. Models are adjusted for image grader, age, sex and ethnicity of the child. *P-value < 0.05

Table 3.4.4. Associations of metabolic and inflammatory markers with retinal vessel calibers in childhood (N = 2,793)

Cardiovascular risk factors	N	Arteriolar caliber (SDS)	Venular caliber (SDS)
Total-cholesterol (SDS)			
Basic models	2786	-0.01 (-0.05, 0.03)	0.00 (-0.03, 0.04)
Body mass index		-0.01 (-0.04, 0.03)	0.00 (-0.03, 0.04)
HDL- cholesterol (SDS)			
Basic models	2788	0.01 (-0.03, 0.05)	0.01 (-0.03, 0.05)
Body mass index		0.01 (-0.03, 0.04)	0.01 (-0.02, 0.05)
LDL- cholesterol (SDS)			
Basic models	2789	0.00 (-0.04, 0.03)	0.01 (-0.03, 0.04)
Body mass index		0.00 (-0.03, 0.04)	0.01 (-0.03, 0.04)
Triglycerides (SDS)			
Basic models	2780	0.00 (-0.04, 0.04)	-0.01 (-0.05, 0.02)
Body mass index		0.01 (-0.03, 0.04)	-0.02 (-0.05, 0.02)
Insulin (SDS)			
Basic models	2760	-0.01 (-0.05, 0.03)	0.00 (-0.04, 0.03)
Body mass index		0.00 (-0.04, 0.04)	0.00 (-0.04, 0.04)
C-peptide (SDS)			
Basic models	2762	-0.02 (-0.06, 0.01)	-0.02 (-0.05, 0.02)
Body mass index		-0.01 (-0.05, 0.03)	-0.02 (-0.06, 0.02)
C-reactive protein (SDS)			
Basic models	2793	0.01 (-0.03, 0.04)	0.10 (0.06, 0.14)*
Body mass index		0.03 (-0.01, 0.06)	0.09 (0.06, 0.13)*

Abbreviations: N; number; SDS; standard deviation scores; Values are linear regression coefficients (95% confidence interval). The estimates represent differences in retinal vessels diameters per standard deviation score of lipids levels, insulin, c-peptide and C-reactive protein. Basic models are adjusted for image grader, age, sex and ethnicity of the child. Full models are additionally adjusted for body mass index of the child. *P-value < 0.05

Discussion

Among a low risk population of school-age children, higher total body and abdominal fat mass measures were associated with narrower retinal arteriolar caliber, but not venular caliber. Lipid and insulin levels were not associated with retinal vessel calibers, whereas higher C-reactive protein levels were associated with wider childhood retinal venular caliber.

Methodological considerations

We used a population-based cohort study design with a large number of subjects. The response rate at baseline for participation in the Generation R Study cohort was 61%. We do not expect that this non-response at baseline led to biased effect estimates because biased estimates in large cohort studies mainly arise from loss to follow-up rather than from non-response at baseline.[24] Of all children participating at the age of 6 years, 64 % participated in the adiposity, cardiovascular and retinal vessels follow-up

studies. This loss to follow-up could lead to selection bias if the associations of body fat distribution, metabolic and inflammatory markers with retinal vessel calibers would differ between children included and not included in the final analyses.[11] This seems unlikely. We obtained detailed measures of childhood adiposity and metabolic and inflammatory markers. DXA quantifies fat content with high precision and has the capacity for regional analysis, but cannot differentiate between abdominal visceral and subcutaneous fat compartments. We measured abdominal fat mass by ultrasound, a valid method for measuring both subcutaneous abdominal fat mass and pre-peritoneal fat mass, as a proxy for visceral abdominal fat mass.[17] Both DXA and abdominal ultrasound have been validated against CT.[16] Use of 30-minutes fasting blood samples may have led to underestimation of the observed associations. However, it has been shown in adults that non-fasting lipid levels can accurately predict increased risks of cardiovascular events in later life.[25] We used validated techniques to measure retinal vessel calibers. We did not take into account other ocular factors that might affect retinal vessel measurements, such as axial length and refractive error.[26, 27] However, it has been previously shown that these factors have only a small impact on the measurement of retinal vessel calibers and did not influence the associations between retinal vessel calibers and cardio-metabolic risk factors.[28] Although, we adjusted for a large number of potential socio-demographic factors, residual confounding in the observed associations might still occur, as in any observational study. Due to the cross-sectional analyses, we were not able to explore directions and causality of the observed associations. Based on previous studies we hypothesized that increased obesity and metabolic risk factors levels might lead to alterations in retinal microvasculature, mainly through increased inflammatory responses and increased total blood volume among obese children.[29, 30]

Interpretation of main findings

Childhood obesity and cardiovascular risk factors at younger ages are associated with higher risks of cardiovascular diseases and premature death in adulthood.[31] It has been hypothesized that microvasculature adaptations involved in the mechanisms linking obesity with cardiovascular diseases.[32, 33] Increased endothelial dysfunction, increased inflammatory responses and anatomic alterations such as medial hyperplasia and hyalinization of micro-vessels are shown to play an important role on the development of hypertension, stroke and premature death.[30, 34] In humans microvasculature can be assessed by retinal photography. Studies among adults have shown that retinal arteriolar narrowing is strongly associated with higher systolic blood pressure, and independently predicts the risk of stroke.[35, 36] In contrast, obesity and metabolic syndrome were associated with wider retinal venular caliber in adults.[4] Whether microvascular adaptations in response to body fatness and metabolic risk factor profiles are already present in childhood is not well known. Few studies have examined the associations of body mass index and retinal vessel calibers among children. A study among 2,353 children aged 12.7 years showed that children in the highest quartile of body mass index had narrower retinal arteriolar caliber and wider retinal venular caliber than children in the lowest quartile of body mass index.[6] In line with these findings, we observed that obese children had narrower retinal arteriolar caliber and tended to have wider venular caliber as compared to normal weight children.

Body mass index does not distinguish lean mass from fat mass. More detailed measures of general and abdominal fat mass distribution might be better predictors of development of cardiovascular and metabolic diseases.[8] Two studies among adults aged 50 years and older reported that higher waist circumference and waist to hip ratio were associated with wider retinal venular caliber.[4, 37] A study among 2,353 children aged 12.7 years also reported wider retinal venular caliber among children in higher quartiles of waist circumference.[6] Another study among 136 children aged between 6 and 16 years observed wider retinal venular caliber among children with an increase triceps skinfold.[38] We observed that total fat mass was associated with narrower retinal arteriolar caliber, but not with venular caliber. We did not observe an association of android/gynoid fat mass and pre-peritoneal fat mass, markers of waist circumference and visceral fat mass, respectively, with childhood retinal vessel calibers. This may be explained by a small accumulation and less pathogenic visceral adipose tissue at younger ages, than at older ages.

Associations of lipid and insulin levels with retinal vessel calibers were mainly studied among adults. Two studies among adults found that lower HDL-cholesterol levels, but not total- and LDL-cholesterol and triglycerides levels, were associated with narrower retinal arteriolar caliber.[37, 39] Inconsistent results for the associations of diabetes and impaired fasting glucose with retinal vessel calibers have been reported among adults.[40, 41] A study among 573 children aged 11 years showed that higher triglyceride and glucose levels were associated with wider retinal venular caliber.[7] In our study, lipid and insulin levels were not associated with retinal arteriolar and venular calibers in school-age children. We did not have glucose levels available in our study. In line with studies among adults and children, we found that higher C-reactive protein levels were associated with wider retinal venular caliber.[7, 9] Increased systemic inflammation among obese adults and children may be thus be a risk factor for development of wider venular caliber.[7, 10] These results suggest that increased adiposity and subsequently increased subclinical inflammation may already be associated with alterations in the microcirculation in childhood.

Excess body fat causes adipocyte dysfunction, which can lead to higher expression of pro-inflammatory proteins and stimulate the hepatic production of acute phase reactants, such as C-reactive protein levels.[42, 43] Increased C-reactive protein levels play an important role in the destruction of venular endothelial layer mediated by the activation of the leucocytes, and subsequently lead to wider retinal venular caliber.[44] Increased inflammatory protein levels also stimulate the endothelial nitric oxide release, which can initiate relaxation of smooth muscle of the vessels and vasodilation.[45] However, the associations of higher C-reactive protein levels with wider retinal vessel calibers remained present after adjustment for body mass index, which suggest that other patho-physiological mechanisms might be involved.

Our findings suggest that microvascular adaptations in response to increased body fat and increased inflammation already occur in childhood. Further studies are needed to examine other potential mechanisms involved in the development of microvasculature alterations. Also, whether microvasculature alterations can affect the risk of cardiovascular disease in later life needs to be further studied.

Conclusions

Among a low risk population of school-age children, higher general and abdominal fat mass measures were associated with narrower retinal arteriolar caliber, and higher C-reactive protein levels were associated with wider retinal venular caliber. Further studies are needed to examine whether the alterations in the microvasculature influence the development of cardiovascular diseases in later life.

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Supplemental Material

Figure S3.4.1. Flow chart of participants in study

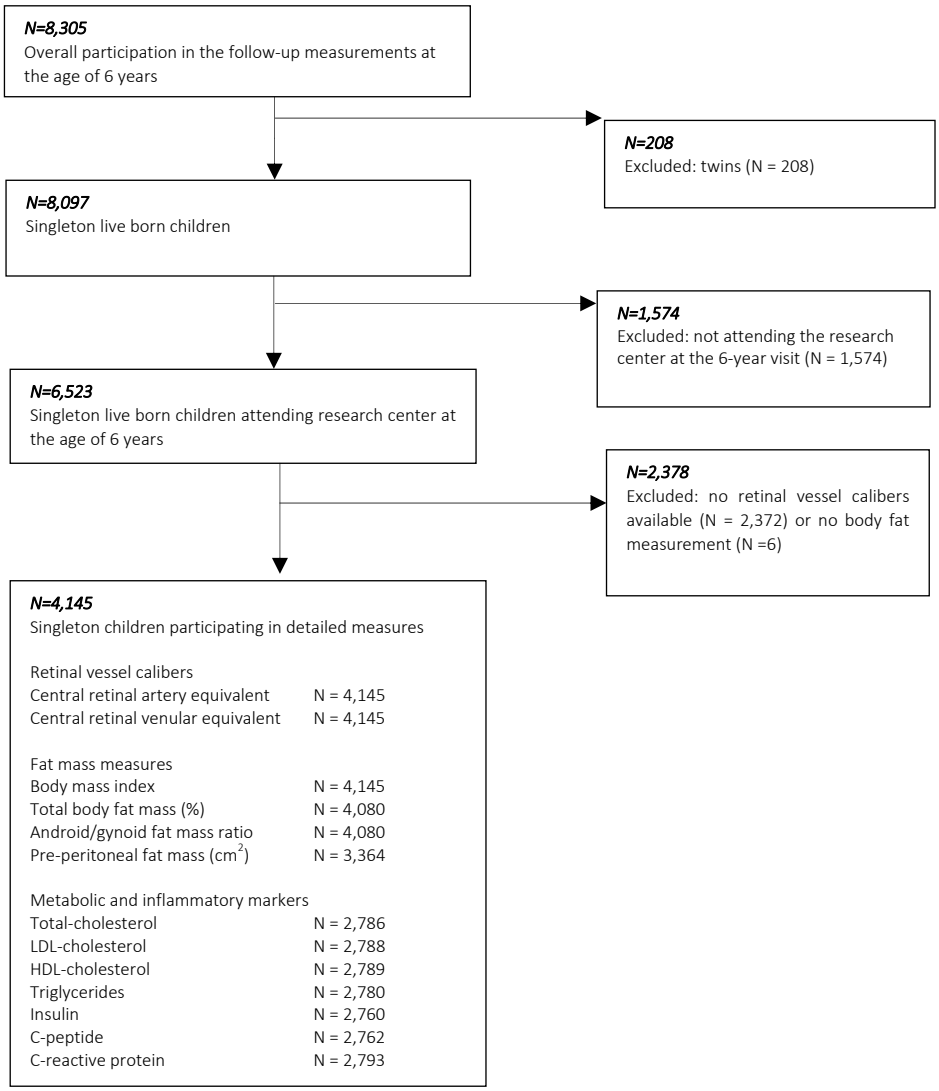

Table S3.4.1. Comparison of subject characteristics between children included and not included in the analyses

Characteristics	Participation	No participation	P value
Childhood characteristics			
Age of the mother (years), mean (SD)	30.4 (5.3)	30.7 (5.0)	*0.03*
Education, Higher, (%)	53.9	47.4	*0.01*
Marital status, Married, (%)	87.5	88.2	*0.23*
Household income, Higher, (%)	67.2	66.3	*0.05*
Multiparous, Yes, (%)	44.2	41.0	*0.09*
Age of the child (years), median (95 % range)	6.0 (5.8, 8.0)	6.0 (5.4, 7.2)	*<0.01*
Sex, Boys, (%)	50	50.6	*0.31*
Gestational age at birth (weeks)	40.1 (36.0, 42.3)	40.1 (35.1, 42.3)	*0.05*
Birth weight (grams), mean (SD)	3437 (542)	3430 (576)	*0.01*
Ethnicity of the child, (%)			
European	62.9	65.3	*0.01*
Non-European	37.1	34.7	
Ever breastfeeding, (%)	92.6	92.0	*0.21*
TV watching >= 2 hours per day, (%)	19.3	19.7	*0.39*

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

Chapter 3.5

Retinal microvasculature and cardiovascular health in childhood

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Abstract

Background: Alterations in retinal microvasculature are associated with increased risks of cardiovascular diseases. We examined the associations of retinal vessel calibers with cardiovascular markers in school-age children.

Methods and Results: Among 4,007 school-age-children we measured retinal vessel calibers from digitized retinal photographs and cardiovascular markers at the median age of 6.0 years. Narrower retinal arteriolar caliber was associated with a higher systolic and diastolic blood pressure (differences: -0.20 standard deviation score (SDS) (95%CI: -0.24, -0.18), and -0.14 SDS (95% CI: -0.17, -0.11), per SDS increase in retinal arteriolar caliber), mean arterial pressure and pulse pressure, but not with carotid-femoral pulse wave velocity, heart rate, cardiac output and left ventricular mass. A wider retinal venular caliber was associated with a lower systolic blood pressure, mean arterial pressure, pulse pressure and a higher carotid-femoral pulse wave velocity (differences in carotid-femoral pulse wave velocity: 0.04 SDS (95% CI: 0.01, 0.07), per SDS increase in retinal venular caliber). Both narrower retinal arteriolar and venular calibers were associated with higher risks of hypertension at the age of 6 years, with the strongest associations for retinal arteriolar caliber (Odds ratio: 1.35 (95% CI 1.21, 1.45) per SDS decrease in arteriolar caliber). Adjustment for maternal and infant socio-demographic factors did not influence the observed associations.

Conclusions: Both retinal arteriolar and venular calibers are associated with childhood blood pressure, whereas retinal venular caliber is associated with carotid-femoral pulse wave velocity in school-age-children. Microvascular adaptations in childhood might influence cardiovascular health and disease from childhood onwards.

Introduction

It has been suggested that the development of hypertension might originate in early life.[1] Early alterations in the microvasculature structure might be part of the underlying mechanism leading to the development of hypertension.[2] Studies in rats have shown that alterations in the microvascular structure and, hence, increased peripheral resistance precede the development of hypertension.[3] Over the last few decades, advances in retinal photography have allowed us to non-invasively assess the microvasculature in humans. Several cross-sectional and longitudinal studies among adults have shown that retinal arteriolar narrowing is associated with an increased risk of hypertension[4, 5], whereas wider retinal venular caliber is associated with an increased risk of metabolic syndrome and inflammation.[6] However, it remains unclear whether microvascular abnormalities also affect cardiovascular risk factors in childhood. Only few studies among children have examined the associations of retinal vessel calibers with cardiovascular risk factors, and suggested that retinal arteriolar narrowing correlates with increased blood pressure levels in children at 6 years and 12 years old.[7, 8] However, these studies did not have information about other cardiovascular markers.

Therefore, we examined among 4,007 children participating, the cross-sectional associations of retinal vessel calibers with detailed cardiovascular markers.

Methods

Study design

This study was embedded in the Generation R Study in Rotterdam, the Netherlands.[9] The study protocol was approved by the Medical Ethical Committee of the University Medical Center Rotterdam. In total, 6,523 children participated in the detailed follow-up measurements at the median age of 6.0 years (95% range 5.7- 8.0). Retinal vessel calibers were available in 4,007 children. Missing retinal vessel measurements were mainly due to the later start of these measurements during the follow-up visits (Supplemental **Figure S3.5.1**). Birth weight was lower among children who were not included in the analyses, as compared to children who were included (Supplemental **Table S3.5.1**).

Retinal microvasculature assessment

At the age of 6 years retinal photographs were taken by a well-trained staff in a dedicated research center. Digital retinal photographs centered on the optic disc were taken in one eye with images resolution 4096 and 3072 pixels, using Topcon digital retinal camera (model TRC, NW300). We use the same methods and protocols to measure retinal vascular caliber from the digitized retinal photographs, as described in previous studies among adults and children.[10, 11] Briefly, a semi-automatic computer imaging program was used to measure the caliber of the 6 largest retinal arterioles and venules located one half to 1 disc diameter from the optic disc margin.[12] Using the revised Knudtson-Parr-Hubbard formula, absolute arteriolar and venular diameter were estimated in micrometers and subsequently were summarized as central retinal arteriolar and venular equivalents, representing the average arteriolar and venular caliber of that eye, respectively.[13] Graders, masked to birth parameters and other participant characteristics, operated the computer program and monitored all retinal measurements. We

constructed grader specific standard deviation scores for both central retinal and arteriolar equivalents. Interclass correlation coefficients between graders were 0.79 for retinal arteriolar caliber and 0.89 for retinal venular caliber, which suggest adequate reproducibility.

Cardiovascular markers

At the age of 6 years we measured blood pressure at the right brachial artery with the child lying quietly in supine position, four times with one-minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus TM (Paramus, NJ, USA).[14] A cuff was selected with a width approximately 40% of the arm circumference and long enough to cover 90% of the arm circumference. We calculated the mean value for systolic and diastolic blood pressure using the last three blood pressure measurement of each participant. We subsequently calculated the mean arterial pressure (mean arterial pressure = (systolic blood pressure + 2×blood pressure)/3) and pulse pressure (pulse pressure = (systolic blood pressure – diastolic blood pressure)). We defined hypertension as systolic and diastolic blood pressure above the 95th percentile, using age and height specific cut-points defined by the National High Blood Pressure Education Program Working Group on High Blood Pressure.[15] Carotid-femoral pulse wave velocity was assessed using the automatic Complior SP device (Complior; Artech Medical, Pantin, France) with participants in supine position, as described in detail.[16] The distance between the recording sites at the carotid (proximal) and femoral (distal) artery was measured over the surface of the body to the nearest centimeter. Carotid-femoral pulse wave velocity was calculated as the ratio of the distance traveled by the pulse wave and the time delay between the waveforms, as expressed in meters per second. Carotid-radial pulse wave velocity can be measured reliably in large pediatric population-based cohorts, with interclass correlation coefficients within- and between-technicians varying higher than 0.96.[17] Two-dimensional echocardiography was used to measure the interventricular end-diastolic septal thickness, left ventricular end-diastolic diameter, left ventricular end-diastolic posterior wall thickness, heart rate and cardiac output. These measurements were performed using methods recommended by the American Society of Echocardiography, and used to calculate the left ventricular mass and (left ventricular relative wall thickness = (2×diastolic posterior wall thickness/left ventricular end-diastolic diameter)).[18, 19]

Covariates

We collected information about maternal age, parity, educational level, pre-pregnancy body mass index (BMI), smoking during pregnancy and folic acid supplement use by questionnaires. Maternal and paternal blood pressure was assessed at enrollment. Information on ethnicity was obtained from the first questionnaire at enrolment in the study [9]. Gestational age at birth was based on fetal ultrasound examination during the first ultrasound visit. Birth weight was obtained from medical records. Information on breastfeeding and average TV watching time was assessed by questionnaire. At the age of 6 years we measured height and weight and calculated BMI (kg/m^2).

Statistical analyses

First, we examined the correlations between all retinal vessel calibers and cardiovascular markers using Pearson correlation coefficients. Second, we used multiple linear regression models to assess the associations of retinal vessel calibers with vascular outcomes (systolic and diastolic blood pressure, mean arterial pressure, pulse pressure and carotid-femoral pulse wave velocity), and cardiac outcomes (heart rate, cardiac output, left ventricular mass and left ventricular relative wall thickness). For these analyses we constructed standard deviation scores (SDS) ((observed value- mean)/SD) for all variables in order to enable comparison in effect size of different outcome measures. Since the outcomes are highly correlated, we regrouped them in three groups and we applied Bonferroni-Holm correction taking account for three groups of outcomes. Third, we used logistic regression models to examine the associations of retinal vessel calibers with the risk of hypertension in childhood. All analyses were adjusted for image grader and age, sex and ethnicity of the child. We additionally adjusted these models for maternal and infant socio-demographic factors, birth outcomes and childhood BMI. We tested for interaction terms between child's sex and BMI with retinal vessel calibers in relation to cardiovascular risk factors in childhood. Since no significant interaction terms were present, no further stratified analyses were performed. Analyses were performed using the Statistical Package of Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Table 3.5.1 shows characteristics of the study population. The mean (SD) arteriolar and venular calibers were 159.0 µm (14.9) and 219.0 µm (20.2), respectively. Supplemental Table S3.5.2 gives the correlation coefficients between the retina vessel calibers and cardiovascular markers.

Retinal vessel calibers and cardiovascular markers in childhood

Table 3.5.2 shows that in the models adjusted for image grader and age, sex and ethnicity of the child, narrower retinal arteriolar caliber was associated with a higher systolic and diastolic blood pressure, mean arterial pressure and pulse pressure (differences: -0.20 SDS (95%CI: -0.24, -0.18), -0.14 SDS (95% CI: -0.17, -0.11), -0.18 SDS (95%CI: -0.21, -0.15) and -0.10 SDS (95%CI: -0.13, -0.06) per SDS increase in retinal arteriolar caliber). No associations were present for retinal arteriolar caliber with carotid-femoral pulse wave velocity. Narrower retinal venular caliber was associated with a higher systolic blood pressure (difference: -0.05 SDS (95% CI: -0.08, -0.02), per SDS increase in retinal venular caliber), mean arterial pressure and pulse pressure, but was not associated with diastolic blood pressure in childhood. A wider retinal venular caliber was associated with a higher carotid-femoral pulse wave velocity (difference: 0.04 SDS (95% CI: 0.01, 0.07), per SDS increase in retinal venular caliber). Additional adjustment for maternal and infant socio-demographic factors and birth outcomes did not materially affect the observed associations. These associations remained significant even after taking multiple testing into account.

Table 3.5.1. Characteristics of study population (N = 4,007)

Characteristics	Values
Maternal characteristics	
Age, median (95% range), y	31.0 (19.7, 39.9)
Height, mean (SD), cm	167.0 (7.4)
Pre-pregnancy weight, mean (SD), kg	67.0 (12.7)
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.7 (18.1, 34.9)
Maternal systolic blood pressure, mean (SD), mm/Hg	115 (12)
Maternal diastolic blood pressure, mean (SD), mm/Hg	68 (10)
Paternal systolic blood pressure, mean (SD), mm/Hg	130 (13)
Paternal diastolic blood pressure, mean (SD), mm/Hg	73 (10)
Parity, nulliparous (%)	55.7
Education, higher (%)	46.1
Folic acid use, never (%)	26.0
Smoked during pregnancy, Yes (%)	25.9
Birth and infant characteristics	
Sex, Boys (%)	50.2
Gestational age at birth, median (95 % range), weeks	40.1 (36.0, 42.3)
Birth weight, mean (SD), grams	3438 (542)
Ethnicity, European (%)	63.2
Ever breastfeeding (%)	92.6
TV watching >= 2 hours per day (%)	19.2
Childhood characteristics	
Age, median (95 % range), y	6.0 (5.7, 8.0)
Body mass index, median (95 % range), kg/m2	15.9 (13.6, 21.4)
Retinal arteriolar caliber, mean (SD), µm	159.0 (14.9)
Retinal venular caliber, mean (SD), µm	219.0 (20.2)
Systolic blood pressure, mean (SD), mm/Hg	103 (8)
Diastolic blood pressure, mean (SD), mm/Hg	61 (7)
Mean arterial pressure, mean (SD), mm/Hg	75 (7)
Pulse pressure, mean (SD), mm/Hg	42 (7)
Carotid-femoral pulse wave velocity, mean (SD), cm/s	5.5 (0.9)
Heart rate, mean (SD), beats/min	82 (12)
Cardiac output, mean (SD), L/min	3.2 (0.7)
Left ventricular mass, mean (SD), g/ m^2	53.6 (11.9)
Left ventricular relative wall thickness, mean (SD)	0.30 (0.05)

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

Table 3.5.2. Associations of retinal arteriolar and venular calibers with vascular outcomes in childhood (N = 4,007)

Retinal vessel calibers in SDS	Vascular outcomes in SDS				
	Systolic blood pressure	Diastolic blood pressure	Mean arterial pressure	Pulse pressure	Carotid femoral pulse wave velocity
Retinal arteriolar caliber	*N = 4,007*	*N = 4,007*	*N = 4,007*	*N = 4,007*	*N = 3,821*
Basic model	-0.20 (-0.24, -0.18)*	-0.14 (-0.17, -0.11)*	-0.18 (-0.21, -0.15)*	-0.10 (-0.13, -0.06)*	0.02 (-0.01, 0.05)
Full model	-0.19 (-0.21, -0.16)*	-0.13 (-0.16, -0.10)*	-0.17 (-0.20, -0.14)*	-0.10 (-0.12, -0.07)*	0.02 (-0.02, 0.05)
Retinal venular caliber	*N = 4,007*	*N = 4,007*	*N = 4,007*	*N = 4,007*	*N = 3,821*
Basic model	-0.05 (-0.08, 0.02)*	-0.01 (-0.04, 0.02)	-0.03 (-0.06, -0.00)*	-0.03 (-0.07, -0.00)*	0.04 (0.01, 0.07)*
Full model	-0.05 (-0.08, 0.02)*	-0.01 (-0.04, 0.02)	-0.03 (-0.06, 0.00)	-0.05 (-0.08, -0.02)*	0.04 (0.01, 0.08)*

Values are linear regression coefficients (95% confidence interval). The estimates represent differences in vascular outcomes per SDS increase in retinal arteriolar and venular calibers. Basic models are adjusted for image grader, age, sex and ethnicity. Full models are additionally adjusted for maternal age, parity, education, pre-pregnancy body mass index, parental blood pressure at intake, smoking and folic acid supplement during pregnancy, and child's breastfeeding, TV watching, gestational age and weight at birth, and childhood body mass index.* $P < 0.05$

Table 3.5.3 shows that no associations were present for retinal arteriolar and venular calibers with heart rate, cardiac output, left ventricular mass and left ventricular relative wall thickness. A sensitivity analysis performed among preterm born children and children born with a low birth weight showed similar results as for the total group (data not shown).

Table 3.5.3. Associations of retinal arteriolar and venular calibers with cardiac outcomes in childhood (N = 4,007)

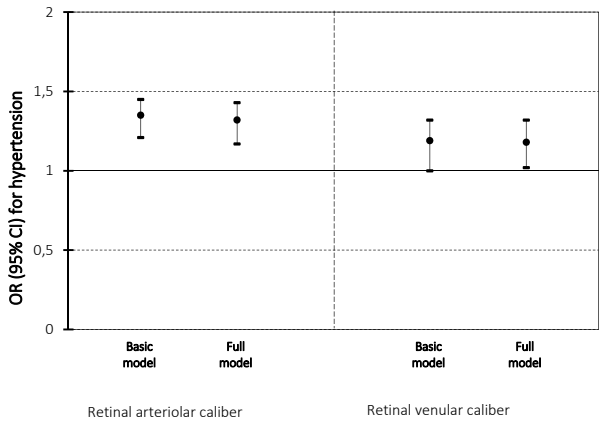
Retinal vessel calibers in SDS	Cardiac outcomes in SDS			
	Heart rate	Cardiac output	Left ventricular mass	Left ventricular relative wall thickness
Retinal arteriolar caliber	*N = 3,537*	*N = 3,520*	*N = 3,817*	*N = 3,809*
Basic model	-0.03 (-0.06, 0.00)	-0.03 (-0.06, 0.00)	0.00 (-0.03, 0.03)	0.00 (-0.03, 0.03)
Full model	-0.03 (-0.06, 0.01)	-0.02 (-0.06, 0.01)	0.00 (-0.02, 0.03)	0.01 (-0.03, 0.04)
Retinal venular caliber	*N = 3,537*	*N = 3,520*	*N = 3,817*	*N = 3,809*
Basic model	0.03 (0.00, 0.06)	0.00 (-0.03, 0.04)	0.01 (-0.02, 0.04)	0.01 (-0.02, 0.04)
Full model	0.03 (-0.00, 0.06)	0.00 (-0.03, 0.03)	0.01 (-0.02, 0.03)	-0.01 (-0.02, 0.04)

Values are linear regression coefficients (95% confidence interval). The estimates represent differences in cardiac outcomes per SDS increase in retinal arteriolar and venular calibers. Basic models are adjusted for image grader, age, sex and ethnicity. Full models are additionally adjusted for maternal age, parity, education, pre-pregnancy body mass index, parental blood pressure at intake, smoking and folic acid supplement during pregnancy, and child's breastfeeding, TV watching, gestational age and weight at birth, and childhood body mass index.* P < 0.05

Retinal vessel calibers and the risk of hypertension

Figure 3.5.1 shows that narrower retinal arteriolar and venular calibers were associated with higher risks of hypertension at the age of 6 years, with the strongest associations for retinal arteriolar caliber (Odds ratios: 1.35 (95% CI 1.21, 1.45), 1.19 (95% CI 1.00, 1.32), per SDS decrease in arteriolar and venular calibers, respectively). Additional adjustment for maternal and infant socio-demographic factors and birth outcomes did not influence the observed associations.

Figure 3.5.1. Associations of retinal vessels with the risk of hypertension in children (N = 4007)



Values are odds ratios (95% confidence interval). The estimates represent risk of hypertension per standard deviation score decrease in retinal vessels calibers. Basic model is adjusted for image grader, age, sex and ethnicity. Full models are additionally adjusted for maternal age, parity, education, pre-pregnancy body mass index, parental blood pressure at intake, smoking and folic acid supplement during pregnancy, and child's breastfeeding, TV watching, gestational age and weight at birth, and childhood body mass index.* P < 0.05

Discussion

We observed in a low-risk community-based population of school-age children, that narrower retinal arteriolar caliber was associated with higher systolic and diastolic blood pressure, mean arterial blood pressure and pulse pressure, whereas wider retinal venular caliber was associated with a lower systolic blood pressure and a higher carotid-femoral pulse wave velocity. Children with narrower retinal arteriolar and venular calibers were more likely to have an increased risk of elevated blood pressure.

Methodological considerations

We used a population-based cohort study design with a large number of subjects. Of all children participating in follow-up measurements at the age of 6 years, 64% participated in the retinal vessels and cardiovascular follow-up studies. The non-response could lead to biased effect estimates if the associations of different obesity measures and cardiovascular risk factors with retinal vessel calibers would be different between children included and not included in the analyses.[9] Birth weight was lower in children who were not included in the current analyses compared to those who were included ($p < 0.01$). We used validated techniques to measure retinal vessels calibers. We did not take into account other ocular factors, such as axial length and refractive error that might affect retinal vessels measurement.[20] However, it has been previously shown among adults that these factors have only a small impact on the measurement of retinal vessel calibers.[21] Due to the cross-sectional nature of the analyses, we were not able to explore directions and causality of the observed associations.

Interpretation of main findings

Cardiovascular risk factors at younger ages are associated with higher risk of disease and premature death in adulthood.[22] Previous studies have hypothesized that microvasculature alterations might be part of the early underlying mechanisms in the development of cardiovascular disease.[2] These studies were mostly conducted among adults and focused on the associations of retinal vessel calibers with blood pressure. Among adults, retinal arteriolar narrowing is strongly associated with higher pressure, and independently predicted the risk of stroke [23, 24], whereas wider retinal venular caliber was associated with increased risks of metabolic syndrome and measures of inflammation.[6] It remains unclear whether microvascular abnormalities are a result of cardiovascular disease or are part of the factors that relate to the development of these diseases. Thus, examining the associations among children, without clinical cardiovascular disease, may provide further insight in the pathways underlying these associations.

Increased blood pressure is the leading factor for cardiovascular disease and pulse pressure has been recognized as a risk factor for stroke.[25] Previously, a study among 5,628 adults showed that narrower retinal arteriolar caliber is associated with increased blood pressure, independent from baseline blood pressure and BMI.[26] Studies among children also observed significant inverse associations between retinal arteriolar caliber and blood pressure.[27, 28] Two cross-sectional studies among 1,953 children aged 6-8 years and 587 children aged 11 years found significant inverse associations between retinal arteriolar caliber and systolic and diastolic blood pressure levels, and mean arte-

rial pressure.[8, 27] We also observed that narrower retinal arteriolar caliber was associated with higher systolic and diastolic blood pressure, mean arterial pressure and pulse pressure independent of maternal and infant socio-demographic factors and childhood BMI. Results from previous studies on the associations of retinal venular caliber with blood pressure are inconsistent.[8, 27, 28] A study among 578 children at the age of 11 years observed that narrower retinal venular caliber was associated with higher diastolic, but not systolic blood pressure.[27] Another cross-sectional study among children aged 4-5 years showed that increased systolic blood pressure was associated with wider venular caliber.[28] In our study we observed that narrower retinal venular caliber was associated with higher systolic blood pressure and pulse pressure, but not with diastolic blood pressure. Retinal arteriolar and venular calibers were positively correlated (r=0.43). Therefore, the observed associations of retinal venular caliber with systolic blood pressure might be explained by confounding by retinal arteriolar caliber.[29]

A meta-analysis showed that retinal arteriolar narrowing, likely indicative of increased peripheral vascular resistance, leads to subsequent development of hypertension in adults.[30] Also, cross-sectional studies among children aged 6-8 and 12.7 years have shown that narrower retinal arterioles were significantly correlated with increased risks of hypertension.[8, 31] In line with these studies, we also found significant associations of narrower retinal arteriolar and venular calibers with increased risks of elevated blood pressure in school age children.

Carotid-femoral pulse wave velocity is a marker of arterial stiffness and it is associated with cardiovascular disease in adults.[32] Among adults, a cross-sectional study among 135 individuals showed that arterial stiffness measured by central pulse pressure was inversely correlated with wall-to-lumen ratio of retinal arterioles.[33] Another study among 197 adults found that retinal arteriolar narrowing was associated with higher carotid-femoral pulse wave velocity.[34] No previous study among children examined the associations of retinal vessel calibers with carotid-femoral pulse wave velocity. In our study we observed that wider retinal venular caliber was associated with a higher carotid-femoral pulse wave velocity, suggesting that microvasculature alterations might affect carotid-femoral pulse wave velocity already from childhood onwards.

Increased left ventricular mass is an early pathogenic process in the development of heart failure.[35] In children increased left ventricular mass is correlated with higher adiposity and hypertension.[36, 37] A longitudinal study among 132 children showed that increased left ventricular mass tends to track between 13 and 27 years.[36] Only few studies examined the associations of retinal microvasculature with left ventricular mass and were all conducted among adults. A study among 4,593 adults showed that narrower retinal arteriolar caliber was associated with left ventricular mass remodeling, independent from traditional risk factors for cardiovascular diseases.[38] Contrary, in our population of school age children we did not find significant associations of retinal vessel calibers with left ventricular mass. These associations may become more apparent at older ages. We also did not find significant associations of retinal vessel calibers with cardiac output and heart rate, which are important cardiac measures and are shown to contribute to cardiovascular disease development. To the best of our knowledge, no previous study examined the associations of retinal vessel calibers with these cardiac measures.

Our findings suggest that microvasculature alterations might be involved in the early mechanisms leading to cardiovascular diseases in later life. The retinal vessels share similar anatomical and physio-logical properties with coronary and cerebral microcirculation and have been suggested as biomarkers to estimate systemic vascular health.[6, 39] Eutrophic remodeling [40], and anatomic alterations such as intimal thickness and hyalinization may be mechanisms leading to narrowing retinal arteriolar vessels, which as a consequence might lead to an increase peripheral resistance and a higher blood pressure in later life.[2] Although the observed effect estimates for the associations of retinal vessel calibers with different cardiovascular markers were small, these findings are important from an etiological perspective. Previous studies have shown that cardiovascular risk factors tend to track from childhood into adulthood.[1, 41] A large meta-analysis showed that blood pressure tracks from childhood into adulthood, and early-life blood pressure is associated with increased cardiovascular risk in later life.[42] Thus, these studies suggest that subclinical differences in risk factors for cardiovascular disease in childhood are related to the development of cardiovascular disease in later life. Further studies are needed to examine the long-term consequences of the observed differences in cardiovascular indices throughout the life course.

Conclusions

We observed that narrower retinal arteriolar caliber was associated with higher systolic and diastolic blood pressure, mean arterial blood pressure and pulse pressure, whereas wider retinal venular caliber was associated with a lower systolic blood pressure and a higher carotid-femoral pulse wave velocity. These results suggest that retinal arteriolar and venular adaptations are markers of microvascular pathways that ultimately lead to the development of cardiovascular risk factors in later life. Further studies are needed to usefulness of examining the predictive value of alterations in the retinal microvasculature in the development of clinical cardiovascular diseases throughout the life course.

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Supplemental Material

Figure S3.5.1. Flow chart of participants in study

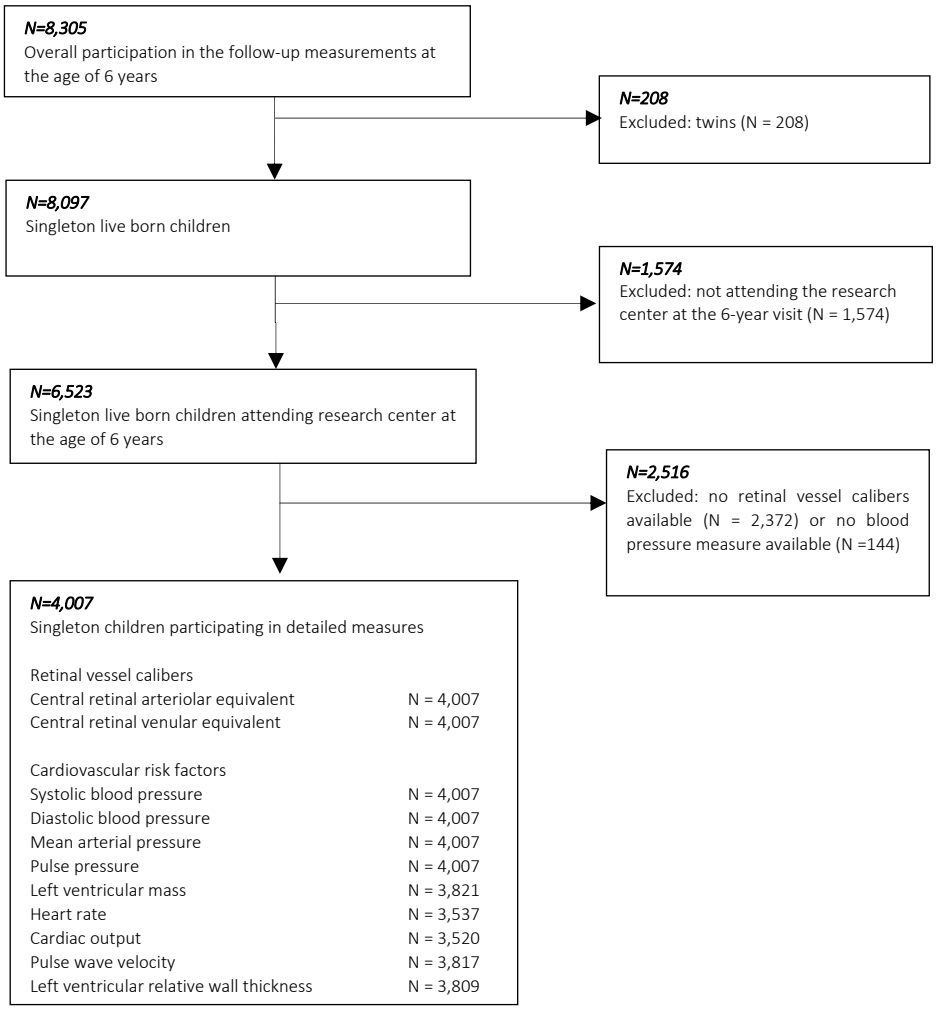

Table S3.5.1. Comparison of subject characteristics between children included and not included in the analyses

Characteristics	Participation N= 4007	No participation N= 2516	P value
Maternal characteristics			
Age, median (95% range), y	31.0 (19.7, 39.9)	31.2 (20.0, 39.8)	*0.12*
Height, mean (SD), cm	167.0 (7.4)	168.0 (7.3)	*0.46*
Pre-pregnancy weight, mean (SD), kg	67.0 (12.7)	66.0 (12.3)	*0.09*
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.7 (18.1, 34.9)	22.6 (17.9, 34.2)	*0.05*
Maternal systolic blood pressure, mean (SD), mm/Hg	115 (12)	116 (12)	*0.23*
Maternal diastolic blood pressure, mean (SD), mm/Hg	68 (10)	68 (9)	*0.33*
Paternal systolic blood pressure, mean (SD), mm/Hg	130 (13)	131 (14)	*0.29*
Paternal diastolic blood pressure, mean (SD), mm/Hg	73 (10)	74 (10)	*0.12*
Parity, nulliparous (%)	55.7	57.7	*0.06*
Education, higher (%)	46.1	47.4	*0.04*
Folic acid use, never (%)	26.0	24.0	*0.29*
Smoked during pregnancy, Yes (%)	25.9	25.7	*0.57*
Childhood characteristics			
Age, median (95% range), y	6.0 (5.7, 8.0)	6.0 (5.5, 7.3)	*<0.01*
Sex, Boys (%)	50.2	50.3	*0.47*
Ethnicity of the child, (%)			
European	63.2	65.8	*0.02*
Non-European	35.8	34.2	
Gestational age at birth, median (95 % range), weeks	40.1 (36.0, 42.3)	40.1 (35.2, 42.3)	*0.06*
Birth weight, mean (SD), grams	3438 (542)	3400 (575)	*<0.01*
Ever breastfeeding (%)	92.6	92.1	*0.59*
TV watching >= 2 hours per day (%)	19.2	19.8	*0.64*

Values are means (SD), percentages (%), or medians (95 % range). P-values were assessed using One-Way ANOVA tests for continued variables, and Chi-square tests for categorical variables

Table S3.5.2. Correlation coefficients between retinal vessel calibers and cardiovascular markers at the age of 6 years (N = 4,007)

	Retinal arteriolar caliber	Retinal venular caliber	Systolic blood pressure	Diastolic blood pressure	Mean arterial pressure	Pulse pressure	Carotid-femoral pulse wave velocity	Heart rate	Cardiac output	Left ventricular mass	Left ventricular relative wall
Retinal arteriolar caliber	1										
Retinal venular caliber	0.43*	1									
Systolic blood pressure	-0.21*	-0.04*	1								
Diastolic blood pressure	-0.13*	0.02	0.62*	1							
Mean arterial pressure	-0.18*	-0.02	0.84*	0.95*	1						
Pulse pressure	-0.12*	-0.05*	0.59*	-0.27*	0.06*	1					
Carotid-femoral pulse wave velocity	0.02*	0.05*	0.13*	0.17*	0.17*	-0.02	1				
Heart rate	-0.01	0.05*	0.17*	0.22*	0.22*	-0.01	0.07*	1			
Cardiac output	-0.05*	-0.02*	0.20*	0.13*	0.17*	0.11*	0.03	0.47*	1		
Left ventricular mass	-0.05*	-0.02	0.13*	0.01	0.06*	0.15*	0.03	-0.18*	0.41*	1	
Left ventricular relative wall	0.00	0.02	0.03*	0.03	0.04*	0.01	-0.06*	0.02	-0.30*	0.30*	1

Values represent Pearson correlation coefficients. * P-value<0.05

Chapter 4 | General discussion

General discussion

Introduction

Cardiovascular disease is the leading cause of death worldwide and is a major public health problem in adult populations. The developmental-origins hypothesis suggests that cardiovascular disease might originate from early life.[1] This hypothesis is supported by studies in animals and humans.[2, 3]

Observational studies in humans have shown that fetal growth restriction and rapid infant growth are associated with cardiac and vascular changes in childhood and an increased risk of cardiovascular disease in adulthood.[4] Also, adverse maternal exposures during fetal life, such as higher maternal blood pressure during pregnancy and the presence of gestational hypertensive disorders are associated with increased risks of fetal growth restriction and long-term cardiovascular consequences in offspring.[5, 6] The increased risk of cardiovascular disease in later life is not only determined by a suboptimal intrauterine environment, may also be the result of a subsequent adverse environmental exposures during the early postnatal period. Suboptimal infant nutrition and increased adiposity levels throughout early childhood are associated with the development of cardiovascular disease in later life.[7, 8] Thus, both a restricted nutritional in utero environment and abundant postnatal environment may lead to cardiovascular disease in later life.

The mechanisms relating these adverse exposures in early life with an increased risk of cardiovascular diseases in later life are not fully understood, but may include early but permanent microvascular structure adaptations.[9] In humans, microvascular adaptations can be assessed by retinal vessel calibers.[10] The retinal vessels share similar anatomical and physiological properties with the coronary and cerebral microcirculation and have been suggested as an adequate proxy measure of systemic microvasculature.[11, 12] Previous studies reported that that retinal arteriolar narrowing is associated with increased risks of hypertension in later life, whereas wider retinal venular caliber is associated with an increased risk of metabolic syndrome and inflammation.[12-14]

The main aim of the studies presented in this thesis was to identify maternal, fetal and infant factors associated with microvasculature alterations and cardiovascular health outcomes in childhood. This chapter provides a general discussion of the main findings of the studies presented in this thesis, discusses general methodological issues and provides suggestions for future research.

Interpretation of the main findings

Maternal factors

Maternal blood pressure and childhood retinal vessel calibers

Results from observational studies suggests that gestational hypertensive disorders are associated with an adverse cardiovascular profile in the offspring.[15] Offspring of moth-

ers with preeclampsia have an increased risk of fetal growth restriction, higher blood pressure, vascular abnormalities and stroke in later life.[6, 16, 17] Gestational hypertensive disorders form the extreme of the spectrum of blood pressure development during pregnancy.[18] Several studies have shown that also higher maternal blood pressure levels within the normal range during pregnancy are associated with offspring cardiovascular risk factors.[19, 20] Early microvasculature adaptations may explain the associations of maternal blood pressure levels during pregnancy with blood pressure levels in the offspring.[16] Results from studies in this thesis showed that a higher maternal blood pressure in early pregnancy was associated with childhood retinal arteriolar narrowing, whereas a higher maternal blood pressure in late pregnancy was associated with childhood retinal venular narrowing. These associations were independent of maternal blood pressure in other pregnancy periods and not explained by birth characteristics or childhood body mass index and blood pressure. Clinically diagnosed maternal gestational hypertensive disorders tended to be associated with retinal arteriolar narrowing in childhood, but these associations were largely explained by birth and childhood factors. Different mechanisms may explain the observed associations for early and late pregnancy. In early pregnancy, the fetal cardiovascular system rapidly develops, whereas late pregnancy is characterized by increased placental blood flow and rapid fetal growth.[21] Higher maternal blood pressure levels during pregnancy reflect a suboptimal placental development and function, which may also affect fetal vascular development[22] and alterations in microvasculature in later life.[23 24]

Maternal angiogenic factors and childhood retinal vessel calibers

Placental and fetal vascular development is a complex process in which several angiogenic factors are involved.[25] Placenta growth factor (PlGF), a pro-angiogenic factor secreted by cytotrophoblast, plays a key role in the development of the fetal vascular system and an adequate utero-placental circulation.[26] Soluble fms-like tyrosine kinase (sFlt-1), is an anti-angiogenic factor secreted by endothelial cells, which can block the effect of PlGF.[25] Previous studies showed that lower PlGF levels can disrupt the normal process of neo-angiogenesis, whereas higher sFlt-1 levels during pregnancy can lead to systemic endothelial dysfunction, leading to a suboptimal feto-placental vascular system.[27,28] For the current study, we hypothesized that angiogenic factors during pregnancy may not only affect placental and fetal vessel development, but also persistently influence microvasculature structures postnatally. Results from studies in this thesis showed that lower second trimester maternal PlGF levels are associated with narrower retinal arteriolar caliber in childhood. This association was not explained by maternal socio-demographic and lifestyle related characteristics, pregnancy complications, birth characteristics or childhood characteristics. Several biological pathways may explain the these associations. Lower pro-angiogenic factors levels decrease nitric oxide (NO) availability, which could lead to endothelial dysfunction and impaired endothelium dependent vasodilatation.[29] Lower PlGF levels also reflect inadequate placental development, which affects oxygen levels in the feto-placental environment.[16] In humans, hypoxia during pregnancy can lead to altered in utero endothelial development by inhibiting endothelial cell proliferation [30], and by stimulating a systemic endothelial dysfunction.[27] Finally, altered PlGF levels inhibit transforming growth factor –β (TGF-β).[31] TGF-β is

required for inhibition of sprouting endothelial cells angiogenesis, and lower TGF-β concentrations can lead to endothelial dysfunction.[32] Thus, retinal arteriolar narrowing in childhood might reflect structural microvasculature changes and pathophysiological processes related to endothelial dysfunction.[33]

Fetal and infant growth

Fetal and infant growth and childhood body fat distribution

Fetal life and infancy have been recognized as critical periods for the development of overweight in later life.[34, 35] Both children with a high birth weight and children with a low birth weight followed by infant growth acceleration tend to have a higher body mass index and are at increased risk of overweight in childhood and adulthood.[36-40] Body mass index is a widely accepted outcome measure of adiposity, but body fat distribution is stronger related with cardio-metabolic risk factors in childhood and adulthood.[41, 42] In this thesis, was observed that weight gain in second and third trimester of fetal life and in early, mid and late infancy was independently and positively associated with childhood body mass index. Only infant weight gain was associated higher childhood fat mass index, android/gynoid fat mass ratio, and abdominal fat mass. Children who had fetal growth deceleration followed by infant growth acceleration had the highest android/gynoid fat mass ratio. These results are consistent with other studies showing that rapid weight gain in infancy is associated with a higher body fat in later life.[43, 44] The mechanisms underlying the associations of fetal growth patterns and rapid infant growth with development of higher adiposity levels in childhood may involve fetal adaptations due to a suboptimal fetal environment exposures.[1] Impaired growth in utero may be associated with increased allocation of nutrients to adipose tissue during development and may then result in accelerated infant growth, which may contribute to a greater risk of obesity and cardiovascular disease in adulthood.[45-49]

Fetal and infant growth and childhood retinal vessel caliber

Longitudinal studies have shown that the risk of cardiovascular disease is highest among individuals born with low birth weight followed by accelerated infant weight gain.[36-40] Studies using more detailed fetal growth measures have also shown that impaired fetal growth, already from first trimester onwards, is associated with a higher risks of adverse cardiovascular profile in later life.[50] Microvasculature alterations might be the mechanisms underlying these associations.[13] Results presented in this thesis showed that children born preterm have narrower retinal arteriolar caliber as compared to children born on term. The associations of birth weight with retinal arteriolar caliber were fully explained by gestational age at birth, suggesting that not birth weight, but preterm birth is a critical factor in the pathogenesis of the microvasculature adaptations. Children with infant growth acceleration had also narrower retinal arteriolar caliber. These associations tended to be stronger among preterm born infants. No associations of length, weight and body mass index in different fetal and childhood periods with retinal vessel calibers were found. Whether the observed differences in microvasculature structure lead to cardiovascular disease in later life should be further studied. The biological mechanisms underlying these associations might involve microvasculature adaptations

due to a suboptimal fetal environment exposures.[1] Fetal hypoxia and suboptimal nutrition might play an important role in early vascular maladaptations. Chronic hypoxia during fetal life is shown to stimulate structural vascular changes and exaggerated neovascularization.[51] Next to adverse fetal influences, early childhood environmental influences might also be important. A premature child has to adapt to a higher extra-uterine oxygen tension, which down-regulates endothelial growth factor release and subsequently inhibits normal growth of the vessels and neo-angiogenesis.[52, 53] All these microvasculature adaptations may contribute to short-term survival, but might lead to an increase risk of cardiovascular disease in later life.[1]

Infant diet

Infant diet and metabolic outcomes

Breastfeeding during infancy may have a protective effect on the development of cardio-metabolic diseases in adulthood.[54-56] Few studies have been performed in children and suggested that shorter duration of breastfeeding and early age at introduction of solid foods are related to higher blood pressure in childhood.[57-59] However, results seem inconsistent.[60-62] Studies in children are important, in order to understand the early structural processes and because of the limited influences of lifestyle related behaviors on the development of cardiovascular disease. Results presented in this thesis showed that the associations of breastfeeding duration and exclusivity with childhood levels of cholesterol, triglycerides or insulin were explained by family related socio-demographic characteristics and maternal life style related factors. Similarly, results from a randomized control trial showed that longer breastfeeding duration and exclusive breastfeeding among healthy term infants, did not influence lipid, glucose and insulin levels at the age of 11.5 years.[59] No consistent results for the associations of age at introduction of solid foods with metabolic outcomes in school-age children were found. Significant associations of shorter and exclusivity breastfeeding with the risk of clustering of cardio-metabolic risk factors at the age of 6 years were found, but these associations attenuated after adjustment for maternal and childhood confounders. Similarly, a systematic review among 10, 912 young adults from five prospective birth-cohort studied showed no evidence that ever breastfeed, longer breastfeeding duration and late introduction of complementary food were associated with risk of adult hypertension, diabetes and overweight.[63] Thus, these results suggest that the previously observed protective associations of breastfeeding duration and exclusivity with higher cardio-metabolic risk factors are likely to reflect residual confounding.

Infant diet and retinal vessel calibers

Early microvasculature structure alterations were hypothesised to be involved in the mechanisms underlying the previous observed associations of breastfeeding patterns with an adverse cardiovascular profile. Results from this thesis showed that never breastfeeding tended to be associated with narrower retinal arteriolar and venular calibers. After adjustment for maternal socio-demographic and lifestyle-related characteristics and childhood factors significant associations were present only for narrower retinal venular caliber. No associations of breastfeeding duration and exclusivity and

age of introduction of solid foods with retinal vessel calibers were present. To the best of our knowledge no previous study examined the associations of breastfeeding patterns with retinal vessel calibers in later life. A study among 159 children aged 11-14 years showed that endothelial function of skin microvasculature was significantly better among breastfed children, as compared to the ones fed with infant formula.[64] Another study among 1,667 young adults found that breastfed men have better brachial endothelial function compared to formula fed men.[65] The observed associations in this thesis may also be explained by residual confounding due to missing detailed information on childhood diet and further lifestyle factors. Thus, careful conclusions are needed about potential causal mechanisms since the effect estimates are small and the significances are borderline. Previous studies suggested that narrower retinal vessel calibers among never breastfed infant might reflect structural microvasculature changes and pathophysiological processes related to endothelial function, due to lower polyunsaturated fatty acid levels concentrations.

Childhood body fat distribution

Fat distribution and cardiovascular risk factors

Childhood obesity is associated with cardiovascular diseases in later life.[66] Body mass index does not distinguish lean mass from fat mass.[67] Among adults, it has been shown that total body fat mass and waist circumference are, independent from body mass index, associated with adverse cardiovascular risk factors and the risk of all-cause mortality. These findings suggest that total body fat mass and abdominal fat mass might be more strongly associated with adverse health outcomes than body mass index.[41, 68] Thus far, studies focused on the associations of detailed total and abdominal fat mass with cardiovascular risk factors in children show inconsistent results.[8, 69] Results presented in this thesis showed that fat mass percentage is associated with higher systolic and diastolic blood pressure, independent from body mass index. Both android/gynoid fat mass ratio and subcutaneous abdominal fat mass area, which reflect waist to hip ratio and waist circumference, respectively, were associated with cardiovascular risk factors, independent from body mass index. As compared to associations of preperitoneal fat mass, a proxy measure of visceral fat mass, we observed stronger associations for subcutaneous abdominal fat mass area with most cardiovascular risk factors. These results suggest that children with higher levels of general and abdominal fat mass have, independent of their body mass index, an adverse cardiovascular risk profile. However, the observed effect estimates for the associations of fat mass measures with cardiovascular diseases were small. The additional clinical value of detailed fat measures as compared to body mass index may be limited. Our findings suggest that detailed body fat distribution measurements are important tools in etiological studies focused on the early origins of cardio-metabolic diseases.

Fat distribution and retinal vessel calibers

Higher body mass index in adults is shown to affect endothelial and microvascular function.[70] A previous study showed that children in the highest quartile of body mass index had narrower retinal arteriolar caliber and wider retinal venular caliber than children in

the lowest quartile of body mass index.[71] Inflammatory responses in obese subjects might be involved in the pathophysiological mechanisms underlying the associations of obesity with microvascular adaptations.[72] Not much is known about the effects of specific fat mass measures on microvasculature development at younger ages.[73] Results described in this thesis showed that obese children had narrower retinal arteriolar caliber and tended to have wider venular caliber than normal weight children. Total fat mass, but not android/gynoid fat mass and pre-peritoneal fat mass, was associated with narrower retinal arteriolar caliber, but not with venular caliber. This may be explained by a small accumulation and less pathogenic visceral adipose tissue at younger ages. No associations of lipid and insulin levels with retinal arteriolar and venular calibers were present, whereas higher C-reactive protein levels were associated with wider retinal venular caliber. These results suggest that increased adiposity and subsequently increased subclinical inflammation may already be associated with alterations in the microcirculation in childhood.

Retinal vessel calibers and cardiovascular health in childhood

In humans, the retinal vessels have been suggested as an adequate measure to estimate systemic microvasculature.[11, 12] The previous studies conducted were mostly among adults and focused on the associations of retinal vessel calibers with blood pressure. These studies found that retinal arteriolar narrowing is strongly associated with higher pressure, and independently predicted the risk of stroke [13, 74], whereas wider retinal venular caliber was associated with increased risks of metabolic syndrome and inflammation.[12] However, it remains unclear whether microvascular abnormalities are a result of cardiovascular disease or are part of the factors that relate to the development of these diseases. Thus, examining the associations among children, without clinical cardiovascular disease, may provide further insight in the pathways underlying these associations. Results presented in this thesis suggest that narrower childhood retinal arteriolar caliber is associated with higher childhood systolic and diastolic blood pressure, mean arterial pressure and pulse pressure, independent of maternal and infant socio-demographic factors and childhood body mass index. Retinal venular caliber was associated with higher systolic blood pressure and pulse pressure, but not with diastolic blood pressure. Also a wider retinal venular caliber was associated with a higher carotid-femoral pulse wave velocity, suggesting that microvasculature alterations might affect carotid-femoral pulse wave velocity already from childhood onwards. Results from previous studies on the associations of retinal venular caliber with blood pressure are inconsistent.[75-77] This might be the result of different ages at outcome measurements or adjustment for different confounders between studies. The mechanisms explaining the associations of retinal vessel calibers with increased blood pressure and other cardiovascular risk factors may include eutrophic remodeling [78], and anatomic alterations such as intimal thickness and hyalinization may be mechanisms leading to narrowing retinal arteriolar vessels, which as a consequence might lead to an increase peripheral resistance and a higher blood pressure in later life [79]. Although the observed effect estimates for the associations of retinal vessel calibers with different cardiovascular markers were small, these findings are important from an etiological perspective.

Methodological considerations

Specific methodological considerations of the present studies have been presented in this thesis have been described in Chapter 2 and Chapter 3 of this thesis. In the following paragraphs, general methodological considerations regarding selection bias, information bias and confounding are discussed.

Selection bias

Selection bias may occur if the association between the determinant and outcome of interest is different in subjects who participate in the study and those who were eligible but do not participate in the study. Of all children eligible at birth, 61% participated in the Generation R Study. This non-response at baseline is not likely to be random. Participants in the Generation R Study generally were more likely to belong to the Dutch ethnicity and had a higher socio-economic class. Also, the percentages of women with gestational hypertensive disorders or children born preterm were lower than expected from the population figures in Rotterdam. This selection towards a more affluent and healthy population may have led to bias in etiological association studies if the selection mechanisms are related to both the determinant and the outcome measures. However, several studies have shown that in cohort studies associations are not strongly influenced by selective non-participation at baseline.[80, 81] The selection towards a more homogeneous population may affect the generalizability of our findings and lead to lower prevalence rates and subsequently reduced statistical power. In addition, selection bias may also occur due to selective loss to follow-up if associations would be different between those included in the analyses and those loss to follow-up. At the age of 6 years, children and their mothers were invited to participate in detailed body fat and cardiovascular follow-up measurements. The response rate at this follow-up was approximately 70%. A lower percentage of children participated in blood sample measurements at the age of 6 years, which was mainly due to non-consent for venous puncture or crying of the child. The lower percentage of children participated in retinal vessel measurements were mainly due to the later start of these measurements during the follow-up visits. Mothers from children who did not visit the research center more frequently had unhealthy lifestyle habits and were less well educated than the total study population. Overall, the selective loss to follow-up towards a more healthy population may have affected the effect estimates presented in this thesis, but this bias is difficult to quantify.

Information bias

Information bias arises because of misclassification of determinant or outcome measurements.[82] Misclassification of either determinant or outcome can be classified as non-differential or differential. In non-differential misclassification the determinant status is not related to the outcome status, and vice versa. In differential misclassification the determinant and outcome status are related. Non-differential misclassification generally leads to an underestimation or dilution of the effect estimates, whereas differential misclassification may lead to either overestimated or underestimated effect

estimates.[82] Exposure data used in our studies were collected prospectively and before assessment of the outcomes. Also, both parents as well as data collectors were unaware of the specific research questions under study. This makes differential misclassification of the exposure less likely. Non-differential misclassification might have occurred. In the studies presented in this thesis, infant feeding patterns were obtained by questionnaires, and it has been proven to be difficult to acquire reliable measurement of adverse lifestyle-related factors by self-reported questionnaires. Also, pregnancy dating for most women was performed using ultrasound measurements of crown-rump length or biparietal diameter at the first visit. This method might be better than dating by last menstrual period, but neglects variation in early fetal growth. As a consequence, growth variation in second and third trimester might be underestimated and random measurement error in estimation of pregnancy duration may have occurred. In most of the studies presented in this thesis, the outcome was assessed using medical records, well-trained research nurses or standardized hands-on assessments of body composition and cardiovascular development. Furthermore, the observers were not aware of the exposure status, which makes differential misclassification of the outcomes less likely.

Confounding

A confounding factor is an extraneous variable that correlates with both the determinant and the outcome, and this variable is not an intermediate variable in the causal pathway between the exposure and the outcome.[82] If a confounding factor is not taken into account, this may lead to a biased effect estimate of the association between the determinant and the outcome. To take account for confounding, we adjusted all analyses for multiple potential confounders. We selected covariates based on previous studies, their associations with the outcomes of interest or a change in effect estimate of more than 10%. Although we had information about a wide range of potential confounders, residual confounding may still be an issue, as in any observational study. Some of the unmeasured confounders might include parental or child diet habits, physical activity levels and specific sedentary habits. Also, information about several confounding variables was self-reported and measurement error of the confounding variables might have occurred. Residual confounding may have led to an overestimation of the observed effect estimates. Also, we assessed the associations of both maternal and paternal blood pressure during pregnancy. Similar effects of maternal and paternal blood pressure on childhood outcomes would suggest that the association of the maternal exposure with childhood outcomes is explained by residual confounding due to unmeasured environmental or family factors, rather than direct intra-uterine mechanisms. As example, results presented in this suggested that higher maternal blood pressure in early pregnancy tended to be more strongly associated with childhood retinal arteriolar narrowing, as compared to paternal blood pressure, which suggests that at least part of the association may be explained by direct intra-uterine mechanisms.

Future research

Cardiovascular disease is the leading cause of death in the adult population worldwide. Previous studies have shown that childhood cardiovascular risk factors tend to track

into adulthood and are associated with an increased risk of cardiovascular disease in adulthood.[83, 84] In this thesis was described that maternal, fetal and infant factors have an impact on cardiovascular risk factors in childhood. These results give more insights in early development of cardiovascular disease and its risk factors. However, the observed effect estimates for the associations were small, and are mainly of interest from a developmental perspective. Subclinical differences in risk factors for cardiovascular disease in childhood may be related to the development of cardiovascular disease in later life. It is important to perform long-term follow up studies from early life into young adulthood in order to examine whether the associations of maternal hemodynamics, growth patterns and childhood adiposity with microvasculature adaptations persist in later life, and whether the observed differences in childhood microvasculature would lead to long-term cardiovascular consequences throughout the life course.

Due to the observational design of our study, we cannot establish causality of the observed associations. Further animal studies are required to test the hypothesis that poor in utero growth, in response to maternal hemodynamics conditions, influences microvascular structure. Experimental manipulations of maternal blood pressure and angiogenic factors during pregnancy, and of in utero growth in animal models might give further understanding whether these exposures would result in structural vessel changes in the offspring. Nonetheless, it is not clear whether the changes observed on retinal vascular calibers are directly related to the causal pathways of cardiovascular disease. Future research in animal models and clinic settings to monitor these retinal vascular changes and their relationship to the onset, progression, and regression of cardiovascular disease is needed to validate this phenotype. Next to experimental studies in animal, randomized control trials are a preferred study design to establish causality. Several trials have been established to assess the influence of improvement of maternal hemodynamic status during pregnancy on childhood cardiovascular outcomes.[85-87] A number of maternal calcium supplementation trials have been conducted on pregnant women in recent years, primarily to investigate the potential for reducing the risk of preeclampsia.[88, 89] Follow-up studies have been conducted on the trial participants mainly focusing on offspring blood pressure as an outcome.[87, 90] A reduction in offspring systolic blood pressure was found among children of mothers with calcium supplementation during pregnancy.[87] Thus, it might be important to develop intervention trial focused on the improvement of maternal cardiovascular status during pregnancy and different childhood outcomes. It is not always possible to develop randomize trials for the exposures presented in this thesis, such as randomizing healthy babies in breastfed and non-breastfeed group. Thus, observational studies that are able to use more sophisticated methods to assess causality are important. Comparing maternal-offspring and paternal-offspring associations provides a method to separate direct intra-uterine mechanisms from the associations explained by residual confounding due to unmeasured environmental or family factors. Sibling comparison studies control for confounders as it is assumed that potential family-based confounders will be similar among siblings. Mendelian randomization studies may also help to establish causality for the observed associations. These studies use genetic variances, which are robustly associated with the exposure of interest and are not affected by confounding, to examine whether the exposure is causally related to outcome.

More detailed assessments of the studied exposures might also provide further insight in the mechanisms that relate maternal and infant factors with childhood microvasculature alterations. Further studies are needed to have more detailed pregnancy-related hemodynamic adaptations, placenta function and fetal development available. It has been suggested that a combination of placental measures including placental morphology, blood flow and placental nutrient transporter activity and expression, provides a better proxy for the intrauterine environment than birth weight, and may give further insight in developmental programming of cardiovascular disease.[91] Due to strong advances in imaging techniques, it is possible to visualize embryonic development in further detail, which may provide further insight for the associations of fetal growth restriction with childhood microvasculature alterations.[92]

Main childhood outcomes studied in this thesis were childhood body composition, retinal vessel calibers, blood pressure, left ventricular mass and cardiovascular biomarkers. Further detailed measurements of fetal and childhood body composition and cardiovascular development might provide further insight in the underlying mechanisms linking early life exposures with retinal vessel calibers alterations and cardiovascular disease in later life. Next to retinal vessel calibers, also other measurement of retinal microvascular architecture might be obtained from retinal pictures. For example, geometric parameters of the retinal vascular network, such as vessel tortuosity, the angle and number of bifurcations, and the length to diameter ratio of vessels appear to convey prognostic information on the risk of hypertension and cardiovascular disease in adults, and might thus be of interest to further study them in childhood populations.[93, 94] The suggestions that both narrower retinal arteriolar and wider retinal venular result from underlying endothelial dysfunction offers scope for further potential assessment.[32] For example, ultrasound assessments make it possible to visualize the endothelial function non-invasively[95] and this might provide further insight in the pathophysiological mechanisms underlying the microvasculature structure and function alterations in childhood. Other functional measurement of retinal microvasculature may well be more relevant to the prediction of cardiovascular risk. Techniques such as scanning laser doppler flowmetry and scanning laser ophthalmoscopy[96, 97] permit in vivo measurement of microcirculatory blood flow that is inaccessible in any other microcirculation. When coupled with imaging of the retinal vessels, estimates of retinal vessel wall thickness may be obtained, providing unique means of determining actual arteriolar and venular wall thickness, which is clearly of value in assessing the health of the microcirculation.[96, 97] Whether these additional measures of retinal vascular hemodynamics will help to identify those at risk of cardiovascular diseases is still needed to be explored. There are also strong indications that retinal vascular changes parallel pathology in the coronary micro and macrocirculations, with retinal arteriolar narrowing strongly associated with the presence of coronary artery occlusion[98] and reduced myocardial perfusion measures on cardiac MRI[99]. Thus, imaging techniques, such as coronary circulation MRI, are of interest to obtain further insight in detailed cardiovascular development.

Epigenetics has been proposed as a potential biological mechanism underlying the developmental origins hypothesis.[100] Epigenetic mechanisms involve three classes of molecular mechanisms such as DNA methylation, histone modifications, and DNA-binding proteins. Environmental influences in early life can permanently alter epigenetic

gene regulation, and thereby affect permanently the risk of cardiovascular disease in later life.[100, 101] This hypothesis is supported from animals studies showing that epigenetic modifications may occur due to suboptimal environmental exposures in early life.[101] In humans, a lower methylation of the IGF2 gene was found in adults exposed to undernutrition during the Dutch Famine.[102] These findings suggest that epigenetic modifications induced by early environmental exposures may have phenotypic consequences throughout the life course. Future studies are needed to obtain further insight in the role of epigenetics, and critical periods for epigenetic variations, as underlying mechanisms for associations of early life exposures with microcirculation adaptations and and cardiovascular risk factors development.

Clinical implications

In this thesis we identified several maternal, fetal and infant factors that might influence microvasculature alterations and risk of adverse cardiovascular outcomes in childhood. These findings may be important for identification of high-risk children and for the development of preventive strategies or interventions already from early stages life onwards. Based on our findings, fetal life and infancy seem to be a critical periods for the microvasculature structures and cardiovascular outcomes in children. Future interventions on health promotion and cardiovascular disease prevention should be focused on improving maternal health status during pregnancy and infant development.

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Chapter 5 | Summary

Summary

Chapter 1 describes the background and hypothesis for the studies presented in this thesis. Cardiovascular disease is a major public health problem in the general adult population. The developmental-origins hypothesis suggests that cardiovascular disease might originate from early life. This hypothesis is supported by experimental studies in animals and observational studies in humans.

Observational studies in humans have shown that fetal growth restriction and rapid infant growth are associated with cardiac and vascular changes in childhood and an increased risk of cardiovascular disease in adulthood. Also, adverse maternal exposures during fetal life, such as higher maternal blood pressure during pregnancy and the presence of gestational hypertensive disorders are associated with increased risks of fetal growth restriction and long-term cardiovascular consequences in offspring. The increased risk of cardiovascular disease in later life is not only determined by a suboptimal intrauterine environment, may also be the result of a subsequent adverse environmental exposures during the early postnatal period. Suboptimal infant nutrition and increased adiposity levels throughout early childhood are associated with the development of cardiovascular disease in later life. Thus, both a restricted nutritional in utero environment and abundant postnatal environment may lead to cardiovascular disease in later life.

The mechanisms relating these adverse exposures in early life with an increased risk of cardiovascular diseases in later life are not fully understood, but may include early but permanent microvascular structure adaptations. In humans, microvascular adaptations can be assessed by retinal vessel calibers. The retinal vessels share similar anatomical and physiological properties with the coronary and cerebral microcirculation and have been suggested as an adequate proxy measure of systemic microvasculature. Previous studies reported that that retinal arteriolar narrowing is associated with increased risks of hypertension in later life, whereas wider retinal venular caliber is associated with an increased risk of metabolic syndrome and inflammation.

The studies presented in this thesis were embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, The Netherlands. The Generation R Study is designed to identify early environmental and genetic determinants of growth, development and health in fetal life and childhood.

In Chapter 2, studies on maternal hemodynamics influences and fetal and infant growth patterns on childhood adiposity and retinal vessel calibers are described. In Chapter 2.1, we found that maternal systolic blood pressure development in early and late pregnancy are associated with childhood retinal vessel calibers. These associations were independent of maternal blood pressure in other pregnancy periods and independent of birth characteristics or childhood body mass index and blood pressure. Thus, maternal blood pressure levels in early and late pregnancy, might critical periods for the development of childhood microvasculature adaptations. Further, we examined the associations of maternal angiogenic factors in first and second trimester of pregnancy with childhood retinal vessel calibers (Chapter 2.2). We observed that lower second

trimester maternal PlGF levels are associated with narrower retinal arteriolar caliber in childhood. We did not observed significant associations of first trimester maternal PlGF and sFlt-1 with childhood microvasculature.

In **Chapter 2.3,** we explored the associations of different fetal and infant growth measurements with childhood body fat distribution. We observed that weight gain in fetal life and infancy was independently and positively associated with childhood body mass index, whereas only infant weight gain was associated higher childhood fat mass index, android/gynoid fat mass ratio, and abdominal fat mass. Also, children who had fetal growth deceleration followed by infant growth acceleration had the highest value for android/gynoid fat mass ratio. These findings suggest that rapid infant growth might influence development of later body composition. Further, in **Chapter 2.4** we described the associations of fetal and infant growth patterns with childhood microvasculature. We observed that children born preterm have narrower retinal arteriolar caliber as compared to children born on term. Children with infant growth acceleration had also narrower retinal arteriolar caliber, especially among preterm born children. Thus, our results suggest that prematurity and accelerated growth in infancy may have an adverse impact on microvasculature structure and function.

In **Chapter 3,** the associations of infant diet patterns and childhood body fat distribution with cardio-metabolic risk factors in childhood are described. In **Chapter 3.1,** we examined the associations of infant diet with lipid levels and insulin concentrations in childhood. We observed that ever breastfeeding, breastfeeding duration and exclusivity are not consistently associated with childhood levels of cholesterol, triglycerides or insulin. We also did not find consistent results for the associations of age at introduction of solid foods with metabolic outcomes in school-age children. Thus, our results do not support the hypothesis that breastfeeding is associated with the development of cardiovascular diseases, and suggest that the previously observed associations may be explained by confounding factors. We also explored whether the early microvasculature structure alterations might be involved in the mechanisms underlying the previous observed associations of breastfeeding patterns with an adverse cardiovascular profile. (**Chapter 3.2**). We found that never breastfeeding was associated with narrower retinal venular caliber. No associations of breastfeeding duration and exclusivity and age of introduction of solid foods with retinal vessel calibers were present. However, these associations might also be explained by residual confounding due to missing detailed information on childhood diet and further lifestyle factors.

In **Chapter 3.3**, we showed that, independent from childhood body mass index, fat mass percentage, android/gynoid fat mass ratio and subcutaneous abdominal fat mass area is associated with higher blood pressure, blood levels of lipids and insulin in childhood. We found stronger associations for subcutaneous abdominal fat mass area with most cardiovascular risk factors, as compared to preperitoneal abdominal fat mass, which is consider as a proxy measure of visceral fat mass. This may be explained by less pathogenic and only a small accumulation of visceral adipose tissue at younger ages. Thus, our findings suggest that detailed body fat distribution measurements are important tools in etiological studies focused on the early origins of cardio-metabolic dis-

eases. In Chapter 3.4, we examined whether microvasculature adaptations are involved in the mechanisms linking obesity with cardiovascular diseases. We observed that higher body mass index and total fat mass, but not android/gynoid fat mass and preperitoneal fat mass, was associated with narrower retinal arteriolar caliber, but not with venular caliber. Lipid and insulin levels were not associated with retinal arteriolar and venular calibers, whereas higher C-reactive protein levels were associated with wider retinal venular caliber. Our results suggest that increased adiposity and subsequently increased subclinical inflammation may already be associated with alterations in the microcirculation in childhood.

In Chapter 3.5, we examined whether the observed retinal vessel calibers alterations were associated with higher cardiovascular outcomes in school-age children. We observed that narrower retinal arteriolar caliber was associated with higher systolic and diastolic blood pressure, mean arterial pressure and pulse pressure independent of maternal and infant socio-demographic factors and childhood body mass index. Narrower retinal venular caliber was associated with higher systolic blood pressure and pulse pressure, but not with diastolic blood pressure. We also found that wider retinal venular caliber was associated with a higher carotid-femoral pulse wave velocity. Thus, our results suggest that microvascular adaptations might influence cardiovascular health from childhood onwards.

In Chapter 4 we provide a general discussion in which the studies described in this thesis are described in broader context, and implications and suggestions for future research are discussed.

In conclusion, findings from this thesis suggest that maternal, fetal and infant factors are associated with microvasculature alterations and cardiovascular health outcomes in childhood. Although the observed associations were relatively small to moderate, they may be important for cardiovascular disease on a population level. Preventive strategies should focus on improving maternal cardiovascular health status during pregnancy and reducing childhood adiposity to improve cardiovascular health status of the offspring.

Samenvatting

Hoofdstuk 1 beschrijft de achtergrond en hypothese voor de studies beschreven in dit proefschrift. Hart- en vaatziekten zijn een belangrijk volksgezondheidsprobleem. De 'developmental-origins' hypothese stelt dat cardiovasculaire aandoeningen op volwassen leeftijd hun grondslag al vinden in het vroege leven. Deze hypothese wordt ondersteund door zowel experimentele studies in dieren als observationele studies in mensen.

Observationele studies in mensen hebben laten zien dat groeirestrictie in de foetale periode, gevolgd door een relatief snellere groei tijdens de peutertijd, is geassocieerd met veranderingen van hart en bloedvaten in de kindertijd, en met een verhoogd risico op hart- en vaataandoeningen op volwassen leeftijd. Blootstelling aan ongunstige maternale factoren tijdens het foetale leven, zoals hypertensieve zwangerschapsaandoeningen, is tevens geassocieerd met een verhoogd risico op foetale groeirestrictie, en heeft lange termijn consequenties op cardiovasculair gebied. Het verhoogde risico op hart- en vaataandoeningen wordt niet alleen bepaald door een suboptimale intrauteriene omgeving, maar kan ook het resultaat zijn van ongunstige blootstellingen tijdens de postnatale periode. Suboptimale voeding in de peutertijd en verhoogde adipositas tijdens de kindertijd zijn geassocieerd met het ontwikkelen van cardiovasculaire aandoeningen later in het leven. Dus niet alleen verminderde placentaire doorbloeding, maar ook een overvloed aan voeding in de postnatale periode kunnen leiden tot een verhoogd risico op cardiovasculaire aandoeningen op latere leeftijd.

De mechanismen waarop deze ongunstige blootstellingen in het vroege leven leiden tot cardiovasculaire ziekte later in het leven zijn nog niet volledig opgehelderd. Waarschijnlijk spelen permanente adaptaties van de microvasculaire structuur in het vroege leven een rol. In mensen kunnen deze microvasculaire adaptaties worden onderzocht door het meten van de grootte van de bloedvaten in de retina. De retinale vaten delen anatomische en fysiologische eigenschappen met de coronaire en cerebrale microcirculatie en zouden daarom een goede reflectie zijn van de systemische microvascularisatie. Eerdere studies hebben aangetoond dat smalle retinale arteriolen zijn geassocieerd met een verhoogd risico op hoge bloeddruk op latere leeftijd, terwijl wijdere retinale venules zijn geassocieerd met het Metabool syndroom en inflammatie.

De studies die gepresenteerd worden in dit proefschrift zijn onderdeel van het Generation R onderzoek, een populatie-gebaseerd prospectief cohort onderzoek vanaf het foetale leven tot in de jonge volwassenheid in Rotterdam, Nederland. Het Generation R onderzoek heeft tot doel om vroege omgevings- en genetische factoren te identificeren, die van invloed zijn op de groei, ontwikkeling en gezondheid in het foetale leven en op de kinderleeftijd.

In Hoofdstuk 2 worden studies over maternale hemodynamische invloeden op retina vaten beschreven, alsmede de invloed van vroege groeipatronen op lichaamssamenstelling en de grootte van de retina vaten. In hoofdstuk 2.1 tonen we aan dat maternale systolische bloeddruk ontwikkeling in de vroege en in de late zwangerschap, maar niet in mid-zwangerschap is geassocieerd met het kaliber van de vaten in de retina in kinderen van schoolleeftijd. Deze associaties zijn onafhankelijk van maternale bloeddruk in eerdere zwangerschapsperioden en onafhankelijk van geboorte karakteristie-

ken, body mass index en bloeddruk op de kinderleeftijd. Maternale bloeddruk in de vroege en late zwangerschap is dus mogelijk een kritieke periode voor het ontwikkelen van micro vasculaire adaptaties, welke te meten zijn in de kinderjaren. Daarnaast hebben we onderzocht of er associaties zijn tussen maternale bloedvatvormende factoren in het eerste en tweede trimester van de zwangerschap met het kaliber van retina vaten op de kinderleeftijd (Hoofdstuk 2.2). We hebben gevonden dat lagere maternale PlGF levels in het tweede trimester zijn geassocieerd met smallere retinale arteriolen op kinderleeftijd. We hebben geen significante associaties gevonden tussen PlGF en sFlt-1 in het eerste trimester en de microvasculatuur op de kinderleeftijd.

In Hoofdstuk 2.3 hebben we de associaties onderzocht van verschillende groeimetingen tijdens de foetale periode en de vroege kindertijd met lichaamsvetverdeling op latere leeftijd. Gewichtstoename op foetale leeftijd en tijdens de vroege kindertijd was positief geassocieerd met body mass index op kinderleeftijd. Deze associatie was onafhankelijk van gewicht op eerdere meetpunten. Gewichtstoename tijdens de vroege kindertijd was ook geassocieerd met een hogere vetmassa index, buikvet/heupvet ratio en abdominale vet massa. Kinderen met een vertraagde foetale groei, gevolgd door een relatief snelle groei in de vroege kindertijd hadden de hoogste waarde voor buikvet/heupvet ratio. Deze bevindingen suggereren dat snelle groei in de vroege kindertijd de ontwikkeling van de latere lichaamssamenstelling kan beïnvloeden. Verder beschrijven we in Hoofdstuk 2.4 de associaties van foetale groeipatronen en groeipatronen in de vroege kindertijd met microvascularisatie op kinderleeftijd. We hebben geobserveerd dat prematuur geboren kinderen smallere retinale arteriolen hebben, vergeleken met á term geboren kinderen. Kinderen met versnelde groei in de vroege kindertijd hadden ook smallere retinale arteriolen, vooral als deze versnelde groei volgde op vroeggeboorte. Onze resultaten suggereren dat prematuriteit en versnelde groei in de eerste levensjaren een negatieve impact hebben op de microvasculaire structuur.

In Hoofdstuk 3 worden de associaties van dieetpatroon in de eerste levensjaren en lichaamsvetverdeling op kinderleeftijd met cardio-metabole risicofactoren beschreven. In Hoofdstuk 3.1 onderzoeken we de associaties van het dieetpatroon in de eerste levensjaren met lipiden- en insuline concentraties op de kinderleeftijd. We zagen dat het ooit krijgen van borstvoeding, de duur van borstvoeding en exclusiviteit van borstvoeding niet consistent geassocieerd zijn met cholesterol, triglyceride en insuline concentraties op de kinderleeftijd. We vonden ook geen consistente associaties voor de leeftijd van introductie van vaste voeding met de metabole uitkomsten op kinderleeftijd. Onze resultaten ondersteunen de hypothese dat borstvoeding is geassocieerd met de ontwikkeling van cardiovasculaire ziekten dus niet. De associaties die eerder werden geobserveerd kunnen verklaard worden door vertekenende factoren. We hebben onderzocht of vroege veranderingen in de microvasculaire structuur de onderliggende mechanismen van de eerder geobserveerde associaties van borstvoeding patronen met een nadelig cardiovasculair profiel beïnvloeden. (Hoofdstuk 3.2). Het nooit krijgen van borstvoeding krijgen was geassocieerd met een smallere retinale venules. Associaties van duur van de borstvoeding, exclusiviteit, en leeftijd van introductie van vaste voeding met het kaliber van retina vaten zijn niet gevonden. De gevonden associaties kunnen verklaard worden door resterende vertekenende factoren, doordat er geen gedetail-

leerde informatie over voedingspatroon op kinderleeftijd of andere lifestyle factoren beschikbaar was.

In Hoofdstuk 3.3 tonen we dat, onafhankelijk van de body mass index, vet massa percentage, buikvet/heupvet ratio en subcutane abdominale vet massa geassocieerd zijn met hogere bloeddruk en serumconcentraties van lipiden en insuline op de kinderleeftijd. We vonden sterkere associaties voor subcutane abdominale vet massa met de meeste cardiovasculaire risicofactoren, in vergelijking met pre-peritoneale abdominale vet massa, wat wordt gezien als een proxy meting van viscerale vet massa. Dit kan worden verklaard door een minder pathogene en kleinere accumulatie van visceraal vet op jongere leeftijd. Onze bevindingen suggereren dat gedetailleerde lichaamsvetmetingen belangrijke instrumenten zijn in etiologische studies die zich richten op de vroege beginselen van cardio-metabole ziekten. In Hoofdstuk 3.4 hebben we onderzocht of aanpassingen in de microcirculatie van belang zijn in de mechanismen waarop obesitas cardiovasculaire ziekten veroorzaakt. We zagen dat hogere body mass index en totale vet massa, maar niet buikvet/heupvet ratio of pre-peritoneale vet massa, zijn geassocieerd met smallere retinale arteriolen, maar niet met het kaliber van de retina venules. Lipide en insuline concentraties zijn niet geassocieerd met de kalibers van arteriolen of venulen in de retina, maar C-reactive proteine concentratie is wel geassocieerd met een wijder retina venule kaliber. Onze resultaten suggereren dat verhoogde adipositas en de daaropvolgende subklinische inflammatie mogelijk al gepaard gaan met veranderingen in de microcirculatie op kinderleeftijd.

In Hoofdstuk 3.5 hebben we onderzocht of de geobserveerde adaptaties in het kaliber van de vaten in de retina zijn geassocieerd met hogere cardiovasculaire uitkomsten op kinderleeftijd. We hebben geobserveerd dat smallere arteriolen in de retina zijn geassocieerd met hogere systolische en diastolische bloeddruk, gemiddelde bloeddruk en polsdruk, onafhankelijk van maternale en socio-demografische factoren tijdens de eerste levensjaren en body mass index op kinderleeftijd. Smallere venules in de retina is geassocieerd met hogere systolische bloeddruk en polsdruk, maar niet met diastolische bloeddruk. We vonden ook dat wijdere venules in de retina is geassocieerd met hogere carotide-femorale drukgolf snelheid. Onze resultaten laten dus zien dat adaptaties in de microcirculatie al van invloed zijn op cardiovasculaire gezondheid vanaf de vroege kinderleeftijd.

In Hoofdstuk 4 worden de studies die staan beschreven in dit proefschrift bediscussieerd en in een bredere context geplaatst, waarbij de implicaties worden besproken en suggesties voor toekomstig onderzoek worden gedaan.

Concluderend suggereren de bevindingen van dit proefschrift dat maternale, foetale en vroege kinderleeftijd factoren zijn geassocieerd met aanpassingen in de microcirculatie en cardiovasculaire gezondheid op kinderleeftijd. Hoewel de geobserveerde associaties klein tot gemiddeld zijn, kunnen ze belangrijk zijn voor cardiovasculaire aandoeningen op een populatie niveau. Preventieve strategieën zouden zich moeten richten op het verbeteren van maternale cardiovasculaire gezondheid tijdens de zwangerschap en het verminderen van adipositas op kinderleeftijd, om zo de cardiovasculaire gezondheid van kinderen te verbeteren.

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12. **Gishti O**, Jaddoe VW, Hofman A, Wong TY, Ikram MK, Gaillard R. Body fat distribution, metabolic and inflammatory markers and retinal microvasculature in school-age children. The Generation R Study. *Submitted*

Co-author

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About the author

Olta Gishti was born November 15th 1986 in Roskovec, Fier, Albania. She graduated from high school at the Bazat e Shkences in Fier, in 2005. In the same year, she started her medical education at the University of Tirana, Tirana, Albania. During the fourth year of her medical education, she was invited to participate in an exchange program at Faculty of Medicine of University of Granada, Granada, Spain. In 2011 she graduated as a medical doctor in Univerity of Tirana, Tirana, Albania. In 2012 she was invited to participate in the Master of Science program Health Sciences, specialisation Public Health, at the Netherlands Institute for Health Sciences. In 2012, she received her Master of Science degree in Public Health and expanded her research project in her current PhD-project entitled 'Microvasculature and cardiovascular risk factors in childhood' under supervision of Prof. dr. V.W.V. Jaddoe (Departments of Epidemiology and Pediatrics), Prof. dr. E.A.P. Steegers (Department of Obstetrics and Gynaecology) and Dr. Romy Gaillard (Department of Epidemiology and Pediatrics). The results of this work are presented in this dissertation. In 2013 she homologated her medical degree in Spain. Now, she is working to achieve the BIG registration in the Netherlands. She would like to start the residency in Ophtalmology.

PhD portfolio

Summary PhD training and teaching activities

Name PhD student: Olta Gishti
Erasmus MC Department: Epidemiology
Research School: Netherlands Institute for Health Sciences
PhD period: August 2012 - June 2015
Promotors: Prof. dr. V.W.V. Jaddoe, Prof. dr. E.A.P. Steegers

1. PhD training

	Year	Workload (ECTS)
General courses		
Master's degree Health Sciences, specialization Public Health, NIHES, Erasmus University Rotterdam, the Netherlands	2012-2013	
Doctor of Science's degree Health Sciences, specialization Epidemiology, NIHES, Erasmus University Rotterdam, the Netherlands	2013-2014	
Clinical decision analyses	2012-2014	0.7
Methods of Clinical Research		0.7
Methods of public health research		0.7
Topics in Meta-analysis		0.7
Pharmaco-epidemiology		0.7
Methods of Health Services Research		0.7
Primary and secondary prevention research		0.7
Social epidemiology		0.7
Causal inference		0.7
Conceptal foundation of epidemiologic study design		0.7
History of epidemiologic ideas		0.7
Advances in epidemiologic analyses		0.4
Causal mediation analyses		0.7
Study Design		4.3
Biostatistical methods 1: Basic principales		5.7
Biostatistical methods 2: Classical regression models		4.3
Public health research methods		5.7
International comparison of health care systems		1.4
Site visit to municipal health service Rotterdam		0.3
Integration module		0.3
English language		1.4
Advanced courses		
Missing values in Clinical Research		0.7
Women's health		0.9
Advanced Topics in Decision-making in Medicine		1.9
From problem to solution in public health		1.1
Public health in low and middle income countries		3.0
Courses for the Quantitative Researcher		1.4
Repeated measurements in clinical studies		1.4
Bayesian statistics		1.4
Planning and evaluation of screening		1.4
Quality of life measurements		0.9
Introduction to medical writing		1.1

General academic skills

Scientific Writing in English for Publication, Erasmus MC, the Netherlands		2.0
Development research proposal		2.5
Oral research presentation		1.4

Seminars and workshops

Generation R Research meetings, Erasmus MC, The Netherlands	2012-2015	1.0
Seminars at the department of Epidemiology, Erasmus MC, The Netherlands	2012-2015	1.0

(Inter)national congresses and presentations

Invited speaker

ICBMS. Tirana, Albania. *Oral presentation*	2013	1.4
Endo 2015, San Diego, California. *Oral presentation*	2015	1.4

Other

Developmental Origins of Health and Disease (DOHaD), Singapore. *Mini oral and Poster presentation*	2013	1.4
Nutrition and Growth Conference, Barcelona, Spain. *Oral and poster presentation*	2014	1.4
Power of Programming, Munich, Germany. *Poster presentation*	2014	0.7

Scholarships and grants

Joineusee exchange student grant	2010
Eraweb master student grant	2012-2013
Vereniging Trustfonds Erasmus Universiteit Rotterdam, several travel grants	2014-2015

Other

Reviewed articles for *Plos One, Hypertension, Eur J Epidemiol, Paediatr Perinat Epidemiol*	
Participation in organizing ICBMS conference, Tirana, Albania.	2013-2015

2. Teaching

Supervising Master's and Bachelor's theses

Aleksandra Jelena, Clinical Epidemiology, Nihes, the Netherlands	2014-2015	4.0
Project title: *Maternal polyunsaturated fatty acid levels during pregnancy and childhood blood pressure. & Maternal polyunsaturated fatty acid levels during pregnancy and childhood adiposity.*		
Dilan Gizem Yesil, Medical student, Erasmus MC, the Netherlands	2014	2.0
Project title: *Influence of maternal gestational hypertensive disorders on microvasculature in school-age children. The Generation R Study.*		

Words of gratitude

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Propositions

1. Higher maternal blood pressure in pregnancy is associated with microvasculature maladaptation in the offspring. (*This thesis*)

2. Both fetal life and infancy are critical periods for the development of childhood overweight and obesity. (*This thesis*)

3. Preterm birth and accelerated growth in infancy have an adverse impact on microvasculature structure and function in childhood. (*This thesis*)

4. The associations of infant feeding with metabolic outcomes in childhood are most likely confounded by family-based socio-demographic and life style related factors. (*This thesis*)

5. General and abdominal fat measures are associated with cardiovascular risk factors in childhood, independent from body mass index. (*This thesis*)

6. Growing awareness that investment in the health, education and nutrition of young people in relation to their responsibilities during pregnancy and parenthood is of fundamental importance. (Gluckman PD, N Engl J Med 2008)

7. There is good scientific evidence that the increased rates of myopia correlates with higher intelligence quotient. (Czepita D, Ann Acad Med Stetin. 2008)

8. Epidemiological methods may be scientific, but their objectives are often thoroughly human. (Broadbent A, Philosophy of Epidemiology 2013)

9. There is no simple choice between either population based or high risk strategies to reduce cardiovascular mortality, but in case of resource limited health services, preventive approaches in healthy populations are of great importance. (Mayor S, BMJ 2015)

10. Population health would grow by leaps and bounds if more women would have access to higher education.

11. To study foreign languages and cultures, is not a great tide to sweep away all differences, but learn us to respect our differences.