Early Life Nutrition, Growth and **Kidney Function** in Children

The Generation R Study

Kozeta Miliku

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ACKNOWLEDGEMENTS

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Early Life Nutrition, Growth and Kidney Function in Children

The Generation R Study

Voeding in het vroege leven, groei en nierfunctie bij kinderen

Het Generation R Onderzoek

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. H.A.P. Pols

and in accordance with the decision of the Doctorate Board.
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by

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MANUSCRIPTS THAT FORM THE BASIS OF THIS THESIS

Miliku K, Voortman T, van den Hooven EH, Hofman A, Franco OH, Jaddoe VW. First-trimester maternal protein intake and childhood kidney outcomes: The Generation R Study. *Am J Clin Nutr. 2015;102(1):123–9*.

Miliku K, Mesu A, Franco OH, Hofman A, Steegers EA, Jaddoe VWV. Maternal and fetal folate, vitamin B_{12} and homocysteine concentrations and childhood kidney outcomes. *Am J Kidney Dis. 2017. doi: 10.1053/j.ajkd.2016.11.014*.

Miliku K, Vinkhuyzen A, Blanken LM, McGrath JJ, Eyles DW, Burne TH, Hofman A, Tiemeier H, Steegers EA, Gaillard R, Jaddoe VW. Maternal vitamin D concentrations during pregnancy, fetal growth patterns, and risks of adverse birth outcomes. *Am J Clin Nutr. 2016;103(6):1514–22*.

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Miliku K, Voortman T, Franco OH, McGrath JJ, Eyles DW, Burne TH, Hofman A, Tiemeier H, Jaddoe VW. Vitamin D status during fetal life and childhood kidney outcomes. *Eur J Clin Nutr. 2015; 70(5):629–34*.

Miliku K, Bergen NE, Bakker H, Hofman A, Steegers EA, Gaillard R, Jaddoe VW. Associations of maternal and paternal blood pressure patterns and hypertensive disorders during pregnancy, with childhood blood pressure *J Am Heart Assoc. 2016; 10.1161/JAHA.116.003884*.

Miliku K, Vogelezang S, Franco OH, Hofman A, Jaddoe VW, Felix JF. Influence of common genetic variants on childhood kidney outcomes. *Pediatr Res. 2016; 80(1):60–6*.

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Miliku K, Bakker H, Dorresteijn EM, Cransberg K, Franco OH, Felix JF, Jaddoe VWV. Childhood estimates of glomerular filtration rate based on creatinine and cystatin C: Importance of body composition. *Am J Nephrol. doi: 10.1159/000463395.*

Chapter 1

General introduction

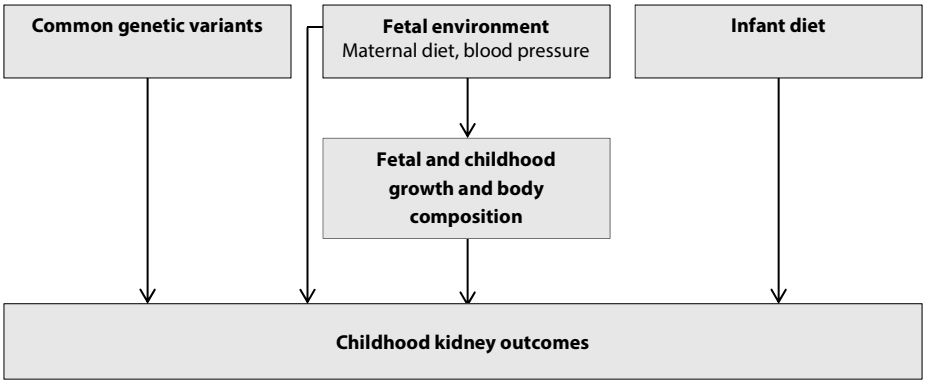
INTRODUCTION

Chronic kidney disease is a major public health problem worldwide, with a global prevalence of 8 to 16%.[1] The "Developmental Origins of Health and Disease Hypothesis" states that various diseases in later life originate from fetal life and early infancy.[2] This hypothesis suggests that a suboptimal fetal environment leads to developmental adaptations that permanently alter growth, physiology and metabolism, which may be beneficial in short-term but with long-term consequences for health.[2] Adverse exposures, such as poor nutrition, in early life may lead to metabolic adaptations to maintain development of key body organs, including the kidney.[3, 4]

Epidemiological evidence suggests that early life events, including low birth weight, have an important role for the susceptibility to develop chronic kidney disease in later life.[5–8] Additionally, historical studies suggest that adult offspring of mothers who were exposed to severe undernutrition during their pregnancies have an increased risk of cardiovascular and renal disease, independent of birth weight.[9, 10] The Dutch Famine studies provide strong evidence that maternal undernutrition during pregnancy increases the risk of high blood pressure and microalbuminuria in the offspring.[11, 12]

Nephrogenesis starts in early pregnancy, continues until the 36th week of gestation, and largely ceases thereafter. Therefore, adverse exposures during critical periods of nephron formation may lead to impaired kidney development.[13, 14] Post mortem studies have observed that infants with a low birth weight have smaller kidneys with a reduced number of nephrons.[15] Brenner's "Hyperfiltration Theory" suggests that adverse fetal adaptations, may lead to smaller kidneys with a reduced number of nephrons, and subsequently to glomerular hyperfiltration and sclerosis, which predisposes to development of impaired kidney function and eventually kidney disease in adulthood.[16–19] Investigating specific nutritional factors during pregnancy and other early life factors may provide new insights into kidney development. Therefore, the studies presented in this thesis focus on identification of early determinants of early growth and kidney function in children (**Figure 1.1**).

Figure 1.1. Framework of the studies included in this thesis



FETAL FACTORS

Maternal diet and other lifestyle related factors during pregnancy are related to growth, cardiovascular and kidney outcomes in the offspring. The Dutch Famine studies provide strong evidence that maternal undernutrition during pregnancy affects birth weight, cardiovascular and renal health of the offspring.[11, 12] However, not much is known about the specific nutrients in modern diets that influence early growth and kidney development in children. Also, the potential mechanisms underlying these associations are not well known. The strongest evidence from the animal studies suggest that especially protein-restriction in pregnant rats can affect offspring kidney health.[20] *Protein* is one of the main nutrient components which provides essential amino acids required for growth. In patients with mild renal insufficiency, a high protein intake is reported to aggravate the severity of the disease.[21] However, experimental studies suggest that maternal protein malnutrition impacts fetal kidney vascular development and prevents achievement of full kidney function in the adults.[8, 20, 22] Whether maternal protein intake in pregnancy influences childhood kidney structure and function in a healthy population is unknown.

Beside macronutrients, previous literature suggests that micronutrients such as B-vitamins and vitamin D status in fetal life may alter metabolism, vasculature, and organ growth and function, leading to increased risk of cardiovascular disorders, adiposity, and altered kidney function in the offspring.[23–25] *Folate* is an essential *B-vitamin*, important for cell growth and replication and together with *vitamin B_{12}*, is an important methyl donor in many reactions, including the production of thymidine for deoxyribonucleic acid (DNA) synthesis, polyamine synthesis, and biosynthesis of methionine from *homocysteine*.[26–28] Folate and vitamin B_{12} contribute to lowering homocysteine concentrations. Adults studies have shown associations of elevated homocysteine concentrations with an accelerated decline in kidney function.[29] However no previous studies have explored the associations of maternal folate, vitamin B_{12} and homocysteine concentrations during pregnancy with childhood kidney structure and function.

Vitamin D is another micronutrient with a known role in differentiation and maturation processes of the cell. Vitamin D deficiency during pregnancy is common.[30] Next to its well-known function in bone health and rickets in offspring,[25] an accumulating body of evidence suggest that vitamin D status during pregnancy may be associated with persistent changes in offspring health including adverse birth outcomes and impaired development.[31–33] Whether maternal vitamin D deficiency is associated with fetal growth patterns besides birth weight is unclear. Some experimental studies suggest a stimulated offspring nephrogenesis and an altered body composition profile from vitamin D deficient mothers.[34, 35] Studies exploring these associations in healthy children are lacking.

Next to maternal nutritional factors in pregnancy, gestational hypertensive disorders are associated with suboptimal placental-fetal nutrition transport and increased risks of delivering preterm and small size for gestational age infants,[36] both of which are risk factors for hypertension in adulthood.[37, 38] However gestational hypertensive disorders do not represent the full spectrum of blood pressure development during pregnancy. Not much is known about the critical periods of maternal blood pressure for the development of childhood blood pressure.

GENETIC AND CHILDHOOD FACTORS

Risk factors for impaired kidney function track from childhood to adulthood.[39, 40] Kidney measures in early life are associated with kidney disease in later life. Besides environmental factors, the associations between early life kidney characteristics and kidney disease in later life may also be explained by common genetic variants. These variants may affect early kidney development and function and thereby predispose individuals to kidney disease in later life. Large genome-wide association studies in adults have identified common genetic variants related to measures of impaired kidney function.[41] Whether these genetic variants also affect kidney function in early childhood is not known.

Next to fetal and genetic factors, early life nutrition can affect kidney development and subsequently lead to chronic kidney disease.[6] Breastfeeding is suggested to have a protective effect on the development of cardiometabolic diseases.[42] Also, the long-term protective effects of breastfeeding on blood pressure, obesity and diabetes are recognized.[43] Whether breastfeeding influences childhood kidney health is unknown.

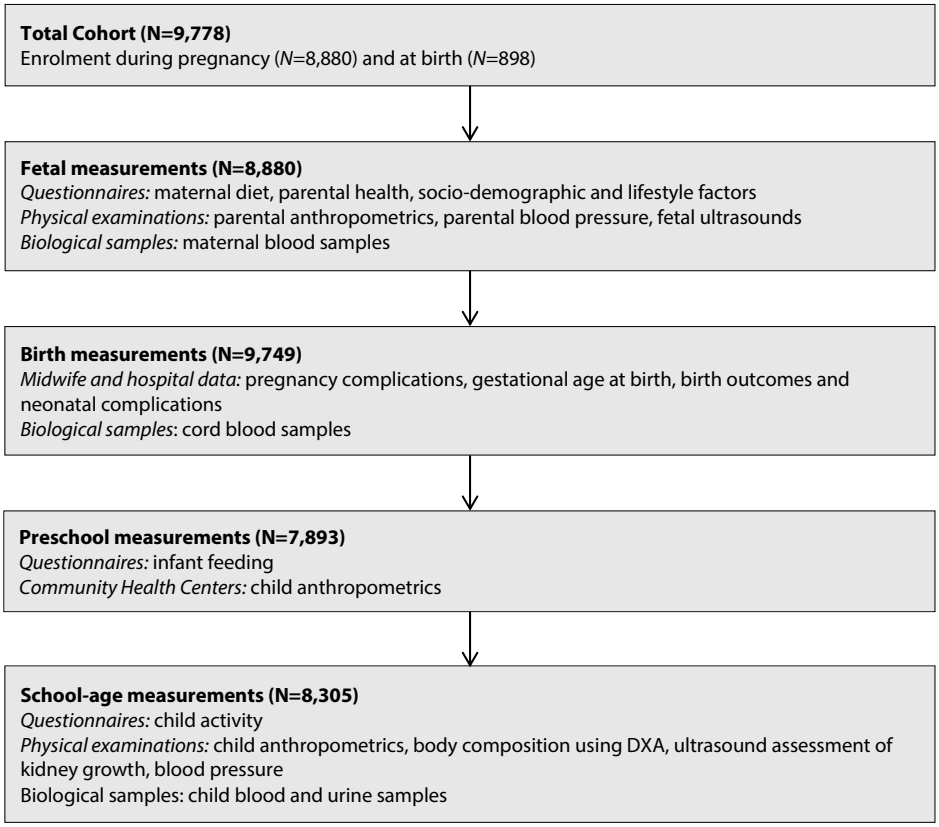
GENERAL AIM OF THIS THESIS

The general aim of this thesis was to identify pathways linking early life nutrition, with early growth and subsequent kidney structure and function in children.

GENERAL DESIGN

The studies described in this thesis are embedded in The Generation R Study, a population-based prospective cohort study from fetal life onwards.[44] Mothers who lived in Rotterdam and had an expected delivery date between April 2002 and January 2006 were enrolled in the study. Enrollment was aimed in early pregnancy, but was allowed until the birth of their child. Partners were also invited to participate. As shown in **Figure 1.2**, 9,778 mothers were enrolled in the study, of which 91% (8,880) were enrolled during pregnancy. Visits were planned in early pregnancy (gestational age <18 weeks), mid-pregnancy (gestational age 18–25 weeks) and late pregnancy (gestational age >25 weeks). Physical and fetal ultrasound examinations were carried out and blood and urine samples were collected during each visit. Before each visit, mothers were asked to fill out postal questionnaires. A food frequency questionnaire was sent out in early pregnancy. Fathers participated in one postal questionnaire and one physical examination. Around the age of 6 years, all children were invited to visit a dedicated research center in the Erasmus MC-Sophia Children's Hospital. Measurements during this visit included anthropometrics, body composition using Dual-energy X-ray Absorptiometry (DXA), blood pressure, kidney ultrasound and blood and urine samples for later determination of kidney function and cardiovascular risk factors.

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



OUTLINE

In **Chapter 2**, studies on fetal factors, especially maternal nutritional factors and their influence on fetal growth and childhood kidney function are presented. **Chapter 2.1** describes the associations of maternal protein intake in early pregnancy and childhood kidney development. In **Chapter 2.2,** we explore the association of maternal folate, vitamin B_{12} and homocysteine concentrations during pregnancy with offspring kidney structure and function. **Chapter 2.3** presents the potential role of maternal vitamin D concentrations during pregnancy in fetal growth and the risk of adverse birth outcomes. In **Chapter 2.4**, we examine the association of maternal and fetal vitamin D status with childhood body composition and cardiovascular outcomes. **Chapter 2.5** describes the relation of maternal vitamin D concentrations during pregnancy with childhood kidney outcomes. **Chapter 2.6** focuses on potential associations of maternal and paternal blood pressure and hypertensive disorders in pregnancy with childhood blood pressure developments. In **Chapter 3** we present studies focused on potential

genetic, infant and childhood determinants of kidney function. In **Chapter 3.1**, the associations of a genetic risk score combining previously identified common genetic variants related to impaired kidney function in adults with kidney outcomes in school-age children are explored. In **Chapter 3.2**, we study the associations of infant breastfeeding with childhood kidney structure and function. **Chapter 3.3** compares the associations of body composition measures with estimates of glomerular filtration rate based on creatinine and cystatin C concentrations. Lastly, **Chapter 4** presents an overall discussion of the main findings of this thesis and also covers suggestions for future research and clinical implications.

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Chapter 2

Fetal factors

Chapter 2.1

First-trimester maternal protein intake and childhood kidney outcomes

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Adapted from Am J Clin Nutr. 2015;102(1):123–9

ABSTRACT

Background: Nutritional exposures during in utero development may have long-lasting consequences for postnatal renal health. Animal studies suggest that specifically maternal dietary protein intake during pregnancy influences childhood kidney function.

Objective: We examined the associations of total, animal and vegetable maternal protein intake during pregnancy with kidney volume and function in school-age children.

Design: This study was performed in 3,650 pregnant women and their children participating in a population-based cohort study from early life onwards. First trimester energy adjusted maternal protein intake was assessed with a food frequency questionnaire. At the child's age of 6 years, we assessed kidney volume, estimated glomerular filtration rate (eGFR) using serum creatinine and cystatin C levels, and microalbuminuria using urine albumin-creatinine ratios.

Results: First trimester maternal total protein intake was associated with a higher childhood creatinine-based eGFR (0.06 (95% CI 0.01, 0.12) ml/min/1.73m^2 per gram of protein intake). This association was mainly driven by vegetable protein intake (0.22 (95% CI 0.10, 0.35) ml/min/1.73m^2 per gram of vegetable protein intake). These associations were not explained by protein intake in early childhood. First trimester maternal protein intake was not significantly associated with childhood kidney volume, cystatin C-based eGFR or the risk of microalbuminuria.

Conclusion: Our findings suggest that higher total and vegetable, but not animal, maternal protein intake during first trimester of pregnancy is associated with a higher eGFR in childhood. Further follow-up studies are needed to investigate whether maternal protein intake in early pregnancy also affects the risk of kidney diseases in later life.

INTRODUCTION

Maternal nutritional exposures during pregnancy may persistently affect offspring kidney development.[1] Results from the Dutch Famine Study suggest that maternal exposure to extreme famine in mid-gestation is linked to an increased risk of microalbuminuria in their adult offspring.[2] The mechanisms by which suboptimal maternal nutrition affects offspring kidney development may include smaller kidneys with a reduced number of nephrons, which in turn leads to glomerular hyperfiltration and sclerosis.[3] These adaptations predispose individuals to subsequent development of higher blood pressure, impaired kidney function and end-stage kidney disease in adulthood.[3] Apart from the evidence from the Dutch Famine Study, not much is known about more common and contemporary adverse nutritional exposures during pregnancy that influence kidney function in the offspring. Animal studies suggest that low maternal protein intake during pregnancy leads to a lower number of nephrons in their offspring and may hence impact the risk of renal disease and hypertension in later life.[1, 4, 5] Nine-months old lambs whose mothers were fed with a 50% nutrient restricted diet during early-mid pregnancy, had fewer renal glomeruli than did those whose mothers were fed a normal diet.[4] In addition, studies showed that a low protein diet in pregnant rats results in a nephron deficit in the offspring at birth which extends into postnatal life.[5] Observational studies in human adults showed that high dietary protein is associated with progression of renal disease.[6] Interestingly, studies in adults also suggest different effects of animal versus vegetable protein on kidney health.[7, 8]

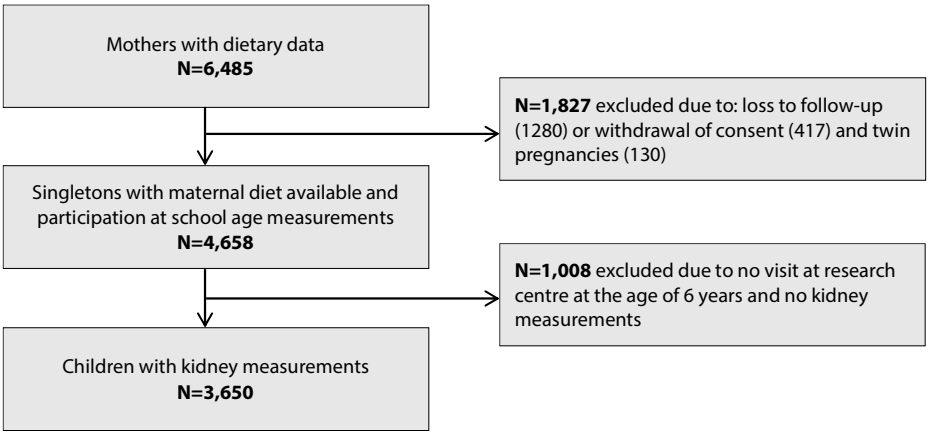
Therefore, we examined, in a population-based prospective cohort study among 3,650 mothers and children, the associations of first-trimester maternal protein intake during pregnancy with child's kidney outcomes at the age of 6 years. Main kidney outcomes were kidney volume, eGFR based on serum creatinine and cystatin C levels and microalbuminuria.

METHODS

Subjects

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onward in Rotterdam, the Netherlands.[9] The study was conducted according to the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all mothers. Enrolment in the study was aimed at early pregnancy, but was allowed until the birth of the child. Information about maternal diet and other lifestyle-related variables during pregnancy was collected at enrolment. At the age of 6 years, all participating children and their mothers were invited to a dedicated research center, to participate in detailed hands-on measurements. Of the 4,658 singleton live-born children with maternal nutritional data available and who participated in the study during childhood, 3,650 (78%) children attended the follow-up visit at the age of 6 years with successful measurements on kidney volume and function (**Figure 2.1.1**).

Figure 2.1.1 Flowchart of the study participants



Maternal dietary assessment

Maternal dietary intake was assessed at enrollment (median 13.5 weeks of gestation, 95% range 9.8–22.9) using a modified version of the validated semi-quantitative food frequency questionnaire (FFQ) of Klipstein-Grobusch et al.[10] The FFQ considered food intake over the previous 3 months, which mostly covered dietary intake in the first trimester of pregnancy. The FFQ consisted of 293 items structured according to the meal pattern. Questions included consumption frequency, portion size, preparation method, and additions. We estimated portion sizes by using Dutch household measures and photographs of foods that showed different portion sizes. We used the Dutch Food-Composition Table 2006 to calculate average daily intakes of total energy, carbohydrates, fat, and of total, animal, and vegetable protein.[11] For this study we split protein into animal (from e.g. meat, fish, and dairy) and vegetable protein (from e.g. grains, nuts, and legumes).

Kidney outcome assessments

Children's kidney outcomes were assessed at a median age of 6.1 years (95% range 5.6 to 7.3) by well-trained staff in a dedicated research center in the Sophia Children's Hospital in Rotterdam.

Our main kidney outcomes were combined kidney volume, estimated glomerular filtration rate (eGFR) and microalbuminuria. Combined kidney volume is a structural developmental outcome. Post-mortem studies showed that kidney volume is correlated with the number of nephrons. We used eGFR, calculated from blood creatinine and cystatin C levels as main measure of kidney function: eGFR is a general marker of glomerular filtration used in both clinical practice and population based cohort studies. Microalbuminuria is an established predictor of chronic kidney disease and end-stage renal disease.

Kidney volume was measured with ultrasound, using an ATL-Philips HDI 5000 instrument (Seattle, WA, USA), equipped with a 2.0–5.0 MHz curved array transducer. We identified the

left and right kidney in the sagittal plane along its longitudinal axis. We performed measurements of maximal bipolar kidney length, width and depth. Kidney width and depth were measured at the level of the hilum. The cross-sectional area in which the kidney appeared symmetrically round at its maximum width was used. Kidney volume was calculated using the equation for a prolate ellipsoid: volume (cm^3) = 0.523 x length (cm) x width (cm) x depth (cm).[12] Combined kidney volume was calculated by summing right and left kidney volume. We previously reported good intra-observer and inter-observer correlation coefficients.[13]

Non-fasting blood samples were drawn by antecubital venipuncture. Blood samples were collected, transported, and stored as described in detail elsewhere.[14] Samples were transported on dry ice to the Erasmus Medical Center where creatinine concentrations were measured with enzymatic methods and cystatin C concentrations with a particle-enhanced immunoturbidimetric assay (using Cobas 8000 analyzers; Roche). Quality-control samples demonstrated intra-assay and interassay coefficients of variation ranging from 0.51% to 1.37% and from 1.13% to 1.65%, respectively.

The eGFR was calculated according to the revised Schwartz 2009 formula: $eGFR_{creat}$ = 36.5 * (height (cm)/serum creatinine (µmol/l)).[15] In addition, we estimated the glomerular filtration rate using Zappitelli's formula based on cystatin C levels: $eGFR_{cystC} = 75.94/[CysC^{1.17}]$.[16] Urine creatinine (µmol/l) and urine albumin (µg/l) levels were determined with a Beckman Coulter AU analyzer, creatinine levels were measured with the Jaffe reaction. In line with clinical cut-offs, microalbuminuria was defined as an albumin-creatinine ratio between 2.5 and 25 mg/mmol for boys and between 3.5 and 25 mg/mmol for girls.[17]

Covariates

Information on maternal age, ethnicity, educational level, smoking during pregnancy, alcohol usage during pregnancy, and folic acid supplementation were obtained using questionnaires.[9] We classified maternal ethnicity into 6 categories (Dutch, Turkish, Moroccan, Surinamese or Dutch Antillean, other Western, and other non-Western).[18] Maternal pre-pregnancy height and weight were self-reported and pre-pregnancy body mass index (BMI) was calculated (kg/m^2). Information on the presence of pre-pregnancy comorbidities (defined as the occurrence of high cholesterol, diabetes, hypertension) was available from a questionnaire administered in the first trimester. Information on child's sex, birthweight and gestational age was available from medical records and hospital registries. Sex and gestational age specific SD scores for birth weight were calculated using existing reference data.[19] Information on breastfeeding was obtained from postnatal questionnaires.[20] Child protein intake at the age of 1 year was measured with a validated semi-quantitative FFQ in a subgroup of 2,193 children.[21,22] At the age of 6 years, child height was determined in standing position to the nearest millimeter without shoes by a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Weight was measured using a mechanical personal scale (SECA, Almere, the Netherlands). We calculated BMI (kg/m^2), and body surface area (BSA) (m^2) (using DuBois formula BSA= weight $(kg)^{0.425}$ x height $(cm)^{0.725}$ x 0.007184).[23] Time spent watching television or using a computer at the age of 6 years was assessed with a questionnaire.[24]

Statistical analysis

We adjusted protein intake for energy intake using the nutrient residual method.[25] For interpretation, the predicted protein intake for the mean energy intake (2,073 kcal/d) was added to the residuals as a constant.[25] Protein intake was analyzed as a continuous variable. We used multivariable linear regression models to assess associations of total, animal, and vegetable protein intake with kidney volume, creatinine, cystatin C and eGFR. For these models, we examined whether the residuals were normally distributed using normal probability plots, whether the variance of the residuals was homoscedastic and whether the regression models were linear. We assessed associations of protein intake with risk of microalbuminuria with multivariable logistic models. All models were adjusted for child's age and sex (basic model). Analyses with vegetable protein intake were additionally adjusted for animal protein intake and vice versa. The adjusted models were further controlled for maternal characteristics and socio-demographic factors (maternal age, BMI before pregnancy, gestational weight gain, gestational age at intake, ethnicity, and education), maternal lifestyle factors (smoking, and alcohol consumption during pregnancy, folic acid supplement use during pregnancy), pre-pregnancy comorbidities and child characteristics (birthweight adjusted for gestational age, breastfeeding, body surface area at 6 years visit, and screen time at the age of 6 years). Covariates were included in the regression models based on previous literature or a change of >10% in effect estimates. To assess whether the associations were different by maternal ethnicity, child sex, birthweight, gestational age, or BSA of the child at the age of 6 years, we evaluated the statistical interaction by adding the product term of the covariate and total protein intake to the models. To examine whether the associations of maternal protein intake with kidney outcomes could be explained by later diet of the child, we performed a sensitivity analysis for a subgroup of our cohort in which information was available about protein intake at the age of 1 year.[26] Also, we performed a sensitivity analysis including Dutch women only (N=2,332). To prevent bias associated with missing data, we used multiple imputations (N=5) for covariates with missing values on the basis of the correlation of missing variables with other participant characteristics, according to the Markov Chain Monte Carlo method.[27] The amount of missing values ranged from 2.2% to 16.1%. Because we found similar results, we report the pooled results. Subjects characteristics before and after imputation are shown in **Supplementary Table 2.1.1**. Statistical analyses were performed using the Statistical Package of Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of the mothers and their children are presented in **Table 2.1.2.** Mean (±SD) maternal energy intake was 2,073 kcal per day (±543). Mean protein intake in these mothers was 76.6 g (±20.4), which provided on average 15% of total energy intake. Animal protein intake provided 9% of total energy intake, and vegetable protein intake provided 6%. No differences were observed in maternal energy and protein intake between mothers of children with and without kidney follow up measurements (data not shown).

Table 2.1.1. Subject characteristics (N=3,650)[1]

Subject characteristics	
Maternal characteristics	
Maternal age (y)	31.1 (4.7)
Gestational age at intake (weeks)	13.5 (9.8, 22.9)
Maternal body mass index at enrolment (kg/m^2)	22.6 (18.4, 34.4)
Nulliparous (%)	59.4
Education level (%)	
- No higher education	44.9
- Higher education	55.1
Ethnicity (%)	
- Dutch	64.3
- Turkish	5.1
- Moroccan	3.5
- Surinamese or Dutch Antilles	7.9
- Other Western	12.5
- Other non-Western	6.7
Smoking during pregnancy (%)	
- Never	76.2
- Until pregnancy was known	9.6
- Continued	14.2
Alcohol during pregnancy (%)	
- Never	37.9
- Until pregnancy was known	14.5
- Continued	47.6
Folic acid supplements use (%)	
- No	23.2
- Start 1st to 10 weeks	31.0
- Start periconceptional	45.8
Pre-pregnancy comorbidities (%)	1.6
Maternal diet	
Total energy intake (kcal)	2,073 (543)
Protein (g/d)[2]	
- Total	76.6 (20.4)
- Animal	46.9 (15.4)
- Vegetable	29.9 (9.2)
Protein intake (E %)	15.0 (2.5)
Carbohydrate intake (E %)	48.5 (6.3)
Fat intake (E %)	36.3 (5.5)
Infant characteristics	
Girls (%)	50.1
Dutch ethnicity (%)	65.8
Gestational age at birth (wk)	40.1 (36.1, 42.4)
Birth weight (g)	3,469 (538)
Breastfeeding (%)	
- Exclusive ≥4 months	25.5
- Partial ≥4 months	64.6
- Never or ≤4 months	9.9

Table 2.1.1. Subject characteristics (N=3,650)[1] (continued)

Subject characteristics	
Child protein intake at 1 y (g/d)	25.1 (29.4)
Child characteristics at 6 y visit	
Age (y)	6.1 (5.6, 7.3)
Height (cm)	119.1 (5.6)
Weight (kg)	22.9 (3.8)
Body mass index (kg/m^2)	16.1 (1.7)
Body surface area (m^2)	0.90 (0.08)
Screen time (hour/day)	1.3 (0.3, 4.6)
Kidney volume (cm^3)	119 (23)
Creatinine (µmol/l)	37.1 (5.4)
Cystatin C (µg/l)	784 (83)
$eGFR_{creat}$ (ml/min/1.73m^2)	120 (16)
$eGFR_{cystC}$ (ml/min/1.73m^2)	103 (15)
Microalbuminuria (%)	7.2

[1] Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. Values are based on imputed data. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine levels; $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C levels. [2]unadjusted for energy intake.

Table 2.1.2 shows that in the multivariable adjusted models higher maternal total protein intake in the first trimester was associated with higher childhood $eGFR_{creat}$ (0.06 (95% CI 0.01, 0.12) ml/min/1.73m^2 per gram of protein intake). Stronger associations were observed for first trimester maternal vegetable protein intake with childhood $eGFR_{creat}$ (0.22 (95% CI 0.10, 0.35) ml/min/1.73m^2 per gram of vegetable protein intake). Maternal animal protein intake was not significantly associated with childhood $eGFR_{creat}$. Furthermore maternal protein intake was not significantly associated with kidney volume, $eGFR_{cystC}$ or risk of microalbuminuria. In line with our eGFR findings, first trimester maternal total protein intake and vegetable protein intake were associated with lower levels of childhood creatinine (-0.02 (95% CI -0.04, -0.01) µmol/l and (-0.07 (95% CI -0.11, -0.03) µmol/l per gram of total and vegetable protein intake), but not with cystatin C (**Supplementary Table 2.1.2**).

After additional adjustment for child protein intake at the age of 1 year, the multivariable associations of maternal protein intake with kidney outcomes did not change (**Supplementary Table 2.1.3**). We did not observe significant interactions between maternal total protein intake and sex, birthweight, gestational age, maternal ethnicity or child BSA in the models with kidney outcomes. Results of the sensitivity analyses in Dutch mothers only are presented in **Supplementary Table 2.1.4** showing similar patterns as in the full group, however in this group we also observed an association between higher animal protein intake and a higher $eGFR_{cystC}$.

Table 2.1.2. Associations of maternal protein intake during pregnancy with childhood kidney outcomes[1]

	Kidney volume cm^3 N=3,344	$eGFR_{creat}$ $ml/min/1.73\ m^2$ N=2,494	$eGFR_{cystC}$ $ml/min/1.73\ m^2$ N=2,500	Microalbuminuria Odds ratio N=3,515
Basic model[2]				
Total protein intake (g)[5]	**0.09 (0.06, 0.11)**[4]	**0.08 (0.06, 0.11)**[4]	0.04 (-0.01, 0.09)	1.00 (0.99, 1.01)
Animal protein intake (g)[5]	**0.08 (0.05, 0.10)**[4]	**0.06 (0.04, 0.09)**[4]	0.04 (-0.01, 0.09)	1.00 (0.99, 1.01)
Vegetable protein intake (g)[5]	**0.18 (0.12, 0.23)**[4]	**0.28 (0.23, 0.33)**[4]	0.03 (-0.08, 0.14)	1.00 (0.99, 1.01)
Multivariable adjusted model[3]				
Total protein intake (g)[5]	0.03 (-0.03, 0.08)	**0.06 (0.01, 0.12)**[4]	0.03 (-0.03, 0.08)	1.00 (0.99, 1.01)
Animal protein intake (g)[5]	0.02 (-0.04, 0.08)	0.05 (-0.01, 0.10)	0.03 (-0.03, 0.08)	1.00 (0.99, 1.01)
Vegetable protein intake (g)[5]	0.05 (-0.08, 0.18)	**0.22 (0.10, 0.35)**[4]	0.01(-0.11, 0.13)	1.00 (0.97, 1.02)

[1]Values are based on multivariable linear or logistic regression models and reflect differences or odds ratios and 95% confidence intervals in kidney volume and function measures per gram increase of protein intake. Basic model[2] is adjusted for child's sex and age at 6-year visit. Multivariable adjusted model[3] is adjusted for child's sex, age at 6 year visit, maternal characteristics (age, body mass index before pregnancy, weight gain during pregnancy, gestational age at intake), socio-demographic factors (ethnicity, education), maternal lifestyle (smoking, and alcohol consumption during pregnancy, folic acid intake during pregnancy), pre-pregnancy comorbidities, and child characteristics (birthweight adjusted for gestational age, breastfeeding, body surface area, and screen time at the age of 6y). Models with animal protein intake were additionally adjusted for vegetable protein intake and vice versa. [4]p <0.05. [5]Protein intakes are energy-adjusted using the nutrient residual method. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine levels; $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C levels.

DISCUSSION

In this large population-based prospective cohort study, we observed that a higher maternal intake of total and vegetable protein, but not animal protein, during first trimester of pregnancy is associated with higher $eGFR_{creat}$, but not with kidney size, $eGFR_{cystC}$ or microalbuminuria in school-age children. The observed differences in eGFR related to maternal protein intake with eGFR were small. These differences may be without clinical consequence at an individual level, but may be relevant on a population level.

Historical cohort studies suggest that adult offspring of mothers who were exposed to severe undernutrition during their pregnancy have increased risk of cardiovascular and renal disease.[28] During the winter 1944–1945 the western part of the Netherlands was struck by a period of severe food scarcity, where the daily rations dropped to 400–800 calories.[29] Follow up studies among adults whose mothers were exposed to the famine during their pregnancy, showed an increased risk for having microalbuminuria in adults whose mothers were exposed to the famine during mid-gestation.[2] Blood pressure was also higher in adults whose pregnant mothers where exposed to the famine. The variation in blood pressure depended on the timing mothers were exposed to the famine, with strongest associations on blood pressure for famine during late gestation.[29] Rooseboom et al. [29] postulated that it might be the macronutrient composition rather than the quantity of a pregnant woman's diet that affects the child's blood pressure in later life. Blood pressure was especially higher in adults whose mothers ate small amounts of protein in relation to carbohydrate during the third trimester of pregnancy.[30] Interestingly, a study in Aberdeen showed higher blood pressure

in adults who were exposed to a low animal protein and corresponding high carbohydrate diet in utero.[31]

The mechanisms by which maternal undernutrition affects kidney development in the offspring may include developmental adaptations that lead to smaller kidneys with a reduced number of nephrons, which in turn lead to glomerular hyperfiltration and sclerosis.[3] Increased filtration through each glomerulus leads to hypertrophy and hyperfiltration injury, which is marked by the onset of microalbuminuria, and may eventually lead to a reduction in renal function.[3, 32, 33] The renal effects of undernutrition depend upon its timing during gestation and the nutrients balance.[2]

Thus far, not much is known about common, contemporary nutritional exposures during pregnancy that affect offspring kidney health. Studies in animals have shown that undernutrition, mainly protein restriction, of pregnant rats raises blood pressure in the offspring permanently.[34, 35] Also, maternal protein restriction in rats influences on offspring kidney structure and function, it promotes a reduction in nephron number that is associated with reduction of GFR in postnatal life.[36] In the same population-based cohort as the current study, we did not observe any association between maternal protein intake during the first trimester of the pregnancy with blood pressure in 6 years old children.[37]

In the current study, we observed that first trimester maternal protein intake was positively associated with $eGFR_{creat}$ in 6 years old children. Our results are in line with results from studies performed in rats[5] and sheep.[38] We did not find an association of maternal first trimester protein intake with childhood microalbuminuria. It may be that the effects of impaired kidney growth on microalbuminuria may not be detectable during childhood, but may become evident later in life. Fetal adverse exposures can be compensated for many years before the adverse outcomes are present.[39]

In our study population, the associations with eGFR were stronger for vegetable than for animal protein intake during pregnancy. Previous studies in adults also reported different associations for animal versus vegetable protein on kidney health.[7, 8] A mechanism through which animal and vegetable protein may differentially affect eGFR is via differences in amino acid composition. Experimental studies have shown that different types of amino acids have different renal impact.[40] However further studies need to explore the mechanisms underlying the associations of specifically vegetable proteins with childhood kidney function outcomes.

Nephrogenesis requires a fine balance of many factors that can be disturbed by intrauterine growth restriction, leading to a low nephron endowment.[41] Because nephrogenesis continues until 36 weeks of gestation and largely ceases thereafter, adverse exposures during this critical period may lead to impaired kidney development.[42, 43] A previous study suggested that adult hypertension programmed by maternal exposure to a low protein diet is linked to marked changes in the renal expression of the glucocorticoid receptor, 11 β -hydroxysteroid dehydrogenase and components of the renin-angiotensin system. In addition, protein restriction during pregnancy could affect the growth hormone-insulin-like growth factor and the prostaglandins axis in the offspring.[38] Welham et al. suggested that maternal diet programs the embryonic kidney, altering cell turnover and gene expression at a time when nephrons and glomeruli have yet to form.[44]

Some methodological issues need to be discussed. A major strength of our study is the prospective design from fetal life onwards within a large population-based cohort. Our analyses were based on 3,650 mother-child pairs. We used FFQs to assess maternal diet during the first pregnancy trimester. Although the FFQ yielded valid estimates of nutrient intakes when validated against 3-days 24-hours recalls, measurement error may still have occurred. One of the limitations in our study is that the FFQ was validated only in Dutch women. However, a sensitivity analysis in Dutch mothers only revealed similar results as in the whole group. Unfortunately, we did not have information about child protein intake at a later stage in childhood. In a subgroup of children of the present study group (N=2,193) we had dietary data at the age of 1 year. When we adjusted our models additionally for child protein intake at the age of 1 year, the regression coefficients remained similar in this subgroup, suggesting that child protein intake at the age of 1 year does not affect the association between maternal protein intake and childhood kidney function. This is in line with previous observations in this population that infant protein intake was not associated with kidney health.[26]

We performed detailed measurements of childhood kidney outcomes. Because nephron number cannot be studied in vivo, we used kidney size as a measure of kidney development employing ultrasound as a reliable method to measure kidney volume.[13] Kidney size is also correlated with the number of glomeruli and can be used in epidemiological studies as a measure of kidney development.[45] Glomerular enlargement due to hyperfiltration may also increase kidney volume.[46] The estimation of GFR in children remains challenging. Blood creatinine is most commonly used to calculate eGFR. We used the Schwartz formula based on creatinine levels and height, which was previously validated in a pediatric population.[15] In addition to blood creatinine levels, we also measured blood cystatin C levels to calculate eGFR based on cystatin C levels using Zappitelli's formula.[16, 47] Interestingly, in our population, maternal protein intake was associated with creatinine-based eGFR, but not with cystatin-based eGFR. Our results are in line with those from a trial in adults with chronic kidney disease, in which protein intake affected creatinine but not cystatin C concentrations.[48] Further studies are needed to evaluate the mechanism explaining these differences. Microalbuminuria was evaluated using urine albumin-creatinine ratio from a random urine sample.[49] Finally, although we performed adjustment for a large number of potential maternal and childhood confounders, residual confounding by other lifestyle factors, might still be present. Residual confounding may also be present because of measurement error in several unhealthy life style behaviors such as underreporting of smoking and alcohol consumption. Since the main outcomes were correlated, we did not adjust for multiple comparisons. However, we may have observed false positive associations due to the multiple tests that were performed.

CONCLUSION

In conclusion, results from this large prospective study suggests that higher first trimester maternal intake of total and vegetable protein is associated with a higher eGFR in their children at school-age. These findings are important from an etiological perspective. Further

studies are needed to investigate the underlying mechanisms. Although longitudinal studies suggest that risks factors for kidney diseases track from childhood to adulthood, follow up studies are needed to explore whether protein intake in pregnancy affects risk of kidney diseases in adulthood.

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Supplementary Table 2.1.1. Observed and imputed subject's characteristics (N=3,650)

	Observed	Imputed
Maternal characteristics		
Maternal age (y)	31.1 (4.7)	31.1 (4.7)
Gestational age at intake (weeks)	13.8 (9.8, 23.5)	13.5 (9.8, 22.9)
Maternal body mass index at enrolment (kg/m^2)	22.5 (18.3, 34.2)	22.6 (18.4, 34.4)
Missing, (%)	*15.3*	
Nulliparous (%)	59.5	59.4
Missing, (%)	*0.2*	
Educational level, (%)		
- No higher education	43.2	44.9
- Higher education	54.1	55.1
Missing, (%)	*2.7*	
Ethnicity (%)		
- Dutch	63.9	64.3
- Turkish	5.0	5.1
- Moroccan	3.5	3.5
- Surinamese or Dutch Antilles	7.8	7.9
- Other Western	12.5	12.5
- Other non-Western	6.7	6.7
Missing, (%)	*0.6*	
Smoking during pregnancy, (%)		
- Never smoked	69.3	76.2
- until pregnancy was known	8.8	9.6
- Continued	12.9	14.2
Missing, (%)	*9.0*	
Alcohol during pregnancy, (%)		
- Never alcohol in pregnancy	34.4	37.9
- Until pregnancy was known	13.1	14.5
- Continued	42.7	47.6
Missing, (%)	*9.8*	
Folic acid supplement use, (%)		
- No	13.8	23.2
- Start 1st to 10 weeks	25.2	31.0
- Start periconceptional	40.2	45.8
Missing, (%)	*20.8*	
Prepregnancy comorbidities (%)	1.6	1.6
Missing, (%)	*28.7*	
Maternal diet		
Total energy intake (kcal)	2,073 (543)	NI
Protein (g/d)[1]		
- Total	76.6 (20.4)	NI
- Animal	46.9 (15.4)	NI
- Vegetable	29.9 (9.2)	NI
Protein (E%)	15.0 (2.5)	NI
Carbohydrates (E%)	48.5 (6.3)	NI

Supplementary Table 2.1.1. Observed and imputed subject's characteristics (N=3,650) (continued)

	Observed	Imputed
Fat (E%)	36.3 (5.5)	NI
Missing, %	-	
Infant characteristics		
Girls, (%)	50.1	NI
Dutch ethnicity (%)	66.2	65.8
Missing, (%)	0.5	
Gestational age at birth (wk)	40.1 (36.1, 42.4)	40.1 (36.1, 42.4)
Missing, (%)	-	
Birth weight, (g)	3,469 (538)	3,469 (538)
Missing, (%)	*0.02*	
Breastfeeding (%)		
- Exclusive ≥4 months	21.6	25.5
- Partial ≥4 months	55.2	64.6
- Never or ≤4 months	7.4	9.9
Missing, (%)	15.8	
Child protein intake at 1 y (g/d)	26.6 (29.6)	NI
Missing, (%)	*39.9*	
Child characteristics at 6 y visit		
Age (years)	6.1 (5.6 - 7.3)	NI
Height (cm)	119.1 (5.6)	119.1 (5.6)
Missing, %	*0.2*	
Weight (kg)	22.9 (3.8)	22.9 (3.8)
Missing, (%)	*0.2*	
Body mass index, (kg/m^2)	16.1 (1.7)	16.1 (1.7)
Missing, (%)	*0.2*	
Body surface area, (m^2)	0.90 (0.08)	0.90 (0.08)
Missing, (%)	*0.2*	
Screen time (hour/day)	1.3 (0.3, 4.7)	1.3 (0.3, 4.6)
Missing, (%)	*20.1*	
Kidney volume combined, (cm^3)	119 (23)	NI
Missing, (%)	*8.4*	
Creatinine, (µmol/l)	37.1 (5.4)	NI
Missing, %	*31.5*	
Cystatin C (µg/l)	784 (83)	NI
Missing, %	*31.5*	
$eGFR_{creat}$, ($ml/min/1.73m^2$)	120 (16)	NI
Missing, (%)	*31.7*	
$eGFR_{cystC}$, ($ml/min/1.73m^2$)	103 (15)	NI
Missing, (%)	*31.5*	
Microalbuminuria, (%)	7.2	NI
Missing, %	*3.7*	

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine levels; $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C levels.[†]unadjusted for energy intake. NI - not imputed.

Supplementary Table 2.1.2. Associations of maternal protein intake during pregnancy with childhood creatinine and cystatin C[1]

	Serum creatinine (umol/l) N=2,500	Serum creatinine (umol/l) adjusted for children protein intake at the age of 1 year N=1,205	Serum cystatin C (µg/l) N=2,500	Serum cystatin C (µg/l) adjusted for children protein intake at the age of 1 year N=1,206
Total protein intake (g)[2]	**-0.02 (-0.04, -0.01)**[3]	**-0.03 (-0.05, -0.01)**[3]	-0.12 (-0.41, 0.17)	-0.03 (-0.46, 0.39)
Animal protein intake (g)[2]	-0.01 (-0.03, 0.004)	**-0.03 (-0.06, -0.01)**[3]	-0.13 (-0.42, 0.16)	-0.11 (-0.49, 0.27)
Vegetable protein intake (g)[2]	**-0.07 (-0.11, -0.03)**[3]	**-0.08 (-0.13, -0.03)**[3]	0.05 (-0.60, 0.69)	0.14 (-0.68, 0.45)

[1]Values are based on multivariable linear regression models and reflect differences or odds ratios and 95% confidence intervals in kidney volume and function measures for an increase in protein intake (g/d). The model is adjusted for child's sex, age at 6 year visit, maternal characteristics (age, body mass index before pregnancy, weight gain during pregnancy, gestational age at intake), maternal lifestyle (alcohol consumption, smoking during pregnancy, folic acid intake during pregnancy), socio-demographic factors (ethnicity, education), prepregnancy comorbidities, and child characteristics (breastfeeding, birthweight adjusted for gestational age, body surface area, and screen time at the age of 6y, child protein intake at 1 year). Models with animal protein intake were additionally adjusted for vegetable protein intake and vice versa. Protein intake is used as continuous variable in the regression models. [2]Protein intakes are energy-adjusted using the nutrient residual method. [3]p <0.05.

Supplementary Table 2.1.3. Associations of maternal protein intake during pregnancy with childhood kidney outcomes, additionally adjusted for child protein intake at the age of 1 year (N=2,193)[1]

	Kidney volume Difference (95% CI) cm^3 N=1,603	$eGFR_{creat}$ Difference (95% CI) ml/min/1.73 m^2 N=1,204	$eGFR_{cystC}$ Difference (95% CI) ml/min/1.73 m^2 N=1,206	Microalbuminuria Odds ratio (95% CI) N=1,713
Total protein intake (g)[2]	0.03 (-0.06, 0.11)	**0.11 (0.02, 0.19)**[3]	0.02 (-0.06, 0.09)	1.00 (0.98, 1.02)
Animal protein intake (g)[2]	0.04 (-0.03, 0.12)	**0.12 (0.05, 0.20)**[3]	0.02 (-0.05, 0.10)	1.00 (0.99, 1.02)
Vegetable protein intake (g)[2]	-0.01 (-0.17, 0.16)	**0.30 (0.13, 0.45)**[3]	0.001 (-0.17, 0.17)	1.00 (0.99, 1.03)

[1]Values are based on multivariable linear or logistic regression models and reflect differences or odds ratios and 95% confidence intervals in kidney volume and function measures for an increase in protein intake (g/d). The model is adjusted for child's sex, age at 6 year visit, maternal characteristics (age, body mass index before pregnancy, weight gain during pregnancy, gestational age at intake), maternal lifestyle (alcohol consumption, smoking during pregnancy, folic acid intake during pregnancy), socio-demographic factors (ethnicity, education), pre-pregnancy comorbidities, and child characteristics (breastfeeding, birthweight adjusted for gestational age, body surface area, and screen time at the age of 6y and child protein intake at 1 year). Models with animal protein intake were additionally adjusted for vegetable protein intake and vice versa. Protein intake is used as continuous variable in the regression models. [2]Protein intakes are energy-adjusted using the nutrient residual method. [3]p <0.05. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine levels; $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C levels.

Supplementary Table 2.1.4. Crude and multivariable adjusted associations of maternal protein intake during pregnancy with childhood kidney outcomes, in Dutch mothers only (N=2,332)

	Kidney volume Difference (95% CI) cm^3 N=2,137	Serum creatinine Difference (95% CI) µmol/l N=1,601	Serum cystatin C Difference (95% CI) µg/l N=1,602	$eGFR_{creat}$ Difference (95% CI) $ml/min/1.73m^2$ N=1,597	$eGFR_{cystC}$ Difference (95% CI) $ml/min/1.73m^2$ N=1,602	Microalbuminuria Odds ratio (95% CI) N=2,233
Basic model[1]						
Total protein intake (g)[3]	**0.08 (0.004, 0.17)**[4]	**-0.04 (-0.06, -0.01)**[4]	-0.36 (-0.72, 0.01)	**0.13 (0.06, 0.19)**[4]	**0.08 (0.01, 0.15)**[4]	1.00 (0.98, 1.02)
Animal protein intake (g)[3]	**0.08 (0.001, 0.17)**[4]	**-0.03 (-0.05, -0.007)**[4]	-0.37 (-0.74, 0.01)	**0.11 (0.04, 0.18)**[4]	**0.08 (0.01, 0.15)**[4]	1.00 (0.99, 1.02)
Vegetable protein intake (g)[3]	0.09 (-0.09, 0.26)	**-0.07 (-0.12, -0.02)**[4]	-0.21 (-0.98, 0.56)	**0.22 (0.07, 0.36)**[4]	0.06 (-0.09, 0.20)	0.99 (0.96, 1.02)
Multivariable adjusted model[2]						
Total protein intake (g)[3]	0.02 (-0.05, 0.10)	**-0.02 (-0.05, -0.004)**[4]	-0.26 (-0.64, 0.12)	**0.08 (0.01, 0.15)**[4]	0.07 (-0.001, 0.14)	1.03 (0.61, 1.73)
Animal protein intake (g)[3]	0.03 (-0.05, 0.10)	-0.02 (-0.05, 0.002)	-0.29 (-0.68, 0.09)	**0.07 (0.002, 0.15)**[4]	**0.07 (0.002, 0.14)**[4]	1.00 (0.98, 1.01)
Vegetable protein intake (g)[3]	-0.05 (-0.22, 0.11)	-0.04 (-0.09, 0.01)	0.09 (-0.74, 0.93)	0.10 (-0.05, 0.26)	0.02 (-0.14, 0.17)	0.97 (0.94, 1.01)

Values are based on multivariable linear or logistic regression models and reflect differences or odds ratios and 95% confidence intervals in kidney volume and function measures for maternal protein intake. Basic model[1] is adjusted for child's sex and age at 6 y visit. Multivariable adjusted model[2] is adjusted for child's sex, age at 6 y visit, maternal characteristics (age, body mass index before pregnancy, weight gain during pregnancy, gestational age at intake), maternal lifestyle (alcohol consumption, smoking during pregnancy, folic acid intake during pregnancy), socio-demographic factors (education), pre-pregnancy comorbidities, and child characteristics (breastfeeding, birth weight adjusted for gestational age, body surface area, and screen time at the age of 6y). Models with animal protein intake were additionally adjusted for vegetable protein intake and vice versa. [3]Protein intakes are energy-adjusted using the nutrient residual method. [4]$p < 0.05$. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine levels, $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C levels.

Chapter 2.2

Maternal and fetal folate, vitamin B_{12} and homocysteine concentrations and childhood kidney outcomes

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ABSTRACT

Background: Folate, vitamin B_{12} and homocysteine concentrations during pregnancy are important factors for early development and may persistently influence kidney function in the offspring. We examined the associations of folate, vitamin B_{12}, and homocysteine concentrations during pregnancy with kidney outcomes in school-aged children.
Study design: Population-based prospective cohort study from fetal life onwards.
Settings & participants: This study was performed among 4,226 pregnant women and their children.
Predictors: Folate, vitamin B_{12} and homocysteine blood concentrations measured in early pregnancy (median gestational age 13.2 weeks (25^{th} to 75^{th} percentiles 12.2, 14.8) and at birth (cord blood).
Outcomes & measurements: At the median age of 6.0 years (25th to 75th percentiles 5.9, 6.3) we measured combined kidney volume with ultrasound, estimated glomerular filtration rate based on creatinine ($eGFR_{creat}$) and cystatin C ($eGFR_{cystC}$) concentrations and microalbuminuria.
Results: We observed that higher maternal folate concentrations were associated with larger childhood combined kidney volume, whereas higher maternal vitamin B_{12} concentrations were associated with higher childhood $eGFR_{cystC}$ (*p*-values <0.05). These associations were independent of homocysteine concentrations. Higher maternal homocysteine concentrations were associated with smaller combined kidney volume and lower childhood $eGFR_{cystC}$ (*p*-values <0.05). The association of maternal homocysteine concentrations with childhood $eGFR_{cystC}$ was largely explained by combined kidney volume. Higher cord blood homocysteine concentrations were associated with larger combined kidney volume and lower $eGFR_{cystC}$ (*p*-values <0.05). Folate, vitamin B_{12} or homocysteine concentrations were not associated microalbuminuria.
Limitations: Observational study, so causality cannot be established.
Conclusion: Our findings suggest that folate, vitamin B_{12} and homocysteine concentrations during fetal life are associated with offspring kidney development. However, the effect sizes are small. Further studies are needed to replicate these findings and assess the causality and consequences for kidney health in later life.

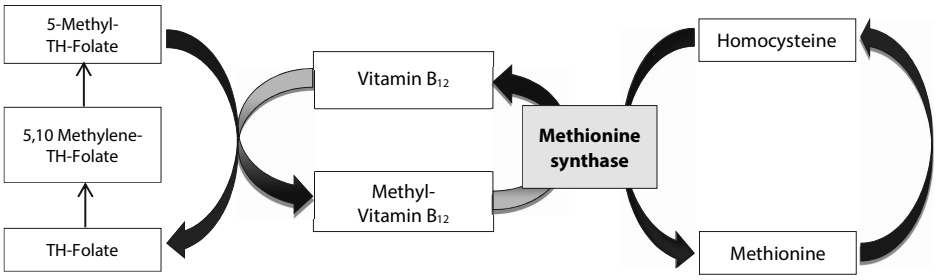
INTRODUCTION

Adverse fetal nutritional exposures may have persistent consequences for kidney health in later life. Specifically, suboptimal nutritional exposures during fetal life may affect nephrogenesis, leading to a reduced number of nephrons and smaller kidneys, which subsequently leads to glomerular hyperfiltration and sclerosis.[1, 2] The importance of early life nutrition for later kidney outcomes is illustrated by studies showing both associations of severe maternal undernutrition, preterm birth, and low birth weight with the risk of kidney disease in adulthood.[3, 4, 5]

Little is known about specific maternal nutritional exposures that persistently influence offspring kidney development.[4, 6] Folate is an essential B-vitamin, important for cell growth and replication; along with vitamin B_{12}, it is a key methyl donor in several reactions, including the production of thymidine for DNA synthesis, polyamine synthesis and biosynthesis of methionine from homocysteine.[7–9] Folate and vitamin B_{12} contribute to lowering homocysteine concentrations (**Figure 2.2.1**).[10] Animal studies indicate that elevated homocysteine concentrations may lead to glomerular damage, and folic acid supplementation lowers creatinine concentration and urinary albumin excretion induced by hyperhomocysteinemia.[11] Also, studies in adults have shown associations of elevated homocysteine concentrations with an accelerated decline in kidney function.[12] Based on these findings, we hypothesized that lower folate and vitamin B_{12} concentrations and higher homocysteine concentrations during fetal life may affect nephrogenesis and lead to smaller kidneys with a lower kidney function.

Thus, we examined, in a population-based prospective cohort study among 4,226 mothers and their children the associations of folate, vitamin B_{12} and homocysteine concentrations during first trimester of pregnancy and at birth with kidney outcomes in school-aged children.

Figure 2.2.1. Folate, vitamin B_{12} and homocysteine metabolism



Schematic representation of homocysteine metabolism. Folate and vitamin B_{12} work closely together on homocysteine metabolism, handing off methyl groups to each other. TH-Folate is converted to 5, 10-Methylene-TH-Folate which is further reduced to 5- Methyl-TH-Folate. With the demethylation of 5-Methyl-TH-Folate, the methyl group is donated into the methionine cycle. Vitamin B_{12} is involved directly in the transfer of the methyl group to homocysteine, through methionine synthase. Methionine synthase is a vitamin B_{12}- dependent enzyme that catalyzes the formation of methionine from homocysteine. The methionine cycle begins with homocysteine that accepts the methyl group from the folate pool through 5- Methyl-TH-Folate. Abbreviations: TH- Folate, Tetrahydro- folate.

METHODS

Subjects

This study was embedded in the Generation R Study, an ongoing population-based prospective cohort study from fetal life onward in Rotterdam, the Netherlands.[13] The study was conducted according to the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of Erasmus University Medical Center, Rotterdam (MEC-2007–413). Written informed consent was obtained from parents. At the age of 6 years, all participating children and their mothers were invited to participate in detailed measurements. Of 8,879 mother prenatally included in the study, 6,128 had measurements on folate, vitamin B_{12} or homocysteine concentrations. Of the 6,057 singleton live-born children from mothers with nutritional data available, 70% of them attended the follow-up visit at the age of 6 years. Children with successful information on at least one of the kidney measurements were included in the study (N=4,226) (**Supplementary Figure 2.2.1**).

Maternal and fetal folate, vitamin B_{12} and homocysteine concentrations

In early pregnancy (median gestational age 13.2 weeks (25^{th} to 75^{th} percentile 12.2, 14.8)) venous samples were drawn and stored at room temperature before being transported to the regional laboratory for processing. Cord blood samples were taken immediately after delivery (40.1 weeks of gestation, 25^{th} to 75^{th} percentiles 39.3–41.0 weeks).[13] To analyze folate, vitamin B_{12} and homocysteine concentrations, ethylenediaminetetraacetic acid plasma samples (folate, homocysteine) and serum samples (vitamin B_{12}) were picked and transported to the Department of Clinical Chemistry at the Erasmus University Medical Centre, Rotterdam. After thawing, folate, homocysteine and vitamin B_{12} concentrations were analyzed using an immunoelectrochemoluminescence assay on the Architect System. These methods are described in detail elsewhere.[14] Folate concentrations were defined as: deficient when <7 nmol/l or normal when ≥7 nmol/l.[15]

Folic acid supplement intake

Information on folic acid supplement use (0.4–0.5 mg) and the initiation of supplementation was obtained by questionnaires at the enrolment of the study. We categorized folic acid supplement use into three groups: 1) periconceptional use; 2) start when pregnancy was known; 3) no use during pregnancy. Detailed information on folic acid supplement intake is described elsewhere.[16]

Childhood kidney outcomes

As previously described, children's kidney outcomes were assessed at a median age of 6.0 years (25^{th} to 75^{th} percentiles 5.9, 6.3) in a dedicated research center in the Sophia Children's Hospital in Rotterdam.[17] Kidney volume was measured with ultrasound, using an ATL-Philips HDI 5000 instrument (Seattle, WA, USA), equipped with a 2.0–5.0 MHz curved array transducer. We calculated kidney volume using the equation for a prolate ellipsoid.[18] Combined kidney volume was calculated by summing right and left kidney volume. Non-fasting blood

samples were drawn by antecubital venipuncture. Creatinine concentrations were measured with enzymatic methods and cystatin C concentrations with a particle enhanced immunoturbidimetric assay (using Cobas 8000 analyzers, Roche, Almere, the Netherlands). Estimated glomerular filtration rate (eGFR) was calculated according to the revised Schwartz 2009 formula: $eGFR_{creat}$ = 36.5 * (height (cm) / serum creatinine (µmol/l)),[19] and Zappitelli's formula based on cystatin C concentrations: $eGFR_{cystC}$ = 75.94/[$CysC^{1.17}$].[20] Urine creatinine (µmol/l) and urine albumin (µg/l) concentrations were determined with a Beckman Coulter AU analyzer, creatinine concentrations were measured with the Jaffe reaction. Microalbuminuria was defined as an albumin-creatinine ratio between 2.5 and 25 mg/mmol for boys and between 3.5 and 25 mg/mmol for girls.[21] Detailed information on kidney measures is described elsewhere.[17]

Covariates

We obtained information on maternal age, ethnicity, educational level, vitamins supplementation, smoking and alcohol usage during pregnancy using questionnaires.[13] We assessed maternal energy intake at enrollment using a validated semi-quantitative food frequency questionnaire.[22] Ethnicity and educational level were defined according to the classification of Statistics Netherlands.[23] Maternal pre-pregnancy height and weight were self-reported and pre-pregnancy body mass index (BMI) was calculated (kg/m^2). We measured maternal blood pressure in early and late pregnancy by using the Omron 907 automated digital oscillometric spygmanometer.[24] Information on child's sex, birthweight and gestational age was available from medical records and hospital registries. We obtained information on breastfeeding from postnatal questionnaires.[17] At the age of 6 years, child height and weight were determined and BMI (kg/m^2), and body surface area (BSA) (m^2) were calculated.[25]

Statistical analysis

First, we performed a non-response analysis by comparing subject characteristics between children with and without follow-up kidney measurements by using T-tests, Chi-square tests and Mann-Whitney tests. Second, we used multivariable linear and logistic regression models to assess the associations of maternal first trimester and fetal cord blood folate, vitamin B_{12} and homocysteine concentrations with combined kidney volume, $eGFR_{creat}$ and $eGFR_{cystC}$, and risk of microalbuminuria in school-aged children. Folate, vitamin B_{12} and homocysteine concentrations were analysed continuously per standard deviation (SD)- increase to enable comparison between effect estimates. The regression models were first adjusted for child's sex, and age at kidney measurements (basic models), and subsequently also for maternal age, education, body mass index, blood pressure, vitamins supplementation, smoking, alcohol use, energy intake during pregnancy, and for child's birth weight, gestational age at birth, breastfeeding, and body surface area at the age of 6 years (confounder model). These covariates were included in the regression models based on previous literature or a change of >10% in effect estimates. To explore if the observed associations of folate and vitamin B_{12} with kidney outcomes were independent of homocysteine concentrations, we additionally adjusted the confounder model for homocysteine concentrations (homocysteine model). In a separate model we additionally adjusted the kidney function measures for kidney volume

(kidney size model). Third, we used the same models to assess the associations between maternal folic acid supplement intake and childhood kidney outcomes. These analyses, were performed among 3,291 mothers who had information on folic acid supplements. Whether the associations of folate or vitamin B_{12} or homocysteine and kidney outcomes differed by sex or birthweight we analyzed the interaction terms. Since the interaction terms were not significant we did not stratify our analyses. To prevent bias associated with missing data, we used multiple imputations (N=5) only for covariates with missing values on the basis of the correlation of missing variables with other participant characteristics, according to the Markov Chain Monte Carlo method.[26] Detailed information on multiple information procedure is given in Supplementary Materials. Subjects characteristics before and after imputation and the percentages of missing values are shown in **Supplementary Table 2.2.1**. Statistical analyses were performed using the Statistical Package of Social Sciences version 21.0 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp).

RESULTS

Subject characteristics

Table 2.2.1 shows the characteristics of the study population stratified on folate concentrations. The values are based on the original data. **Supplementary Table 2.2.2** shows folate, vitamin B_{12} and homocysteine concentrations per supplement group of folic acid use. The correlation coefficients of the investigated variables are given in **Supplementary Table 2.2.3**. Maternal and cord blood folate, vitamin B_{12} and homocysteine concentrations correlation coefficients ranged from r = 0.34 to 0.46. Maternal folate and vitamin B_{12} concentrations were weakly negatively correlated with child height and weight, whereas homocysteine concentrations were weakly positively correlated with child height and weight (**Supplementary Table 2.2.3**). Results from the non-response analyses are given in **Supplementary Table 2.2.4**. Mothers whose children had kidney follow-up measurements had higher folate and vitamin B_{12}, and lower homocysteine concentrations compared to mothers whose children did not have kidney follow-up measurements.

Maternal and fetal folate, vitamin B_{12} and homocysteine concentrations and childhood kidney outcomes

Table 2.2.2 shows that a 1-SD higher maternal folate concentration was associated with a 1.16 cm^3 (95% confidence interval (CI) 0.47, 1.85) larger childhood combined kidney volume. No other associations were observed of maternal folate concentrations with other kidney outcomes. A 1-SD higher maternal vitamin B_{12} concentration was associated with 1.00 ml/min/1.73m^2 (95% CI 0.43, 1.57) higher childhood $eGFR_{cystC}$. The effects estimates were similar when we adjusted for maternal homocysteine concentrations (homocysteine models) and childhood combined kidney volume (kidney size model). Similarly, a 1-SD higher maternal homocysteine concentration was associated with a -1.44 cm^3 (95% CI -2.09, -0.79) smaller combined kidney volume and a -0.57 ml/min/1.73m^2 (95% CI -1.13, -0.02) lower childhood

Table 2.2.1. Subject characteristics according to folate levels (N=4,149)

	Folate deficient (<7 nmol/l) N=277	Normal folate (≥7 nmol/l) N=3,872
Maternal characteristics		
Maternal age (y)	27.5 (5.6)	30.6 (4.8)
Pre-pregnancy body mass index(kg/m^2)	23.7 (21.0, 27.0)	22.6 (20.8, 25.2)
Missing, n (%)	*60 (22)*	*629 (16)*
Gestational age at intake (wk)	14.2 (12.6, 16.1)	13.2 (12.1, 14.6)
Early pregnancy systolic blood pressure (mmHg)	114 (12)	116 (12)
Missing, n (%)	*3 (1)*	*21 (0.5)*
Early pregnancy diastolic blood pressure (mmHg)	67 (10)	68 (10)
Missing, n (%)	*3 (1)*	*21 (0.5)*
Late pregnancy systolic blood pressure (mmHg)	119 (12)	119 (12)
Missing, n (%)	*14 (5.1)*	*120 (3.1)*
Late pregnancy diastolic blood pressure (mmHg)	69 (10)	69 (9)
Missing, n (%)	*14 (5.1)*	*120 (3.1)*
Education level, n (%)		
- No higher education	204 (73.6)	1,824 (47.1)
- Higher education	44 (15.9)	1,867 (48.2)
Missing, n (%)	*29 (10.5)*	*181 (4.7)*
Ethnicity, n (%)		
- European	99 (35.7)	2,555 (66.0)
- Non-European	167 (60.3)	1,264 (32.6)
Missing, n (%)	*11 (4)*	*53 (1.4)*
Smoking during pregnancy, n (%)		
- Never & until pregnancy was known	154 (55.6)	2,940 (75.9)
- Continued	90 (32.5)	545 (14.1)
Missing, n (%)	*33 (11.9)*	*387 (10)*
Alcohol during pregnancy, n (%)		
- Never & until pregnancy was known	171 (61.7)	1,939 (50.1)
- Continued	59 (21.3)	1,515 (39.1)
Missing, n (%)	*47 (17)*	*418 (10.8)*
Folic acid supplements use, n (%)		
- No	166 (59.9)	515 (13.3)
- Start 1st to 10 weeks	23 (8.3)	1,022 (26.4)
- Start periconceptional	15 (5.4)	1,492 (38.5)
Missing, n (%)	*73 (26.4)*	*843 (21.8)*
Maternal calories intake (kcal)	2,010 (599)	2,050 (549)
Missing, n (%)	*76 (27.4)*	*700 (18.1)*
Vitamin supplements use, n (%)		
- No	211 (76.2)	2,193 (56.6)
- Yes	17 (6.1)	1,114 (28.8)
Missing, n (%)	*49 (17.7)*	*565 (14.6)*
Folate plasma concentrations (nmol/l)	6.1 (5.4, 6.6)	17.9 (11.6, 25.4)
Vitamin B_{12} serum concentrations (pmol/l)	154.5 (115.8, 200.8)	173.0 (131.0, 232.0)
Missing, n (%)	*7 (2.5)*	*235 (6.1)*

Table 2.2.1. Subject characteristics according to folate levels (N=4,149) (continued)

	Folate deficient (<7 nmol/l) N=277	**Normal folate (≥7 nmol/l) N=3,872**
Homocysteine plasma concentrations (µmol/l)	8.4 (7.1, 10.0)	6.8 (6.0, 7.8)
Missing, n (%)	*7 (2.5)*	*62 (1.6)*
Infant characteristics		
Girls, n (%)	135 (48.7)	1,941 (50.1)
Gestational age at birth (wk)	40.1 (39.3, 40.9)	40.1 (39.3, 41.0)
Birth weight (g)	3,309 (596)	3,447 (548)
Missing, n (%)	-	*4 (0.1)*
Breastfeeding, n (%)		
- No	20 (7.2)	231 (6.0)
- Yes	178 (64.3)	2,940 (75.9)
Missing, n (%)	*79 (28.5)*	*701 (18.1)*
Cord blood folate concentrations (nmol/l)	16.6 (13.2, 21.2)	21.1 (16.5, 27.3)
Cord blood vitamin B_{12} concentrations (pmol/l)	267.5 (210.3, 379.8)	302.0 (220.0, 422.0)
Child characteristics at 6y visit		
Age (y)	6.1 (5.9, 6.5)	6.0 (5.9, 6.2)
Height (cm)	120.0 (6.9)	119.3 (5.9)
Missing, n (%)	-	*6 (0.2)*
Weight (kg)	23.0 (20.8, 27.5)	22.4 (20.4, 25.0)
Missing, n (%)	-	*6 (0.2)*
Body mass index (kg/m^2)	16.1 (15.3, 18.1)	15.8 (15.0, 16.9)
Missing, n (%)	-	*6 (0.2)*
Body surface area (m^2)	0.9 (0.1)	0.9 (0.1)
Missing, n (%)	-	*6 (0.2)*
Combined kidney volume (cm^3)	122.1 (28.6)	120.0 (23.2)
Creatinine (µmol/l)	38.2 (5.2)	37.3 (5.6)
Cystatin C (mg/l)	783.1 (74.8)	784.4 (81.8)
$eGFR_{creat}$ (ml/min/1.73m^2)	117.1 (14.8)	119.2 (16.3)
$eGFR_{cystC}$ (ml/min/1.73m^2)	102.4 (13.5)	102.5 (14.7)
Microalbuminuria, n (%)	24 (8.7)	279 (7.2)

Values are frequency counts and percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (25th to 75th percentiles) for continuous variables with a skewed distribution. Values are based on the original data. Folate deficiency was defined as a folate concentration <7 nmol/l. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels: $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

$eGFR_{cystC}$. The association of maternal homocysteine concentrations with childhood $eGFR_{cystC}$ was largely explained by combined kidney volume. None of the exposures were associated with the risk of microalbuminuria. The results from basic models are given in **Supplementary Table 2.2.5**.

Table 2.2.3 shows that a 1-SD higher fetal cord blood homocysteine concentrations was associated with a 1.27 cm^3 (95% CI 0.46, 2.08) larger childhood combined kidney volume and a -1.02 ml/min/1.73m^2 (95% CI -1.76, -0.28) lower $eGFR_{cystC}$. The effect estimates on $eGFR_{cystC}$ re-

mained similar after additional adjustment for kidney size. Higher cord blood homocysteine concentrations was associated with lower $eGFR_{creat}$ after additional adjustment for kidney size -0.91 ml/min/1.73m^2 (95% CI -1.71, -0.12). No other associations were observed of cord blood folate and vitamin B_{12} concentrations with kidney outcomes. The results from basic models are given in **Supplementary Table 2.2.6**.

Table 2.2.4 shows that there was no association between folic acid supplement intake and kidney outcomes.

Table 2.2.2. Associations of maternal folate, vitamin B_{12} and homocysteine concentrations during pregnancy with kidney outcomes at the age of 6 years (N=4,226)

	Difference in outcome measure (95% Confidence Interval)			
First trimester maternal concentrations	**Combined kidney volume (cm^3)**	**$eGFR_{creat}$ (ml/min/1.73m^2)**	**$eGFR_{cystC}$ (ml/min/1.73m^2)**	**Microalbuminuria (odds ratio)**
Folate				
Confounder Model	**1.16 (0.47, 1.85)**** N=3,818	0.01 (-0.65, 0.67) N=2,792	-0.03 (-0.63, 0.58) N=2,792	0.97 (0.85, 1.10) N=4,011
Homocysteine Model	**1.02 (0.32, 1.72)**** N=3,757	-0.06 (-0.74, 0.61) N=2,741	-0.11 (-0.73, 0.51) N=2,741	0.96 (0.84, 1.10) N=3,944
Kidney size Model		-0.24 (-0.90, 0.42) N=2,601	0.01 (-0.59, 0.61) N=2,601	0.95 (0.83, 1.09) N=3,686
Vitamin B_{12}				
Confounder Model	0.47 (-0.17, 1.11) N=3,666	0.20 (-0.43, 0.83) N=2,663	**1.00 (0.43, 1.57)**** N=2,663	1.06 (0.95, 1.19) N=3,849
Homocysteine Model	0.20 (-0.45, 0.85) N=3,563	0.22 (-0.42, 0.85) N=2,579	**0.95 (0.36, 1.53)**** N=2,579	1.07 (0.96, 1.20) N=3,737
Kidney size Model		0.09 (-0.53, 0.71) N=2,481	**0.96 (0.39, 1.52)**** N=2,482	1.08 (0.96, 1.21) N=3,538
Homocysteine				
Confounder Model	**-1.44 (-2.09, -0.79)**** N=3,779	-0.55 (-1.15, 0.05) N=2,755	**-0.57 (-1.13, -0.02)*** N=2,755	1.08 (0.98, 1.20) N=3,969
Kidney size Model		-0.35 (-0.97, 0.28) N=2,566	-0.52 (-1.10, 0.06) N=2,556	1.10 (0.99, 1.23) N=3,649

Values are linear and logistic regression coefficients (95% confidence interval). Confounder model is adjusted for maternal characteristics (age, body mass index before pregnancy, blood pressure in early pregnancy, ethnicity, education, vitamins supplementation, smoking, alcohol consumption, energy intake during pregnancy), and child characteristics (birthweight, gestational age, sex, breastfeeding, age, and body surface area at 6-year visit). Homocysteine model is confounder model additionally adjusted for homocysteine concentrations during pregnancy. Kidney size model is confounder model additionally adjusted for child combined kidney volume. *p <0.05, **p <0.01. Maternal folate, vitamin B_{12} and homocysteine concentrations were analyzed per 1 standard deviation in folate, vitamin B_{12} and homocysteine. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine concentrations; $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C concentrations.

Table 2.2.3. Associations of cord blood folate, vitamin B_{12} and homocysteine concentrations with kidney outcomes at the age of 6 years (N=2,674)

	(Difference in outcome measure (95% Confidence Interval)			
Cord blood concentrations	**Combined kidney volume (cm^3)**	**$eGFR_{creat}$ ($ml/min/1.73m^2$)**	**$eGFR_{cystC}$ ($ml/min/1.73m^2$)**	**Microalbuminuria (odds ratio)**
Folate				
Confounder Model	0.36 (-0.46, 1.18) N=2,384	0.41 (-0.36, 1.17) N=1,753	0.51 (-0.19, 1.22) N=1,753	0.97 (0.83, 1.13) N=2,517
Homocysteine Model	0.74 (-0.12, 1.59) N=2,308	0.30 (-0.50, 1.10) N=1,702	0.31 (-0.43, 1.06) N=1,702	1.00 (0.85, 1.17) N=2,439
Kidney size Model		0.37 (-0.39, 1.14) N=1,625	0.48(-0.24, 1.20) N=1,625	0.97 (0.82, 1.14) N=2,306
Vitamin B_{12}				
Confounder Model	-0.47 (-1.27, 0.34) N=2,413	-0.52 (-1.28, 0.25) N=1,776	0.35 (-0.36, 1.05) N=1,776	0.99 (0.84, 1.15) N=2,548
Homocysteine Model	-0.05 (-0.90, 0.80) N=2,271	-0.76 (-1.56, 0.05) N=1,674	0.17 (-0.58, 0.92) N=1,674	1.00 (0.85, 1.17) N=2,403
Kidney size Model		-0.48 (-1.25, 0.29) N=1,645	0.42 (-0.30, 1.14) N=1,645	1.00 (0.85, 1.18) N=2,335
Homocysteine				
Confounder Model	**1.27 (0.46, 2.08)**** N=2,311	-0.74 (-1.53, 0.06) N=1,705	**-1.02 (-1.76, -0.28)**** N=1,705	1.09 (0.95, 1.26) N=2,443
Kidney size Model		**-0.91 (-1.71, -0.12)*** N=1,579	**-1.14 (-1.89, -0.39)**** N=1,579	1.09 (0.93, 1.27) N=2,236

Values are linear and logistic regression coefficients (95% confidence interval). Confounder model is adjusted for maternal characteristics (age, body mass index before pregnancy, blood pressure in late pregnancy, ethnicity, education, vitamins supplementation, smoking and alcohol consumption, energy intake during pregnancy), and child characteristics (birthweight, gestational age, sex, breastfeeding, age and body surface area at the age of 6year visit). Homocysteine model is confounder model additionally adjusted for homocysteine concentrations at birth. Kidney size model is confounder model additionally adjusted for child combined kidney volume. *p <0.05, **p <0.01. Cord blood folate, vitamin B_{12} and homocysteine concentrations were analyzed per 1 standard deviation in folate, vitamin B_{12} and homocysteine. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine concentrations; $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C concentrations.

DISCUSSION

Results from this population-based prospective cohort study suggest that maternal higher folate and lower homocysteine concentrations are associated with larger childhood combined kidney volume, whereas maternal higher vitamin B_{12} and lower homocysteine concentrations are associated with higher childhood $eGFR_{cystC}$. Lower cord blood homocysteine concentrations were associated with smaller childhood combined kidney volume and higher eGFR.

Interpretation of main findings

Various lines of investigation suggest that an adverse fetal nutrition may have persistent consequences for kidney health in later life. Because nephrogenesis continues until 36 weeks of gestation and mainly stops thereafter, adverse fetal nutritional exposures may have a persistent impact on kidney function in later life.[1, 2] For this study, we specifically hypothesized

Table 2.2.4. Associations of maternal folic acid supplements intake during pregnancy with kidney measurements at the age of 6 years (N=3,291)

	Difference in outcome measure (95% Confidence Interval)			
Folic acid supplement use	**Combined kidney volume (cm^3) N=3,025**	**$eGFR_{creat}$ ($ml/min/1.73m^2$) N=2,211**	**$eGFR_{cystC}$ ($ml/min/1.73m^2$) N=2,215**	**Microalbuminuria (odds ratio) N=3,179**
Basic Model				
No (N=696)	-1.63 (-3.78, 0.53)	0.18 (-1.58, 1.94)	0.50 (-1.14, 2.14)	0.77 (0.54, 1.10)
Started when pregnancy was known (N=1,065)	-0.89 (-2.74, 0.97)	-0.64 (-1.94, 0.66)	0.86 (-0.56, 2.28)	0.71 (0.52, 0.97)
Started periconceptional (N=1,530)	*Reference*	*Reference*	*Reference*	*Reference*
Confounder Model				
No (N=696)	-2.08 (-4.32, 0.15)	1.47 (-0.61, 3.55)	1.14 (-0.79, 3.08)	0.82 (0.54, 1.25)
Started when pregnancy was known (N=1,065)	-1.49 (-3.16, 0.18)	-0.27 (-1.83, 1.30)	1.17 (-0.29, 2.63)	0.75 (0.54, 1.03)
Started periconceptional (N=1,530)	*Reference*	*Reference*	*Reference*	*Reference*

Values are linear and logistic regression coefficients (95% confidence interval). Basic model is adjusted for child's sex and age at 6-year visit. Confounder model is additionally adjusted for maternal characteristics (age, body mass index before pregnancy, blood pressure in early pregnancy, ethnicity, education, vitamin supplements, smoking, alcohol consumption, energy intake during pregnancy), and child characteristics (birthweight, gestational age, breastfeeding, age and body surface area at the age of 6y). *p <0.05. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine concentrations; $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C concentrations.

that low folate and vitamin B_{12} concentrations and higher homocysteine concentrations during fetal life may affect nephrogenesis and lead to persistently smaller kidneys with a lower kidney function.

Not many studies have explored the effect of maternal folate concentrations or folic acid supplements during pregnancy on childhood kidney measures. Results from a trial follow-up study in Rural Bangladesh among 3,267 mother and their 4 to 5 year old children indicated no effect of early maternal multiple micronutrient supplementation or food supplementation with iron and folate on offspring's kidney volume.[27] A study exploring the effect of maternal folic acid supplements during pregnancy has suggested that folic acid supplements alone are a risk factor for congenital anomalies of kidney and urinary tract.[28] In our study only few children (N=6) had evidence of congenital kidney abnormalities. We did not observe an association of maternal folic acid supplements or cord blood folate concentrations with childhood kidney volume. However, our results suggest that higher maternal folate concentrations in early pregnancy are associated with a larger childhood combined kidney volume, independent of homocysteine concentrations. Specifically, folate concentrations in early pregnancy may affect child kidney volume. Compared to selfreported folic acid intake, folate concentrations are more directly linked to the body's biological processes; because they can be used to measure actual folate status with greater precision, slight effects could potentially be detected. This could account for maternal folate concentrations being associated with childhood kidney volume as opposed to folic acid supplementation. Furthermore, statisti-

cally, a continuous measure of folate concentration has greater power to detect differences compared to the categorical approach of folic acid supplementation. A study among young adults suggested that folic acid supplementation in individuals with low dietary folic acid content was associated with decreased creatinine concentrations and subsequently higher eGFRs.[29] Another study among 6 to 8 year old children in rural Nepal suggested that maternal folic acid supplementation reduced the risk of microalbuminuria.[30]

Folate is important for homocysteine metabolism. Animal studies have shown that folic acid supplementation attenuates glomerular damage induced by hyperhomocysteinemia.[11] In our study, we did not observe any association of maternal or cord blood folate concentrations or folic acid supplementations with eGFR or microalbuminuria in 6 year old children. The differences in results may be explained by the different study populations. Together with folate, vitamin B_{12} leads to lower homocysteine concentrations. We observed that higher vitamin B_{12} concentrations during fetal life were associated with a higher $eGFR_{cystC}$. To our knowledge, no previous studies have explored the relationship of vitamin B_{12} with kidney function measures in children. Epidemiological studies in patients with kidney failure suggested that parenteral vitamin B_{12} lowers homocysteine concentrations independent of serum vitamin B_{12} concentrations.[31] Previous studies have shown that after adjustment for homocysteine concentrations, higher vitamin B_{12} concentrations were associated with an increased risk of albuminuria. In individuals with high baseline homocysteine concentrations, increased vitamin B_{12} was associated with reduced kidney function.[32] Specifically, among patients with hyperhomocysteinemia, vitamin B supplementation modulated cystatin C concentrations.[33] In our study the observed associations of vitamin B_{12} with eGFR were independent of homocysteine concentrations. We did not observe any association of vitamin B_{12} concentrations with childhood microalbuminuria. Further research is needed to observe the effect of early life folate and vitamin B_{12} with kidney outcomes in later life.

Our results suggest that higher maternal homocysteine concentrations were associated with smaller combined kidney volume and lower eGFR. Nephrogenesis starts in early pregnancy; therefore high homocysteine concentrations since early pregnancy may influence on nephron formation. As we have previously reported, kidney size is positively correlated with eGFR.[34] We observed that an increased kidney volume explained the associations of maternal homocysteine concentrations with kidney function. Higher cord blood homocysteine concentrations were associated with larger combined kidney volume and lower eGFR. Our findings suggest time specific effects of homocysteine concentrations on kidney volume. It may be that a constant exposure to high homocysteine concentration induces kidney hypertrophy in originally smaller kidneys.

To our knowledge, there are no other studies in children to compare these findings. In line with our findings, in adults, elevated homocysteine concentrations are associated with an accelerated decline in eGFR.[12, 35] Our findings are also supported by experimental studies in rats suggesting that elevated homocysteine concentrations can be an important pathogenic factor in glomerular damage.[11] Results from a study among 340 adults aged 50–75 years, suggested that increased homocysteine concentrations influence on the development of microalbuminuria.[36] In our study, we did not observe any association of homocysteine con-

centrations with the risk of microalbuminuria. Perhaps the effects of impaired kidney growth on microalbuminuria are undetectable during childhood, and become apparent in later life. Altogether, these findings suggest time specific effects of homocysteine concentrations on kidney development. Previous studies using data from the same cohort have observed that low folate and high homocysteine but not vitamin B_{12} concentrations were associated with fetal growth restriction.[14] However, a previous study did not observe any association of maternal folate, vitamin B_{12} or homocysteine concentrations with blood pressure at the age of 6 years.[16]

Although we observed small effect sizes, our results may be important from a population-based perspective. In the Netherlands, food supplies are not fortified with folic acid, but women are advised to use folic acid supplements (400 µg/day) prior to and up to week 10 to12 of pregnancy. Although results presented in this study do not provide a basis to make causal statements, they support population strategies to increase folate and vitamin B_{12} concentrations and subsequently lower homocysteine concentrations in pregnant women.

Potential mechanisms

From our observational study, it is not possible to establish the causality for the observed associations. However, some biological mechanisms may link maternal folate, vitamin B_{12} and homocysteine concentrations with childhood kidney outcomes. Both observational and experimental studies relate low folate and high homocysteine concentrations with endothelial dysfunction and altered vascular development.[12, 37–39] High homocysteine concentrations negatively affect endothelial vasodilatation via inhibition of the generation of endothelial mediators and promotion of adhesion between neutrophil and endothelial cells.[40] It is also possible that homocysteine decreases adenosine levels in plasma and interstitial tissue and induces proliferation and apoptosis of glomerular mesangial cells, renal vascular injury would directly result.[41] Suboptimal vascular development and endothelial dysfunction could lead to hypertension in childhood which could be a predictor to chronic kidney diseases in later life.[2, 4] Also, maternal diet predetermines the embryonic kidney by changing cell turnover and gene expression during a period when nephrons and glomeruli are as yet unformed.[42] The concentration of the methyl donors folate and vitamin B_{12} having a beneficial effect on childhood kidney outcomes supports the hypothesis that epigenetic changes program future kidney health.

Methodological considerations

To our knowledge, this is the largest prospective population-based cohort study examining the associations of folate, vitamin B_{12} and homocysteine concentrations during fetal life with childhood kidney outcomes. We measured folate, vitamin B_{12} and homocysteine concentrations during early pregnancy and at birth, assessing critical periods of kidney development. Cord blood folate and vitamin B_{12} concentrations were higher compared to maternal concentrations. This has also been reported previously in literature.[43] According to Dutch recommendations, we assume that after gestational week 10 to12, women did not take folic acid supplements. We obtained information for maternal folic acid supplementa-

tion using questionnaires. Mothers not taking folic acid supplements in pregnancy had lower folate and higher homocysteine concentrations compared with mothers who started folic acid supplements periconceptional. Furthermore, these mothers had lower folate and vitamin B_{12} and higher homocysteine concentrations as compared with normal ranges of these concentrations during pregnancy.[44] Of the total group of singleton live-born children 70% had available information on kidney measurements. Selection bias in follow-up studies largely results from loss to follow-up rather than from non-response at baseline. Mothers of children without follow-up kidney measurements had lower folate and higher homocysteine concentrations, were younger, smoked more frequently and had lower alcohol intake during pregnancy and there were more with a non-European origin compared with mothers of children with available kidney measurements. Loss to follow-up would lead to selection bias if the associations of maternal first trimester micronutrient concentrations with childhood kidney outcomes were different between those included and those not included in the final analyses. Because this is difficult to determine, selection bias cannot be excluded.

We performed detailed measurements on kidney outcomes. Kidney size was used as a measure of kidney development. Ultrasonography is reliable for measuring kidney volume.[18] Kidney size correlates with the number of glomeruli; in epidemiologic studies, it can be used to measure kidney development.[1] BSA is a well-known predictor of kidney volume. Replacement of BSA with BMI did not change the results. Similar effect estimates to combined kidney volume were observed when we explored Kidney volume/BSA as an additional outcome.[45] To estimate GFR, we used the Schwartz formula based on creatinine concentrations and height, and also Zappitelli's formula based on cystatin C concentrations.[19, 20] In our analyses we observed small differences in effect estimates between $eGFR_{creat}$ and $eGFR_{cystC}$, with slightly stronger effect estimates for $eGFR_{cystC}$. A random urine sample was used to evaluate the presence of microalbuminuria.[46] Our results apply to a relatively healthy sample of pregnant women and children. It may be that our findings could underestimate the true effect measures. Therefore, the generalizability of our results to other populations should be interpreted with caution. Last, although we adjusted for many potential maternal and childhood confounders, residual confounding (eg, like child nutritional status) can still be present.

In conclusion, results from our prospective study, suggest that folate, vitamin B_{12}, and homocysteine concentrations during fetal life affect offspring kidney measures. However, the effect sizes presented in the study are small, and results could reflect confounding. Our findings should be considered as hypothesis generating and require further replication. Additional follow-up studies are warranted to examine the long term consequences for the risk of kidney diseases in later life.

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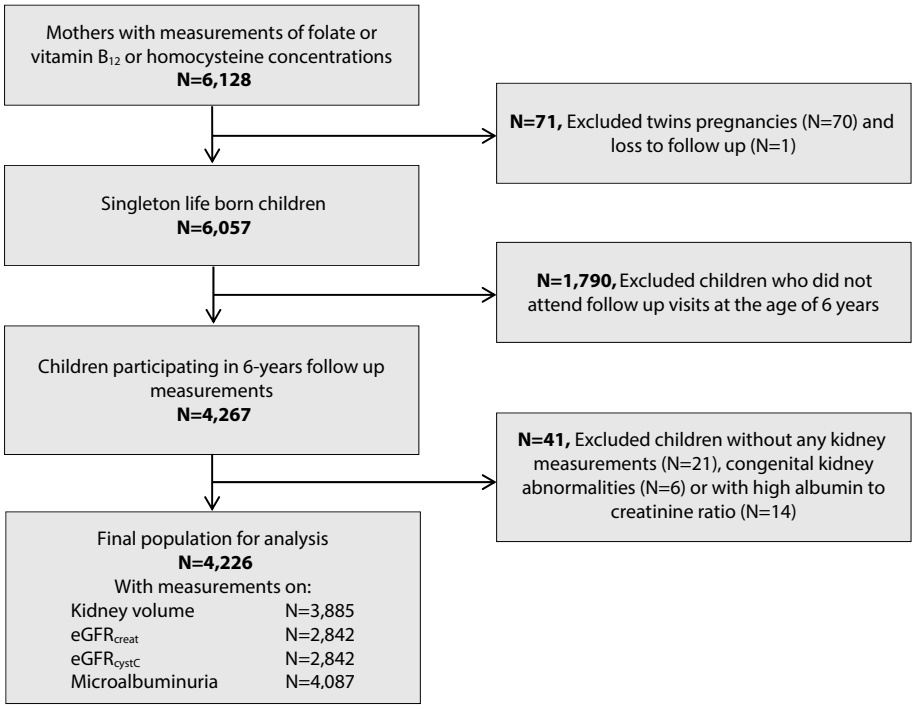
Supplementary Information. Statistical analyses.

Multiple Imputation: We imputed missing data of the covariates using multiple imputations.[1] The percentages of missing values for the covariates within the population for analysis were lower than 20%. Five imputed datasets were created and analyzed.[1–3] The efficiency of 5 imputations is >96%. For the multiple imputation, we used Fully Conditional Specification, an iterative of the Markov chain Monte Carlo approach. For each variable, the fully conditional specification method fits a model using all other available variables in the model as predictors, and then imputes missing values for the specific variable being fit. In the imputation model, we included all covariates, plus maternal height and weight measured at enrollment, household income, paternal age, ethnicity and education level, breastfeeding duration and child height, weight and BMI. These variables were added to increase plausibility of missing at random assumption. Additionally, in the imputation model, we added the studied determinants and outcomes as prediction variables only; they were not imputed themselves. Therefore, numbers of participant are different in each model.

Supplementary Figure 2.2.1. Flowchart of study participants



Supplementary Table 2.2.1. Subject characteristics (N=4,226)

	Observed	Imputed
Maternal characteristics		
Maternal age (y)	30.4 (4.9)	NI
Pre-pregnancy body mass index(kg/m^2)	22.6 (20.8, 25.4)	22.6 (20.8, 25.4)
Missing, n (%)	*701 (16.6)*	-
Gestational age at intake (wk)	13.2 (12.2, 14.8)	NI
Early pregnancy systolic blood pressure (mmHg)	116 (12)	116 (12)
Missing, n (%)	*27 (0.6)*	-
Early pregnancy diastolic blood pressure (mmHg)	68 (10)	68 (10)
Missing, n (%)	*27 (0.6)*	-
Late pregnancy systolic blood pressure (mmHg)	119 (12)	119 (12)
Missing, n (%)	*137 (3.2)*	-
Late pregnancy diastolic blood pressure (mmHg)	69 (9)	69 (9)
Missing, n (%)	*137 (3.2)*	-
Education level, n (%)		
- No higher education	2,060 (48.7)	2,223 (52.6)
- Higher education	1,952 (46.2)	2,003 (47.4)
Missing, n (%)	*214 (5.1)*	-
Ethnicity, n (%)		
- European	2,701 (63.9)	2,738 (64.8)
- Non-European	1,460 (34.5)	1,488 (35.2)
Missing, n (%)	*65 (1.6)*	-
Smoking during pregnancy, n (%)		
- Never & until pregnancy was known	3,148 (74.5)	3,509 (83.0)
- Continued	647 (15.3)	717 (17.0)
Missing, n (%)	*431 (10.2)*	-
Alcohol during pregnancy, n (%)		
- Never & until pregnancy was known	2,147 (50.8)	2,440 (57.7)
- Continued	1,602 (37.9)	1,786 (42.3)
Missing, n (%)	*477 (11.3)*	-
Folic acid supplements use, n (%)		
- No	696 (16.5)	NI
- Start 1st to 10 weeks	1,065 (25.2)	NI
- Start periconceptional	1,530 (36.2)	NI
Missing, n (%)	*935 (22.1)*	-
Maternal calories intake (kcal)	2,045 (553)	2,037 (558)
Missing, n (%)	*795 (18.8)*	-
Vitamin supplements use, n (%)		
- No	2,443 (57.8)	2,889 (68.4)
- Yes	1,152 (27.3)	1,337 (31.6)
Missing, n (%)	*631 (14.9)*	-
Folate plasma concentrations (nmol/l)	16.8 (10.7, 24.8)	NI
Vitamin B_{12} serum concentrations (pmol/l)	171 (129.0, 228.0)	NI
Homocysteine plasma concentrations (µmol/l)	6.9 (6.0, 8.0)	NI
Infant characteristics		
Girls, n (%)	2,121 (50.2)	NI
Gestational age at birth (wk)	40.1 (39.3, 41.0)	NI
Birth weight (g)	3,437 (551)	3,437 (551)
Missing, n (%)	*4 (0.1)*	-

Supplementary Table 2.2.1. Subject characteristics (N=4,226) (continued)

	Observed	Imputed
Breastfeeding, n (%)		
- No	257 (6.1)	329 (7.8)
- Yes	3,172 (75.1)	3,897 (92.2)
Missing, n (%)	*797 (18.8)*	-
Cord blood folate concentrations (nmol/l)	20.8 (16.2, 27.0)	NI
Cord blood vitamin B_{12} concentrations (pmol/l)	299.0 (219.0, 419.0)	NI
Cord blood homocysteine concentrations (µmol/l)	9.0 (7.5, 10.7)	NI
Child characteristics at 6y visit		
Age (y)	6.0 (5.9, 6.3)	NI
Height (cm)	119.3 (5.9)	119.3 (5.9)
Missing, n (%)	*6 (0.1)*	-
Weight (kg)	22.4 (20.4, 25.0)	22.4 (20.4, 25.0)
Missing, n (%)	*6 (0.1)*	-
Body mass index (kg/m^2)	15.8 (15.0, 16.9)	15.8 (15.0, 16.9)
Missing, n (%)	*6 (0.1)*	-
Body surface area (m^2)	0.9 (0.1)	0.9 (0.1)
Missing, n (%)	*6 (0.1)*	-
Combined kidney volume (cm^3)	120.0 (23.6)	NI
Creatinine (µmol/l)	37.4 (5.6)	NI
Cystatin C (mg/l)	784.4 (81.1)	NI
$eGFR_{creat}$ (ml/min/1.73m^2)	119.1 (16.3)	NI
$eGFR_{cystC}$ (ml/min/1.73m^2)	102.4 (14.6)	NI
Microalbuminuria, n (%)	307 (7.3)	NI

Values are frequency counts and percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (25th to 75th percentiles) for continuous variables with a skewed distribution. Percentage of missing variables: maternal pre-pregnancy body mass index (16.6%), early pregnancy systolic and diastolic blood pressure (0.6%), late pregnancy systolic and diastolic blood pressure (3.2%), educational level (5.1%), ethnicity (1.6%), smoking (10.2%), alcohol consumption (11.3%), calories intake (18.8%), vitamin supplements use (14.9%), birth weight (0.1%), breastfeeding (18.8%), child height at measurement (0.1%), weight at measurement (0.1%), body mass index at measurement (0.1%), and body surface area at measurement (0.1%). Maternal age, gestational age at intake, gestational age at birth and gender had no missing values. Abbreviations: GFR_{creat} estimated glomerular filtration rate calculated based on creatinine blood levels: $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels. NI not imputed.

Supplementary Table 2.2.2. Biomarkers concentrations per supplement group of folic acid (N=3,291)

	Concentrations during 1st trimester		
	Folate (nmol/l)	**Vitamin B_{12} (pmol/l)**	**Homocysteine (µmol/l)**
Folic acid supplement use			
No (N=696)	8.7 (7.0, 11.2)	158.0 (122.0, 213.0)	7.4 (6.3, 8.9)
Started when pregnancy was known (N=1,065)	17.7 (12.1, 23.7)	177.0 (131.0, 233.5)	6.9 (6.1, 8.0)
Started periconceptional (N=1,530)	22.7 (16.4, 28.7)	177.0 (134.0, 239.0)	6.7 (5.9, 7.6)
	Cord blood concentrations		
	Folate (nmol/l)	**Vitamin B_{12} (pmol/l)**	**Homocysteine (µmol/l)**
Folic acid supplement use			
No (N=425)	18.8 (14.6, 23.2)	289.0 (204.0, 404.0)	9.4 (8.0, 11.3)
Started when pregnancy was known (N=684)	20.2 (15.8, 26.5)	297.0 (216.0, 422.0)	9.1 (7.5, 216.0)
Started periconceptional (N=980)	22.7 (17.6, 28.6)	304.0 (224.8, 424.0)	8.8 (7.2, 10.4)

Values are medians (25th to 75th percentiles).

Supplementary Table 2.2.3. Correlation coefficients of the investigated measures

	Folate	Vitamin B_{12}	HCY	CB Folate	CB Vitamin B_{12}	CB HCY	Height	Weight	BMI	BSA	Kidney Volume	$eGFR_{creat}$	$eGFR_{cystC}$
Folate	1,00												
Vitamin B_{12}	0.14**	1,00											
HCY	-0.21**	-0.17**	1,00										
CB Folate	0.39**	0.12**	-0.15**	1,00									
CB Vitamin B_{12}	0.08**	0.46**	-0.16**	0.18**	1,00								
CB HCY	-0.17**	-0.20**	0.34**	-0.26**	-0.23**	1,00							
Height	-0.07**	-0.01	0.05**	-0.03	-0.04*	-0.01	1,00						
Weight	-0.14**	-0.04*	0.04*	-0.06**	-0.03	0	0.76**	1,00					
BMI	-0.15**	-0.05**	0.01	-0.06**	0	0.01	0.27**	0.83**	1,00				
BSA	-0.12**	-0.03	0.04**	-0.05**	-0.03	0	0.89**	0.97**	0.67**	1,00			
Kidney Volume	-0.01	0.01	-0.05**	-0.01	-0.04	0.04*	0.49**	0.53**	0.36**	0.55**	1,00		
$eGFR_{creat}$	0.04*	0.02	-0.06**	0.04	-0.02	-0.06*	0.06**	0	-0.04*	0.02	0.24**	1,00	
$eGFR_{cystC}$	0.02	0.08**	-0.05**	0.05*	0.02	-0.08**	-0.03	-0.04*	-0.03	-0.04*	0.12**	0.28**	1,00

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Abbreviations: CB- cord blood, HCY- homocysteine, $eGFR_{creat,}$- estimated glomerular filtration rate based on creatinine concentrations; $eGFR_{cystC}$- estimated glomerular filtration rate based on cystatin C concentrations.

Supplementary Table 2.2.4. Subject characteristics with and without kidney measurements (N=6,043)

	Kidney measurements available N=4,226	Without kidney measurements N=1,817	*p*-value
Maternal characteristics			
Maternal age (y)	30.4 (4.9)	28.4 (5.2)	**<0.001**
Pre-pregnancy body mass index(kg/m^2)	22.6 (20.8, 25.4)	22.4 (20.5, 25.4)	0.5
Missing, n (%)	*701 (16.6)*	*371 (20.4)*	
Gestational age at intake (wk)	13.2 (12.2, 14.8)	13.4 (12.2, 15.1)	**<0.001**
Early pregnancy systolic blood pressure (mmHg)	116 (12)	115 (12)	**0.03**
Missing, n (%)	*27 (0.6)*	*15 (0.8)*	
Early pregnancy diastolic blood pressure (mmHg)	68 (10)	68 (10)	0.06
Missing, n (%)	*27 (0.6)*	*15 (0.8)*	
Late pregnancy systolic blood pressure (mmHg)	119 (12)	118 (12)	**<0.001**
Missing, n (%)	*137 (3.2)*	*177 (9.7)*	
Late pregnancy diastolic blood pressure (mmHg)	69 (9)	69 (10)	**<0.001**
Missing, n (%)	*137 (3.2)*	*177 (9.7)*	
Education level, n (%)			**<0.001**
- No higher education	2,060 (48.7)	1,037 (57.1)	
- Higher education	1,952 (46.2)	567 (31.2)	
Missing, n (%)	*214 (5.1)*	*213 (11.7)*	
Ethnicity, n (%)			**<0.001**
- European	2,701 (63.9)	850 (46.8)	
- Non-European	1,460 (34.5)	792 (43.6)	
Missing, n (%)	*65 (1.6)*	*175 (9.6)*	
Smoking during pregnancy, n (%)			**<0.001**
- Never & until pregnancy was known	3,148 (74.5)	1,226 (67.5)	
- Continued	647 (15.3)	356 (19.6)	
Missing, n (%)	*431 (10.2)*	*235 (12.9)*	
Alcohol during pregnancy, n (%)			**<0.001**
- Never & until pregnancy was known	2,147 (50.8)	1,085 (59.7)	
- Continued	1,602 (37.9)	471 (25.9)	
Missing, n (%)	*477 (11.3)*	*261 (14.4)*	
Folic acid supplements use, n (%)			**<0.001**
- No	696 (16.5)	458 (25.2)	
- Start 1st to 10 weeks	1,065 (25.2)	405 (22.3)	
- Start periconceptional	1,530 (36.2)	456 (25.1)	
Missing, n (%)	*935 (22.1)*	*498 (27.4)*	
Maternal calories intake (kcal)	2,045 (553)	2,003 (586)	**0.03**
Missing, n (%)	*795 (18.8)*	*583 (32.1)*	
Vitamin supplements use, n (%)			**<0.001**
- No	2,443 (57.8)	1,107 (60.9)	
- Yes	1,152 (27.3)	363 (20.0)	
Missing, n (%)	*631 (14.9)*	*347 (19.1)*	
Folate plasma concentrations (nmol/l)	16.8 (10.7, 24.8)	13.2 (8.8, 21.8)	**<0.001**
Vitamin B_{12} serum concentrations (pmol/l)	171.0 (129.0, 228.0)	165.0 (123.0, 222.0)	0.05
Homocysteine plasma concentrations (µmol/l)	6.9 (6.0, 8.0)	7.0 (6.0, 8.1)	**0.02**

Supplementary Table 2.2.4. Subject characteristics with and without kidney measurements (N=6,043) (continued)

	Kidney measurements available N=4,226	Without kidney measurements N=1,817	*p*-value
Infant characteristics			
Girls, n (%)	2,121 (50.2)	863 (47.5)	0.07
Gestational age at birth (wk)	40.1 (39.3, 41.0)	40.0 (39.0, 41.0)	**<0.001**
Birth weight (g)	3,437 (551)	3,379 (592)	**<0.001**
Missing, n (%)	*4 (0.1)*	*34 (1.9)*	
Breastfeeding (%)			**<0.001**
- No	257 (6.1)	102 (5.6)	
- Yes	3,172 (75.1)	924 (50.9)	
Missing, n (%)	*797 (18.8)*	*791 (43.5)*	
Cord blood folate concentrations (nmol/l)	20.8 (16.2, 27.0)	20.3 (16.0, 25.9)	0.08
Cord blood vitamin B_{12} concentrations (pmol/l)	299.0 (219.0, 419.0)	301.0 (211.8, 432.0)	0.4
Cord blood homocysteine concentrations (µmol/l)	9.0 (7.5, 10.7)	9.3 (7.6, 11.3)	**<0.001**

Values are frequency counts and percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (25^{th} to 75^{th} percentiles) for continuous variables with a skewed distribution. Values are based on the original data.

Supplementary Table 2.2.5. Associations of maternal folate, vitamin B_{12} and homocysteine concentrations during pregnancy with kidney outcomes at the age of 6 years (N=4,226)

	Difference in outcome measure (95% Confidence Interval)			
First trimester maternal concentrations	**Kidney volume (cm^3)**	**$eGFR_{creat}$ ($ml/min/1.73m^2$)**	**$eGFR_{cystC}$ ($ml/min/1.73m^2$)**	**Microalbuminuria (odds ratio)**
Folate	N=3,818	N=2,788	N=2,792	N=4,011
Basic Model	**0.83 (0.10, 1.55)***	0.30 (-0.31, 0.91)	0.21 (-0.35, 0.76)	1.01 (0.90, 1.14)
Vitamin B_{12}	N=3,666	N=2,659	N=2,663	N=3,849
Basic Model	0.21 (-0.52, 0.94)	0.28 (-0.34, 0.89)	**1.11 (0.55, 1.67)****	1.07 (0.96, 1.20)
Homocysteine	N=3,779	N=2,755	N=2,751	N=3,969
Basic Model	**-1.66 (-2.40, -0.93)****	**-0.78 (-1.37, -0.18)***	**-0.75 (-1.29, -0.20)****	1.07 (0.97, 1.19)

Values are linear and logistic regression coefficients (95% confidence interval). Basic model is adjusted for child's sex and age at 6-year visit. *p <0.05, **p <0.01. Maternal folate, vitamin B_{12} and homocysteine concentrations were analyzed per 1 standard deviation in folate, vitamin B_{12} and homocysteine. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine concentrations; $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C concentrations.

Supplementary Table 2.2.6. Associations of cord blood folate, vitamin B_{12} and homocysteine concentrations with kidney outcomes at the age of 6 years (N=2,674)

	Difference in outcome measure (95% Confidence Interval)			
Cord blood concentrations	**Kidney volume (cm^3)**	**$eGFR_{creat}$ ($ml/min/1.73m^2$)**	**$eGFR_{cystC}$ ($ml/min/1.73m^2$)**	**Microalbuminuria (odds ratio)**
Folate	N=2,384	N=1,750	N=1,753	N=2,517
Basic Model	0.04 (-0.87, 0.94)	0.51 (-0.24, 1.25)	**0.72 (0.03, 1.41)***	0.98 (0.84, 1.14)
Vitamin B_{12}	N=2,413	N=1,772	N=1,776	N=2,548
Basic Model	**-0.93 (-1.82, -0.04)***	-0.43 (-1.18, 0.33)	0.41 (-0.28, 1.10)	1.01 (0.88, 1.17)
Homocysteine	N=2,311	N=1,702	N=1,705	N=2,443
Basic Model	**1.25 (0.33, 2.16)****	**-1.06 (-1.85, -0.28)****	**-1.22 (-1.95, -0.50)****	1.07 (0.93, 1.23)

Values are linear and logistic regression coefficients (95% confidence interval). Basic model is adjusted for child's sex and age at 6-year visit. *p <0.05, **p <0.01. Cord blood folate, vitamin B_{12} and homocysteine concentrations were analyzed per 1 standard deviation in folate, vitamin B_{12} and homocysteine. Abbreviations: $eGFR_{creat}$, estimated glomerular filtration rate based on creatinine concentrations; $eGFR_{cystC}$, estimated glomerular filtration rate based on cystatin C concentrations.

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Chapter 2.3

Maternal vitamin D concentrations during pregnancy, fetal growth patterns, and risks of adverse birth outcomes

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ABSTRACT

Background: Maternal vitamin D deficiency during pregnancy may impact fetal outcomes.
Objective: To examine whether maternal 25-hydroxyvitamin D (25(OH)D) concentrations in pregnancy affect fetal growth patterns and birth outcomes.
Design: Population-based prospective cohort in Rotterdam, the Netherlands among 7,098 mothers and their offspring. We measured 25(OH)D concentrations at a median gestational age of 20.3 weeks (range 18.5–23.3 weeks). Vitamin D concentrations were analyzed continuously and in quartiles. Fetal head circumference, length and weight were estimated by repeated ultrasounds, preterm birth (gestational age <37 weeks) and small-size for gestational age (less than the fifth percentile) were determined.
Results: Adjusted multivariate regression analyses showed that as compared to mothers with second trimester 25(OH)D concentrations in the highest quartile, those with 25(OH)D concentrations in the lower quartiles had offspring with third trimester fetal growth restriction leading to a smaller birth head circumference, shorter birth length and lower weight at birth (all *P* values <0.05). Mothers who had 25(OH)D concentrations in the lowest quartile had increased risks of preterm delivery (Odds Ratio (OR) 1.72, 95% Confidence Interval (CI) 1.14, 2.60) and small-size for gestational age children (OR 2.07, 95% CI 1.33, 3.22). The estimated population attributable risks of 25(OH)D concentrations <50 nmol/L for preterm birth or small-size for gestational age were 17.3% and 22.6%, respectively. The observed associations were not based on extreme 25(OH)D deficiency, but present within the common ranges.
Conclusions: Low maternal 25(OH)D concentrations are associated with proportional fetal growth restriction and with increased risks of preterm birth and small-size for gestational age at birth. Further studies are needed to investigate the causality of these associations and the potential for public health interventions.

INTRODUCTION

Vitamin D deficiency is common and related to various non-communicable disease.[1] An accumulating body of evidence suggest that vitamin D is also crucial for fetal development because of its important role during cell proliferation, differentiation and maturation processes.[2, 3] Suboptimal vitamin D concentrations may affect early organogenesis and subsequently affect later health and disease.[4] Also, vitamin D is important for placental function, calcium homeostasis, and bone mineralization, which are all important determinants for fetal growth and development.[5, 6] Thus far, most published studies focused on the associations of maternal vitamin D status during pregnancy with fetal development have been mainly based on birth weight and have shown inconsistent results.[7–9] However, birth weight is just a proxy for fetal growth and development. Different fetal growth patterns and body proportions may lead to the same birth weight. Not much is known about the direct effects of maternal vitamin D status on fetal growth and development patterns in healthy populations.[8, 10–13] The inconsistent results from previous studies may be explained by differences between study populations. Because ethnic differences in both vitamin D concentrations and birth outcomes have been reported, the effects of maternal vitamin D status on fetal outcomes may differ between specific populations.[14, 15] Based on previous studies suggesting that low vitamin D concentrations, may lead to suboptimal placentation and fetal skeletal growth, we hypothesized that low vitamin D concentrations may lead to fetal growth restriction and increased risks of adverse birth outcomes.

Therefore, we examined the associations of circulating 25-hydroxyvitamin D (25(OH)D) concentrations with repeatedly measured fetal growth characteristics during second and third trimester and the risks of adverse birth outcomes, in a multi-ethnic population-based prospective cohort study in 7,098 mother-offspring pairs. In addition, we explored whether adverse birth outcomes were associated with cord blood 25(OH)D concentrations.

METHODS

Design and study population

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[16] All children were born between April 2002 and January 2006. Enrolment in the study was aimed at early pregnancy, but was allowed until the birth of the child. The study protocol was approved by the local Medical Ethical Committee. Written consent was obtained from all participating mothers. Second trimester 25(OH)D concentrations were measured in 7,176 mothers. For the present study, we excluded pregnancies leading to twin births (N=77) and loss to follow-up at birth (N=1). Thus, the cohort for analysis comprised 7,098 mothers with any fetal or birth outcome available. (**Supplementary Figure 2.3.1.** Flowchart).

Maternal and cord 25(OH)D blood concentrations

Maternal venous blood samples were collected in second trimester (median 20.3 weeks of gestation, range 18.5–23.3 weeks), whereas umbilical cord blood samples were collected immediately after delivery (median 40.1 weeks of gestation, range 35.9–42.3 weeks).

Cord blood vitamin D concentrations represent neonatal vitamin D status at birth. Measurements of 25(OH)D concentrations were conducted at the Eyles Laboratory at the Queensland Brain Institute, University of Queensland, Australia, in 2014.

Total 25(OH)D was calculated as the sum of $25(OH)D_2$ and $25(OH)D_3$ measured in plasma as previously described.[17] Samples were quantified using isotope dilution liquid chromatography-tandem mass spectrometry. The linearity of 25(OH)D concentration was assessed using matrix-matched calibration standards, with R^2 values of >0.99 across the calibration range (10–125 nmol/L). Inter-assay inaccuracy and imprecision were assessed at four concentration levels for $25(OH)D_3$ (48.3, 49.4, 76.4, 139.2 nmol/L) and a single level (32.3 nmol/L) for $25(OH)D_2$ using certified reference materials and were excellent at all concentration levels tested. Inter-assay inaccuracy and imprecision were both <10% for $25(OH)D_3$ and <17% for $25(OH)D_2$, respectively. We categorized vitamin D status into quartiles (quartile 1: median (full range) 14.7 nmol/L (1.5 to 24.1); quartile 2: 35.1 nmol/L (24.2 to 46.6); quartile 3: 59.0 nmol/L (46.7 to 73.7); quartile 4: 91.6 nmol/L (73.8 to 193.2)). Because optimal vitamin D concentrations remain a subject of debate,[18, 19] we performed a sensitivity analysis by using cut-offs concentrations according to previously used cut-offs and recommendations (severely deficient: <25.0 nmol/L; deficient: 25.0 to 49.9 nmol/L; sufficient: 50.0 to 74.9 nmol/L; optimal ≥75.0 nmol/L).[18, 20–24]

Fetal growth measurements

Fetal ultrasound examinations were carried out in two dedicated research centers in second (median 20.5 weeks of gestation, 95% range 18.6–24.3) and third trimester (median 30.3 weeks of gestation, 95% range 28.4–32.8).[25] The first trimester ultrasound was primarily used for establishing gestational age.[26] In second and third trimester, we measured fetal head circumference, abdominal circumference and femur length to the nearest millimeter using standardized ultrasound procedures.[27] Estimated fetal weight was subsequently calculated using the formula of Hadlock et al.[28] Longitudinal growth curves and gestational-age-adjusted standard deviation scores (SDS) were constructed for all fetal growth measurements.[26] These gestational-age-adjusted SDS were based on reference growth curves from the whole study population, and represent the equivalent of z-scores.[26]

Birth outcomes

We obtained information about offspring sex, gestational age, head circumference, length and weight at birth from medical records.[29] Because head circumference and length were not routinely measured at birth in each delivery center, fewer measurements were available (N=3,681 and N=4,533 for head circumference and length at birth, respectively), as compared to birth weight. Gestational-age-adjusted SDS for head circumference, length and weight were constructed using North European growth standards.[30] We defined preterm birth as a gestational age of <37 weeks at birth, low birth weight as a birthweight <2500 grams, and

small-size for gestational age at birth as a gestational-age-adjusted birthweight below the 5th percentile (-1.79 SD).

Covariates

We used questionnaires at enrollment in the study to collect information about maternal age, ethnicity, educational level, parity, the presence of anorexia, smoking, alcohol usage, folic acid and vitamins supplementation.[29] Maternal energy, iron, zinc and calcium dietary intake during pregnancy was measured at enrollment with a validated semi-quantitative food frequency questionnaire.[31] Ethnicity and educational level were defined according to the demographic classification of Statistics Netherlands.[32] Ethnicity was categorized in the following groups: European, Cape Verdean, Dutch Antillean, Moroccan, Surinamese, Turkish and Others. We measured maternal height and weight at enrollment and calculated body mass index (kg/m^2). Information about gestational hypertensive disorders (gestational hypertension, preeclampsia) and gestational diabetes was available from medical records.[33] The date of blood sampling was categorized into summer, fall, winter, and spring, based on the European seasons.

Statistical analysis

First, we performed regression analyses to relate second trimester maternal 25(OH)D concentrations with fetal growth characteristics in second, and third trimester separately. Second, we assessed the associations of maternal 25(OH)D concentrations with longitudinally measured fetal head circumference, fetal femur length and body length at birth, and fetal weight, expressed as SDS, with the use of unbalanced repeated measurement regression analyses. These analyses enable optimal use of available data, taking into account correlations within subjects and assessing both time dependent and independent associations.[34] Because body length cannot be estimated during fetal life, we used femur length in second and third trimester and body length at birth to assess length growth. We measured femur length to the nearest millimeter using standardized ultrasound procedures.[27] For weight we used estimated fetal weight in second and third trimester and birth weight. Third, we used logistic regression models to assess the associations of maternal 25(OH)D concentrations with the risks of preterm birth, low birth weight, and small-size for gestational age. For all the analyses, 25(OH)D concentrations were analyzed both continuously and by using quartiles. As sensitivity analysis, we also analysed 25(OH)D concentrations in groups of previously used and recommended cut-offs instead of quartiles.[18, 20–24] We calculated the population attributable risk (PAR) for these outcomes with the following equation: PAR% = $[(Rate_{total\ population} - Rate_{unexposed})/(Rate_{total\ population})]*100\%$. Finally, we explored the associations of birth characteristics with cord blood 25(OH)D concentrations using linear regression models. All regression models were first adjusted for season when blood samples were drawn and maternal ethnicity (basic models), and subsequently in addition for maternal age; education; parity; BMI at enrolment; smoking; alcohol use; the presence of anorexia; folic acid and vitamins supplement use; energy and dietary iron, zinc and calcium dietary intake during pregnancy; gestational hypertensive disorders; and gestational diabetes (adjusted models). These covariates were

included in the models based on their associations on fetal and birth outcomes in previous studies, or a change in effect estimates of >10%. Adding maternal nutritional data (dietary iron, zinc and calcium intake and vitamin supplements) did not materially change the effect estimates but slightly improved the model fit (R^2, -2log Likelihood or the Nagelkerk R^2 values). Because of the strong associations of ethnicity with both 25(OH)D concentrations with and fetal outcomes, we first adjusted the regression models for maternal ethnicity and second, we restricted the analyses to Europeans only, the largest ethnic subgroup in our cohort.[35, 36] *P value* of <0.05 was considered as statistically significant. To adjust for multiple testing in the analyses of adverse birth outcomes with cord blood 25(OH)D concentrations, we applied Bonferroni correction considering a *P value* <0.025 (0.05/2) to be significant. To diminish potential bias associated with attrition, missing values of covariates, were multiple imputed by generating 5 independent datasets using the Markov Chain Monte Carlo (MCMC) method. The multiple imputation procedure was based on the correlation between each variable with missing values and the other subject characteristics.[37, 38] Statistical analyses were performed using SPSS version 21.0 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). Subject characteristics before and after imputation, including the percentage of missing values are given in the **Supplementary Table 2.3.1**. The unbalanced repeated-measurements analysis, including the Prox Mixed module, was performed with the Statistical Analysis System (version 9.3; SAS Institute Inc, Cary NC).

RESULTS

Subject characteristics

The median value (95% range) for maternal 25(OH)D was 46.7 nmol/L (7.0, 119.4 nmol/L) (**Table 2.3.1)** Maternal and cord blood 25(OH)D concentrations were correlated (r = 0.62, *P value* <0.01). Growth characteristics during second and third trimester and at birth are shown in **Table 2.3.2**.

Supplementary Table 2.3.2 gives the subject characteristics for each quartile of 25(OH)D concentrations. We observed that mothers with a Turkish or Moroccan ethnicity had the lowest 25(OH)D concentrations.

Maternal 25(OH)D concentrations and fetal growth characteristics

The associations of maternal 25(OH)D concentrations during pregnancy with longitudinally measured fetal head circumference, length and weight are shown in **Figure 2.3.1**. Compared with mothers with 25(OH)D concentrations in the highest quartile (range 73.8 to 193.2 nmol/L), those with 25(OH)D concentrations in the first (range 1.5 to 24.1 nmol/L) and second (range 24.2 to 46.6 nmol/L) quartiles had offspring with restricted fetal head circumference growth from second trimester onwards, leading to a smaller head circumference at birth (differences: -0.20 SDS 95% Confidence Interval (CI) (-0.29, -0.12) and -0.09 SDS 95% CI (-0.17, -0.01) for first and second quartile of 25(OH)D concentrations, respectively). The associations of second trimester maternal 25(OH)D concentrations with fetal length growth and weight growth tended

Table 2.3.1. Subject characteristics (N=7,098)[1]

Characteristics	
Maternal Characteristics	
Age, mean (SD), y	29.7 (5.2)
Body mass index at enrolment, median (95% range), kg/m^2	23.7 (18.7, 36.3)
Gestational age at enrollment, median (95% range), wk	13.9 (9.9, 22.9)
Nulliparous, No. (%)	3,987 (56.2)
Education level, No. (%)	
- No higher education	4,204 (59.2)
- Higher education	2,894 (40.8)
Ethnicity, No. (%)	
- European	4,069 (57.3)
- Cape Verdean	311 (4.4)
- Dutch Antillean	253 (3.5)
- Moroccan	471 (6.6)
- Surinamese	643 (9.1)
- Turkish	651 (9.2)
- Other	700 (9.9)
Presence of anorexia, No. (%)	
- No	6,352 (89.5)
- Yes	503 (7.1)
- Maybe	243 (3.4)
Smoking during pregnancy, No. (%)	
- Never	5,131 (72.3)
- Until pregnancy was known	665 (9.4)
- Continued	1,302 (18.3)
Alcohol consumption during pregnancy, No. (%)	
- Never	3,493 (49.2)
- Until pregnancy was known	974 (13.7)
- Continued	2,631 (37.1)
Folic acid supplement use, No. (%)	
- No	2,204 (31.0)
- Start in the first 10 weeks	2,219 (31.3)
- Start periconceptional	2,675 (37.7)
Vitamin supplement use, No. (%)	
- Yes	5,049 (71.1)
- No	2,049 (28.9)
Maternal energy intake (kcal)	2,039 (490)
Maternal zinc dietary intake (mg)	9.6 (1.6)
Maternal iron dietary intake (mg)	11.1 (2.1)
Maternal calcium dietary intake (mg)	1,087 (418)
Maternal 25(OH)D concentrations, median (95% range), nmol/L	46.7 (7.0, 119.4)
- Severely deficient (<25.0 nmol/L), No. (%)	1,855 (26.1)
- Deficient (25.0 to 49.9 nmol/L), No. (%)	1,919 (27.1)
- Sufficient (50.0 to 74.9 nmol/L), No. (%)	1,619 (22.8)
- Optimal (≥75.0 nmol/L), No. (%)	1,705 (24.0)

Table 2.3.1. Subject characteristics (N=7,098)[1] (continued)

Characteristics	
Season when maternal blood sample was take, No. (%)	
- Spring	2,097 (29.5)
- Summer	1,622 (22.9)
- Autumn	1,702 (24.0)
- Winter	1,677 (23.6)
Pregnancy complications, No. (%)	
- Gestational hypertensive disorders	421 (5.9)
- Gestational diabetes	67 (0.9)
Birth characteristics	
Female sex, No. (%)	3,529 (49.7)
Preterm birth (<37 wk of gestation), No. (%)	370 (5.2)
Low birth weight (<2500 g), No. (%)	342 (4.8)
Small-size for gestational age at birth (<5th percentile), No. (%)	355 (5.0)
25(OH)D concentration in cord blood at birth, median (95% range), nmol/L	27.4 (4.7, 81.4)
Season when cord blood sample was taken, No. (%)	4.264
- Spring	1,130 (26.5)
- Summer	1,164 (27.2)
- Autumn	996 (23.4)
- Winter	977 (22.9)

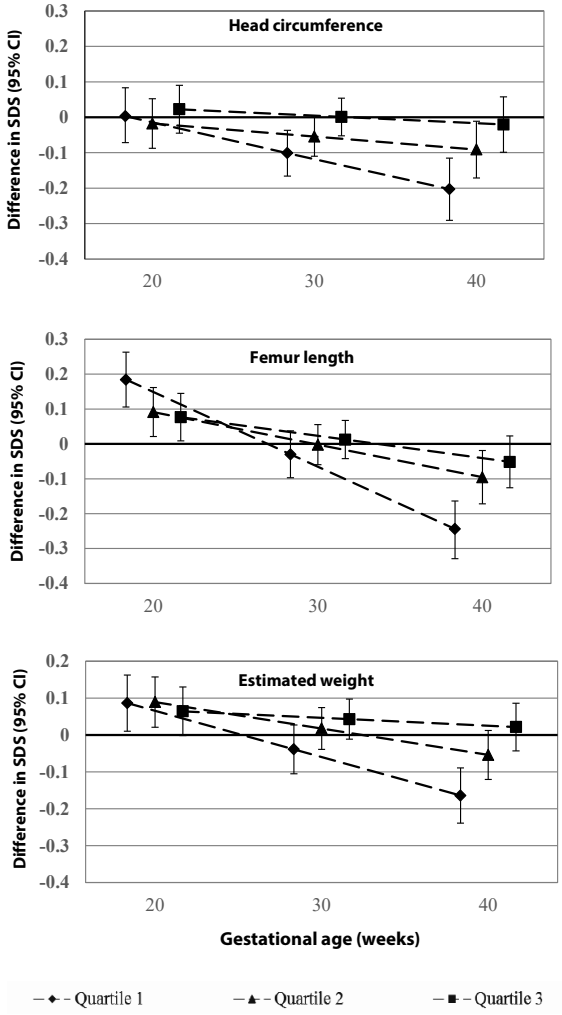
[1]Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.

Table 2.3.2. Fetal growth characteristics (N=7,098)[1]

Fetal growth characteristics (N=7,098)	
Second trimester (N=7,098)	
- Gestational age, median (95% range), wk	20.5 (18.6, 23.4)
- Head circumference, mean (SD), cm	17.9 (1.5)
- Femur length, mean (SD), mm	33.5 (3.6)
- Estimated fetal weight, mean (SD), g	382 (94)
Third trimester (N=7,018)	
- Gestational age at birth, median (95% range), wk	30.3 (28.4, 32.8)
- Head circumference, mean (SD), cm	28.5 (1.2)
- Femur length, mean (SD), mm	57.4 (3.0)
- Estimated fetal weight, mean (SD), g	1,614 (253)
Birth (N=7,046)	
- Gestational age, median (95% range), wk	40.1 (35.6, 42.3)
- Head circumference, mean (SD), cm	33.8 (1.7)
- Body length, mean (SD), cm	50.2 (2.4)
- Weight, mean (SD), g	3,414 (561)

[1]Values are means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.

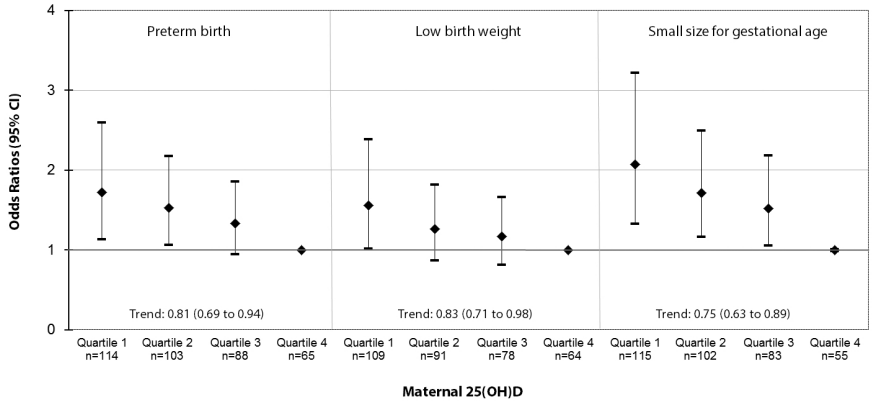
Figure 2.3.1. Associations of maternal second trimester 25(OH)D concentrations with fetal growth patterns (N=7,098)



Values are estimates based on repeated linear regression models and reflect the standard deviation score for each growth characteristic in offspring of mothers whose 25(OH)D concentrations during pregnancy were in the first, second and third quartile compared to offspring from mothers who had 25(OH)D concentrations in the fourth quartile. Length at birth represents the full body length and weight at birth is the measured birth weight.

to be similar as those observed for head circumference. Results from the regression models focused on the associations of 25(OH)D concentrations with one fetal growth measure at each time point showed similar results (**Supplementary Table 2.3.3**). We observed similar associations when using vitamin D cut-offs according to previously used clinical cut-offs and recommendations (**Supplementary Table 2.3.4**). Also, although we observed tendencies for similar effect estimates when we restricted the analyses to Europeans only (N=4069), not

Figure 2.3.2. Associations of maternal second trimester 25(OH)D concentrations with the risks of adverse birth outcomes (N=7,098)



Values are logistic regression coefficients (95% confidence interval) and reflect the risk of adverse birth outcomes compared to the reference group. Continuous analyses reflect the risks of being preterm, having a low birth weight or being small-size for gestational age at birth per 1 SDS increase in maternal 25(OH)D. Multivariable model is adjusted for maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and vitamin supplements, energy, iron, zinc, and calcium dietary intake during pregnancy, education, ethnicity, gestational hypertensive disorders, gestational diabetes, parity, season when blood samples were drawn and the presence of anorexia).

all associations reached statistical significance due to smaller sample sizes (**Supplementary Table 2.3.5**).

Maternal 25(OH)D concentrations and risks of adverse birth outcomes

Compared with mothers who had 25(OH)D concentrations in the highest quartile (range 73.8 to 193.2 nmol/L), those who were in the lowest quartile of 25(OH)D concentrations (range 1.5 to 24.1 nmol/L) had increased risks of children born preterm birth (Odds Ratio (OR) 1.72 (95% CI 1.14, 2.60)), with a low birth weight (OR 1.56 (95% CI 1.02, 2.39) and with a small-size for gestational age at birth (OR 2.07 (95% CI 1.33, 3.22)) (**Figure 2.3.2**). We observed dose-response associations suggesting that higher maternal 25(OH)D concentrations across the full range were associated with lower risks of preterm birth, low birth weight and small-size for gestational age at birth (all *P value*s <0.05). We observed tendencies for similar associations when using 25(OH)D cut-offs according to previously used clinical cut-offs and recommendations instead of quartiles (**Supplementary Figure 2.3.2**). Similar effect estimates were observed when we restricted our analyses to European subjects only, but not all associations were significant (**Supplementary Figure 2.3.3**). The estimated population attributable risks of vitamin D concentrations <50 nmol/L for preterm birth or low birth weight or for small-size for gestational age in our population were 17.3%, 18.4% and 22.6%, respectively.

Adverse birth outcomes and cord blood 25(OH)D concentrations

Higher weight at birth and gestational age adjusted birth weight were associated with higher cord blood 25(OH)D concentrations (differences 0.04 SDS (95% CI 0.01, 0.07)) and 0.03 SDS

Table 2.3.3. Associations of adverse birth outcomes with cord blood 25(OH)D concentrations (N=4,262)[1]

Birth characteristics	N	Cord blood 25(OH)D (nmol/L)
Gestational age	**4,262**	
<37.0 weeks	130	0.02 (-0.13, 0.17)
37.0-41.9 weeks	3849	Reference
≥42 weeks	283	-0.06 (-0.16, 0.05)
Trend		*-0.01 (-0.03, 0)*
Birth weight	**4,258**	
<2,000 grams	15	**-0.49 (-0.91, -0.06)[2]**
2,000–2,499 g	94	-0.01 (-0.18, 0.17)
2,500–2,999 g	607	-0.02 (-0.11, 0.06)
3,000–3,499 g	1,535	Reference
3,500–3,999 g	1,412	0.01 (-0.05, 0.07)
4,000–4,499 g	502	-0.01 (-0.10, 0.08)
≥4,500 grams	93	0.06 (-0.12, 0.23)
Trend		***0.04 (0.01, 0.07)[2,3]***
Birth weight for gestational age	**4,257**	
Small	164	-0.09 (-0.22, 0.05)
Normal	3,881	Reference
Large	212	0.06 (-0.06, 0.18)
Trend		***0.03 (0.01, 0.06)[2]***

[1]Values are linear regression coefficients (95% confidence interval) and reflect the change in standard deviation (SDS) of cord blood 25(OH)D concentrations for each birth weight or gestational age group, compared to the reference group. Trend estimates represent the effect estimates for the continuous associations per SDS change in birth characteristics. Multivariable model is adjusted for fetal sex, maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and vitamin supplements, energy, iron, zinc and calcium dietary intake during pregnancy, education, ethnicity, gestational hypertensive disorders, gestational diabetes, parity, and the presence of anorexia). [2]*P value* <0.05. [3]Also significant after applying Bonferroni correction (*P value* <0.025).

(95% CI 0.01, 0.06) per 1 SD increase in birth weight and gestational age adjusted birth weight, respectively) (**Table 2.3.3**). The association of higher gestational age at birth with cord blood 25(OH)D concentrations was of borderline significance (difference -0.01 SDS (95% CI -0.03, 0) per 1SD increase in gestational age. Tendencies for similar effect estimates were observed when we restricted our analyses to European subjects only (**Supplementary Table 2.3.6**).

DISCUSSION

Results from this large population-based prospective cohort study suggest that second trimester lower maternal 25(OH)D concentrations are associated with third trimester fetal head, length, and weight growth restriction and with increased risks of preterm birth, low birth weight and small-size for gestational age at birth. These associations were not restricted to the extremes, but tended to be present across the full spectrum of maternal 25(OH)D concentrations. Also, a smaller size at birth was associated with lower cord blood 25(OH)D concentrations.

We hypothesized that suboptimal maternal 25(OH)D concentrations affect fetal development leading to an increased risk for adverse birth outcomes. Previous studies reported inconsistent results on the associations of maternal 25(OH)D concentrations with birth outcomes.[2, 7–9, 39] A previous study among 2,473 mother-children pairs, in a multi-center cohort in the United States reported that low concentrations of maternal 25(OH)D are associated with low weight at birth.[9] In line with these findings, a study among 2,146 mother-children pairs in the United States observed that maternal 25(OH)D concentrations were positively associated with weight at birth.[40] Results from another population-based cohort study in the Netherlands among 3,730 mother-children pairs, also suggested that lower maternal 25(OH)D concentrations are associated with lower birth weight.[7] A recent meta-analysis of randomized controlled trials suggested that vitamin D supplementation during pregnancy was associated with increased circulating 25(OH)D concentrations and a higher birth weight.[40] Thus, altogether previous studies suggest that higher vitamin D concentrations in pregnancy lead to higher birth weight.

The use of birth weight as the main fetal outcome has strong limitations. Birth weight does not give information about longitudinal fetal growth and development patterns and fetal body proportions. We examined the impact of maternal 25(OH)D concentrations on head circumference, length and estimated fetal weight during second and third trimester of pregnancy and at birth. We used repeatedly and directly measured fetal growth characteristics and observed that lower maternal 25(OH)D concentrations were associated with restricted fetal head circumference growth from second trimester onwards, correlating with a small head circumference at birth. Similar associations were observed for fetal length growth and fetal weight growth. In line with our findings on head circumference at birth, a multi-center cohort in the United States suggested that lower maternal 25(OH)D concentrations during mid-pregnancy are associated with a smaller head circumference at birth.[39] In contrast, a study among 2,382 mother-child pairs in Spain, showed that higher maternal 25(OH)D concentrations in early pregnancy are associated with a smaller head circumference at birth.[8] Regarding birth length, results from a recent meta-analysis of randomized controlled trials suggested that neonates in the maternal vitamin D supplemented group had a higher birth length compared with the control group.[40] In contrast to these findings, a study among 559 mother-children pairs in India, did not observe any association of maternal 25(OH)D concentrations and length at birth.[41]These differences may be due to differences in study populations. Results from the longitudinal analyses of our large study, suggest that low maternal 25(OH)D concentrations are associated with a proportional growth restriction, though the largest effect may be present on fetal length growth.

We observed that lower maternal 25(OH)D concentrations during mid-pregnancy are associated with a higher risk of preterm birth. A previous study among 2,382 mother-child pairs in Spain did not observe any association of maternal 25(OH)D concentrations during early pregnancy with the risk of preterm birth.[8] This difference in outcome may be due to the specific study population, and smaller sample size in the earlier study, which limited the ability to detect these associations. Our results regarding the risk of low birth weight and of small-size for gestational age are in line with findings from another cohort study in the Neth-

erlands, which observed that lower maternal 25(OH)D concentrations during early pregnancy was associated with an increased risk of low birth weight and small-size for gestational age.[7] Also, a previous study among 3,658 Chinese mothers, whose 25(OH)D concentrations were assessed at different time points during pregnancy, observed that lower maternal 25(OH)D concentrations throughout pregnancy elevated the risk of small-size for gestational age at birth.[42] The observations linking low maternal vitamin D to preterm birth, low birth weight and small-size for gestational age at birth are important. All three outcomes are associated with perinatal mortality and later-life chronic disease.[43] We also observed that children born preterm or with a small-size at birth have lower cord blood 25(OH)D concentrations. Results from a study in Boston among 471 infants, suggested that preterm born infants have a higher risk of having lower 25(OH)D cord blood concentrations.[44] Thus, preterm birth and small size at birth may be both a consequence and a risk factor of vitamin D deficiency. Low vitamin D concentrations in early life may affect health in later life.[45, 46]

Form our observational study, it is not possible to establish the causality for the observed associations. However, several biological mechanisms have been suggested linking maternal 25(OH)D concentrations to fetal development. Maternal vitamin D may affect placental vascularisation.[47] It has been suggested that vitamin D receptors and 1,25-dihydroxyvitaminD ($1,25(OH)_2D$) regulate placental secretion of human placental lactogen and other hormones that affect maternal glucose and fatty acid metabolism, which provide energy for fetal needs.[44, 47] Further studies that focus on the causality and mechanisms explaining the observed associations are needed.

Our findings are important from a population-based perspective. The upper limit of first 2 quartiles of maternal 25(OH)D concentrations correspond to the value of <50 nmol/L, which is defined as 25(OH)D deficiency.[19, 20] In our study, 53% of all mothers had 25(OH)D deficient concentrations, leading to high estimated population attributable risks. In the Netherlands, pregnant women are advised to use folic acid supplements (400 µg/day) prior to and up to week 10–12 of pregnancy and vitamin D supplements (10 µg/day). Therefore, results from our study support population-strategies to improve vitamin D concentrations in pregnant women.

Some methodological considerations need to be considered. To our knowledge, this is the largest multi-ethnic population-based prospective cohort study focused on the associations of maternal 25(OH)D concentrations with directly measured fetal growth characteristics in different periods of pregnancy. In mothers who had available 25(OH)D concentrations, we had a limited loss to follow-up; therefore, we do not expect biased results from selective follow-up. We used 25(OH)D concentration, which is the best and most widely used indicator of vitamin D status.[48] We analyzed vitamin D concentrations as continuous data, quartiles and previously used clinical cut-offs.[18, 20–24] The cut offs remain subject of debate.[18, 19] In line with recommendations of the Endocrine Society and based on previous results from our and other cohort studies, we created four vitamin D groups, including severely deficient (<25.0 nmol/L), deficient (25.0 to 49.9 nmol/L), sufficient (50.0 to 74.9 nmol/L) and optimal (≥75.0 nmol/L) (18, 20–24). The Institute of Medicine (IOM) defines vitamin D as being deficient (<50nmol/L), and sufficient (≥50nmol/L), which would lead to categorizing our severely deficient and deficient groups as deficient, and categorizing the sufficient and optimal groups as sufficient

or adequate. We consider that an advantage of our categories over the IOM categories is that we have more groups and can compare our results with previous pregnancy studies. Also, our findings suggest that the effects of vitamin D on fetal outcomes are not restricted to IOM deficient groups, but present across the full common range. 25(OH)D concentrations were assessed during second trimester and in cord blood at birth. The correlation coefficient between maternal serum 25(OH)D concentrations during second trimester and in cord blood was r = 0.62 (*P value* <0.01). This correlation varies between studies. Some studies report a higher degree of correlation, but still report a difference between maternal 25(OH)D concentrations during third trimester of pregnancy and 25(OH)D concentrations in cord blood at birth.[49] A limitation of our study may be that we do not have maternal 25(OH)D concentrations measured during first trimester. We measured fetal development by direct second- and third trimester fetal ultrasound examinations. Because the main outcomes were correlated, we did not adjust the main analyses for multiple comparisons. Another limitation of our study is the lack of detailed information on vitamin D supplementation and on conditions that may influence vitamin D status, such as bariatric surgery or different gastrointestinal diseases. However, other nutritional factors may influence the observed associations. As previous studies suggested that iron, zinc, calcium and other vitamin supplements influence fetal growth and birth outcomes, we included these nutritional factors in our regression models. Adjustment for these factors did not change the effect estimates materially. Several trials suggested that supplementation of these micronutrients lowers the risks of pregnancy complications in women at risk of deficiencies or adverse outcomes.[50–55] Because, the intake of these micronutrients was estimated from a food frequency questionnaire, we may not have achieved the precision in these concentrations compared to studies with concentrations. Maternal ethnicity is related to both vitamin D concentrations and fetal growth patterns.[35, 36] The study cohort was a multi-ethnic sample in the city of Rotterdam, the Netherlands. Compared with the overall population distribution in the city of Rotterdam, the percentage of Europeans in our study was higher whereas the percentage of Moroccans was lower.[35] We used two approaches to explore the role of maternal ethnicity. First, all main analyses were adjusted for maternal ethnicity. Second, we observed tendencies for similar associations when we restricted the analyses to Europeans only. However, not all effect estimates were significant, probably because of smaller numbers. Unfortunately, we did not have enough numbers in the other ethnic subgroups, to perform ethnic specific analyses. The results of these additional analyses suggest that the associations of vitamin D concentrations with fetal outcomes are not explained by maternal ethnicity. Further studies are needed to explore differences in effect estimates between ethnic subgroups. Finally, although we performed adjustment for a large number of potential maternal confounders, residual confounding by other lifestyle factors, might still be present, as in any observational study. The causality for the associations of vitamin D with fetal developmental outcomes cannot be established from observational studies only. Therefore, future studies are needed to establish causal relationships.

CONCLUSION

Our findings suggest that second trimester low maternal 25(OH)D concentrations are associated with third trimester fetal grow restriction and with increased risks of preterm birth, low birth weight, and small-size for gestational age at birth. These associations were not restricted to the extremes, but tended to be present across the full spectrum of maternal 25(OH)D concentrations. Although the causality of the observed associations need to be further established, these findings support current strategies to increase 25(OH)D concentrations in pregnant women.

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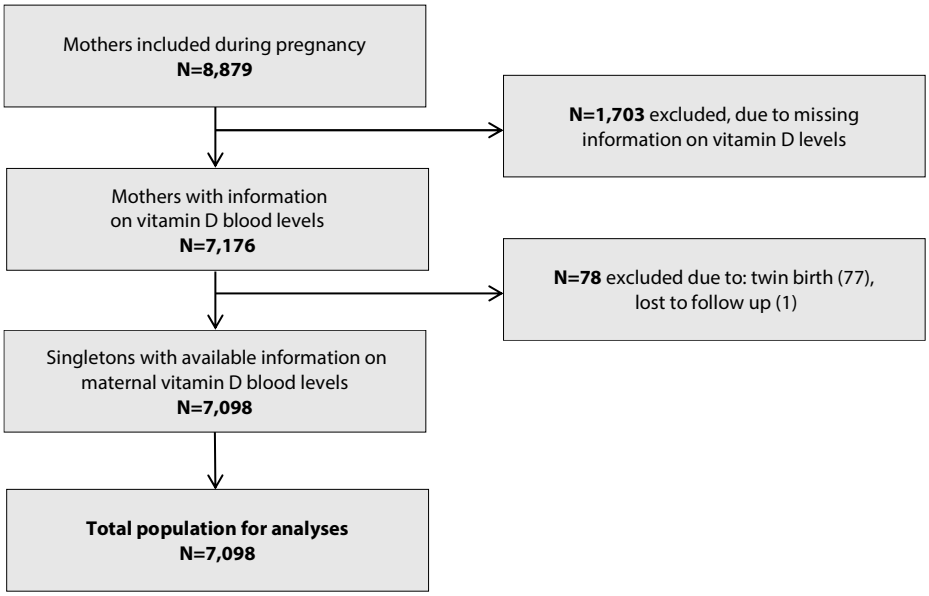
Supplementary Figure 2.3.1. Flowchart of the study participants



Supplementary Table 2.3.1. Subject characteristics observed and imputed[1]

	Original	Imputed
Maternal characteristics		
Age, mean (SD), y	29.7 (5.2)	NI
Body mass index at enrolment, median (95% range), kg/m^2	23.8 (18.7, 36.3)	23.7 (18.7, 36.3)
Missing, No. (%)	*46 (0.6)*	
Gestational age at enrollment, median (95% range), wk	13.9 (9.9, 22.9)	NI
Nulliparous, No. (%)	3,955 (55.7)	3,987 (56.2)
Missing, No. (%)	*67 (0.9)*	
Education level,No. (%)		
- No higher education	3,756 (52.9)	4,204 (59.2)
- Higher education	2,786 (39.3)	2,894 (40.8)
Missing, No. (%)	*556 (7.8)*	
Ethnicity, No. (%)		
- European	3,897 (54.9)	4,069 (57.3)
- Cape Verdean	294 (4.1)	311 (4.4)
- Dutch Antillean	242 (3.4)	253 (3.5)
- Moroccan	447 (6.3)	471 (6.6)
- Turkish	630 (8.9)	651 (9.2)
- Surinamese	610 (8.6)	643 (9.1)
- Other	665 (9.4)	700 (9.9)
Missing, No. (%)	*313 (4.4)*	
Presence of anorexia, No. (%)		
- No	5,254 (74.0)	6,352 (89.5)
- Yes	244 (3.4)	503 (7.1)
- Maybe	114 (1.6)	243 (3.4)
Missing, No. (%)	1,486 (20.9)	
Smoking during pregnancy, No. (%)		
- Never	4,536 (63.9)	5,131 (72.3)
- Until pregnancy was known	551 (7.8)	665 (9.4)
- Continued	1,179 (16.6)	1,302 (18.3)
Missing, No. (%)	*832 (11.7)*	
Alcohol consumption during pregnancy, No. (%)		
- Never	3,001 (42.3)	3,493 (49.2)
- Until pregnancy was known	863 (12.2)	974 (13.7)
- Continued	2,315 (32.6)	2,631 (37.1)
Missing, No. (%)	*919 (12.9)*	
Folic acid supplement use, No. (%)		
- No	1,515 (21.4)	2,204 (31.0)
- Start in the first 10 weeks	1,677 (23.6)	2,219 (31.3)
- Start periconceptional	2,123 (29.9)	2,675 (37.7)
Missing, No. (%)	*1,783 (25.1)*	
Vitamin supplement use, No. (%)		
- Yes	4,128 (58.2)	5,049 (71.1)
- No	1,730 (24.4)	2,049 (28.9)
Missing, No. (%)	*1,240 (17.5)*	

Supplementary Table 2.3.1. Subject characteristics observed and imputed[1] (continued)

	Original	Imputed
Maternal energy intake (kcal)	2,041 (566)	2,039 (490)
Missing, No. (%)	*1,813 (25.5)*	
Maternal zinc intake (mg)	9.6 (1.7)	9.6 (1.6)
Missing, No. (%)	*1,813 (25.5)*	
Maternal iron intake (mg)	11.1 (2.1)	11.1 (2.1)
Missing, No. (%)	*1,813 (25.5)*	
Maternal calcium intake (mg)	1,100 (453)	1,087 (418)
Missing, No. (%)	*1,813 (25.5)*	
Maternal 25(OH)D concentrations, median (95% range), nmol/L	46.7 (7.0, 119.4)	NI
Season when maternal blood sample was take, No. (%)		
- Spring	2,097 (29.5)	NI
- Summer	1,622 (22.9)	NI
- Autumn	1,702 (24.0)	NI
- Winter	1,677 (23.6)	NI
Pregnancy complications, No. (%)		
- Gestational hypertensive disorders	388 (5.5)	421 (5.9)
- Gestational diabetes	67 (0.9)	67 (0.9)
Missing, No. (%)	*422 (5.9)*	
Birth characteristics		
Female sex, No. (%)	3,529 (49.7)	3,529 (49.7)
Missing, No. (%)	*1 (0.01)*	
Preterm birth (<37 wk of gestation), No. (%)	370 (5.2)	NI
Low birth weight (<2500 g), No. (%)	342 (4.8)	NI
Small-size for gestational age at birth (<5th percentile), No. (%)	355 (5.0)	NI
25(OH)D concentration in cord blood at birth, median (95% range), nmol/L	27.4 (4.7, 81.4)	NI
Season when cord blood sample was taken, No. (%)		
- Spring	1,130 (26.5)	NI
- Summer	1,164 (27.2)	NI
- Autumn	996 (23.4)	NI
- Winter	977 (22.9)	NI

[1]Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. NI- not imputed.

Supplementary Table 2.3.2. Subject characteristics according to quartiles of 25(OH)D[1]

	Full group N=7,098	Quartile 1 N=1,774	Quartile 2 N=1,773	Quartile 3 N=1,777	Quartile 4 N=1,774
Maternal characteristics					
Age, mean (SD), y	29.7 (5.2)	27.7 (5.5)	29.6 (5.4)	30.5 (4.8)	31.3 (4.4)
Body mass index at enrolment, median (95% range), kg/m^2	23.7 (18.7, 36.3)	25.0 (18.5, 38.3)	24.0 (18.5, 36.0)	23.6 (18.8, 35.8)	23.0 (18.7, 33.4)
Gestational age at enrollment, median (95% range), wk	13.9 (9.9, 22.9)	15.2 (10.3, 23.5)	13.9 (9.8, 22.7)	13.4 (9.8, 22.1)	13.2 (9.5, 21.9)
Nulliparous, No. (%)	3,987 (56.2)	829 (46.8)	960 (54.1)	1,085 (61.1)	1,113 (62.7)
Education level, No. (%)					
- No higher education	4,204 (59.2)	1,499 (84.5)	1,110 (62.0)	879 (49.5)	726 (40.9)
- Higher education	2,894 (40.8)	275 (15.5)	673 (28.0)	898 (50.5)	1,048 (59.1)
Ethnicity, No. (%)					
- European	4,069 (57.3)	327 (18.5)	940 (53.0)	1,259 (71)	1,543 (87)
- Cape Verdean	311 (4.4)	105 (5.9)	126 (7.1)	62 (3.4)	18 (1.1)
- Dutch Antillean	253 (3.5)	106 (5.9)	88 (4.9)	37 (2.1)	22 (1.2)
- Moroccan	471 (6.6)	331 (18.5)	87 (4.9)	40 (2.2)	13 (0.7)
- Turkish	651 (9.2)	379 (21.5)	148 (8.5)	98 (5.6)	26 (1.5)
- Surinamese	643 (9.1)	321 (18.1)	201 (11.3)	92 (5.2)	29 (1.6)
- Other	700 (9.9)	205 (11.6)	183 (10.3)	189 (10.6)	123 (6.9)
Presence of anorexia, No. (%)					
- No	6,352 (89.5)	1,522 (85.8)	1,578 (89.0)	1,630 (91.7)	1,620 (91.3)
- Yes	503 (7.1)	142 (8.0)	130 (7.3)	111 (6.2)	120 (6.8)
- Maybe	243 (3.4)	110 (6.2)	64 (3.6)	36 (2.1)	34 (1.9)
Smoking during pregnancy, No. (%)					
- Never	5,131 (72.3)	1,226 (69.0)	1,243 (70.0)	1,318 (74.2)	1,344 (75.8)
- Until pregnancy was known	665 (9.4)	145 (8.2)	182 (10.3)	171 (9.6)	169 (9.5)
- Continued	1,302 (18.3)	403 (22.8)	348 (19.7)	288 (16.2)	261 (14.7)
Alcohol consumption during pregnancy, No. (%)					
- Never	3,493 (49.2)	1,297 (73.1)	899 (50.7)	725 (40.8)	571 (32.2)
- Until pregnancy was known	974 (13.7)	164 (9.2)	254 (14.3)	266 (15.0)	291 (16.4)
- Continued	2,631 (37.1)	313 (17.6)	620 (35.0)	786 (44.2)	912 (51.4)

Supplementary Table 2.3.2. Subject characteristics according to quartiles of 25(OH)D[1] (continued)

	Full group N=7,098	Quartile 1 N=1,774	Quartile 2 N=1,773	Quartile 3 N=1,777	Quartile 4 N=1,774
Folic acid supplement use, No. (%)					
- No	2,204 (31.0)	1092 (61.6)	633 (35.7)	310 (17.4)	169 (9.5)
- Start in the first 10 weeks	2,219 (31.3)	415 (23.4)	591 (33.3)	622 (35.0)	591 (33.3)
- Start periconceptional	2,675 (37.7)	267 (15.0)	549 (31.0)	845 (47.6)	1,014 (57.2)
Vitamin supplement use, No. (%)					
- Yes	5,049 (71.1)	1,578 (89.0)	1,353 (76.3)	1,131 (63.6)	987 (55.6)
- No	2,049 (28.9)	196 (11.0)	420 (23.7)	646 (36.4)	787 (44.4)
Maternal energy intake (kcal)	2,039 (490)	1,954 (484)	2,022 (490)	2,081 (499)	2,102 (472)
Maternal zinc intake (mg)	9.6 (1.6)	9.1 (1.6)	9.5 (1.6)	9.7 (1.7)	9.8 (1.6)
Maternal iron intake (mg)	11.1 (2.1)	10.4 (1.9)	11.0 (2.1)	11.3 (2.1)	11.6 (2.1)
Maternal calcium intake (mg)	1,087 (418)	966 (405)	1,060 (408)	1,144 (426)	1,176 (400)
Maternal 25(OH)D concentrations, median (95% range), nmol/L	46.7 (7.0, 119.4)	14.7 (4.7, 23.6)	35.1 (24.6, 46.0)	59.0 (47.1, 73.0)	91.7 (74.5, 136.8)
Season when maternal blood sample was take, No. (%)					
- Spring	2,097 (29.5)	565 (31.8)	570 (32.2)	530 (29.8)	432 (24.4)
- Summer	1,622 (22.9)	179 (10.1)	307 (17.3)	418 (23.5)	718 (40.4)
- Autumn	1,702 (24.0)	434 (24.5)	428 (24.1)	454 (25.5)	386 (21.8)
- Winter	1,677 (23.6)	596 (33.6)	468 (26.4)	375 (21.2)	238 (13.4)
Pregnancy complications, No. (%)					
- Gestational hypertensive disorders	421 (5.9)	91 (5.1)	103 (5.8)	122 (6.9)	105 (5.9)
- Gestational diabetes	67 (0.9)	22 (1.2)	22 (1.2)	11 (0.6)	12 (0.7)
Birth characteristics					
Female sex, No. (%)	3,529 (49.7)	882 (49.7)	884 (49.9)	898 (50.5)	865 (48.8)
Preterm birth (<37 wk of gestation), No. (%)	370 (5.2)	114 (6.4)	103 (5.8)	88 (5.0)	65 (3.7)
Low birth weight (<2500 g), No. (%)	342 (4.8)	109 (6.1)	91 (5.1)	78 (4.4)	64 (3.6)
Small-size for gestational age at birth (<5th percentile), No. (%)	355 (5.0)	115 (6.5)	102 (5.8)	83 (4.7)	55 (3.1)
25(OH)D concentration in cord blood at birth, median (95% range), nmol/L	27.4 (4.7, 81.4)	11.2 (3.0, 52.8)	23.0 (6.0, 70.3)	32.7 (9.0, 81.0)	45.0 (14.3, 93.4)
Season when cord blood sample was taken, No. (%)	4264	1003	1066	1119	1076
- Spring	1,130 (26.5)	329 (32.8)	306 (28.7)	296 (26.4)	199 (18.5)
- Summer	1,164 (27.2)	376 (37.5)	327 (30.7)	271 (24.2)	187 (17.4)
- Autumn	996 (23.4)	152 (15.2)	234(22.0)	286 (25.6)	324 (30.1)
- Winter	977 (22.9)	146 (14.5)	199 (18.6)	266 (23.8)	366 (34.0)

[1]Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.

Supplementary Table 2.3.3. Associations of maternal second trimester 25(OH)D concentrations with fetal growth patterns from multiple regression models (N=7,098)[1]

	Head circumference SDS (95% Confidence Interval)		
25(OH)D concentrations	**2nd trimester N=6,967**	**3rd trimester N=6,800**	**Birth N=3,681**
Quartile 1 (N=1,774)	-0.01 (-0.10, 0.08) N=1,734	-0.07 (-0.15, 0.02) N=1,633	**-0.14 (-0.28, -0.01)[2]** N=919
Quartile 2 (N=1,773)	-0.05 (-0.12, 0.03) N=1,736	-0.03 (-0.10, 0.05) N=1,692	-0.07 (-0.19, 0.04) N=940
Quartile 3 (N=1,777)	0.01 (-0.06, 0.08) N=1,750	0 (-0.07, 0.07) N=1,722	-0.03 (-0.13, 0.07) N=983
Quartile 4 (N=1,774)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.01 (-0.02, 0.04)*	*0.02 (-0.01, 0.05)*	***0.05 (0.01, 0.10)*[2]**
	Length SDS (95% Confidence Interval)		
25(OH)D concentrations	**2nd trimester N=6,988**	**3rd trimester N=6,851**	**Birth N=4,533**
Quartile 1 (N=1,774)	-0.01 (-0.10, 0.08) N=1,741	0.01 (-0.08, 0.10) N=1,674	**-0.20 (-0.33, -0.07)[2,3]** N=1,038
Quartile 2 (N=1,773)	-0.01 (-0.08, 0.07) N=1,745	0.04 (-0.04, 0.11) N=1,705	-0.09 (-0.19, 0.02) N=1,119
Quartile 3 (N=1,777)	0.01 (-0.06, 0.08) N=1,751	0.05 (-0.02, 0.12) N=1,732	-0.09 (-0.18, 0.01) N=1,164
Quartile 4 (N=1,774)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.01 (-0.02, 0.04)*	*-0.01 (-0.04, 0.02)*	***0.06 (0.02, 0.11)*[2,3]**
	Weight SDS (95% Confidence Interval)		
25(OH)D concentrations	**2nd trimester N=6,952**	**3rd trimester N=6,828**	**Birth N=7,043**
Quartile 1 (N=1,774)	-0.07 (-0.15, 0.02) N=1,737	-0.04 (-0.13, 0.05) N=1,666	**-0.17 (-0.26, -0.09)[2,3]** N=1,751
Quartile 2 (N=1,773)	-0.02 (-0.09, 0.06) N=1,731	0.02 (-0.06, 0.09) N=1,701	**-0.07 (-0.14, -0.01)[2]** N=1,759
Quartile 3 (N=1,777)	-0.01 (-0.07, 0.06) N=1,742	0.07 (-0.01, 0.13) N=1,728	-0.02 (-0.09, 0.05) N=1,766
Quartile 4 (N=1,774)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.02 (-0.01, 0.06)*	*0.01 (-0.02, 0.04)*	***0.06 (0.03, 0.09)*[2,3]**

[1]Values are linear regression coefficients (95% confidence interval) and reflect the differences in fetal growth compared to the reference group. Continuous analyses reflect the differences in head circumference, femur length, estimated fetal weight during second and third trimester of pregnancy and head circumference, length and weight at birth per 1 SDS increase in maternal 25(OH)D. Multivariable model is adjusted for maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, vitamin supplements, folic acid, iron, calcium, zinc and energy intake during pregnancy, education, ethnicity, gestational hypertensive disorders, gestational diabetes, parity and season when blood samples were drawn, and the presence of anorexia). [2]*P value* <0.05. Abbreviations: SDS standard deviation scores. [3]Also significant after applying Bonferroni correction (*P value* <0.025).

Supplementary Table 2.3.4. Associations of maternal second trimester 25(OH)D concentrations in clinical cut-off groups with fetal growth patterns (N=7,098)[1]

	Head circumference SDS (95% Confidence Interval)		
25(OH)D concentrations	**2nd trimester N=6,967**	**3rd trimester N=6,800**	**Birth N=3,681**
<25.0 nmol/L (N=1,828)	-0.02 (-0.11, 0.07)	-0.07 (-0.16, 0.02)	**-0.13 (-0.27, 0)**
25.0 to 49.9 nmol/L (N=1,893)	-0.03 (-0.11, 0.04)	-0.02 (-0.09, 0.05)	-0.08 (-0.19, 0.03)
50.0 to 74.9 nmol/L (N=1,604)	0.01 (-0.06, 0.08)	-0.01 (-0.08, 0.06)	-0.04 (-0.14, 0.07)
≥75.0 nmol/L (N=1,693)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.01 (-0.02, 0.04)*	*0.02 (-0.01, 0.05)*	***0.05 (0.01, 0.10)*[2,3]**
	Length SDS (95% Confidence Interval)		
25(OH)D concentrations	**2nd trimester N=6,988**	**3rd trimester N=6,851**	**Birth N=4,533**
<25.0 nmol/L (N=1,828)	-0.01 (-0.09, 0.08)	0.02 (-0.07, 0.11)	**-0.18 (-0.30, -0.05)[2,3]**
25.0 to 49.9 nmol/L (N=1,893)	-0.01 (-0.09, 0.06)	0.04 (-0.04, 0.11)	-0.08 (-0.19, 0.02)
50.0 to 74.9 nmol/L (N=1,604)	0 (-0.07, 0.07)	0.06 (-0.01, 0.12)	-0.08 (-0.18, 0.02)
≥75.0 nmol/L (N=1,693)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.01 (-0.02, 0.04)*	*-0.01 (-0.04, 0.02)*	***0.06 (0.02, 0.11)*[2,3]**
	Weight SDS (95% Confidence Interval)		
25(OH)D concentrations	**2nd trimester N=6,952**	**3rd trimester N=6,828**	**Birth N=7,043**
<25.0 nmol/L (N=1,828)	-0.07 (-0.16, 0.01)	-0.06 (-0.15, 0.03)	**-0.16 (-0.25, -0.08)[2,3]**
25.0 to 49.9 nmol/L (N=1,893)	-0.03 (-0.10, 0.05)	0.02 (-0.06, 0.09)	**-0.07 (-0.14, 0)**
50.0 to 74.9 nmol/L (N=1,604)	-0.01 (-0.08, 0.06)	0.05 (-0.02, 0.12)	-0.05 (-0.11, 0.02)
≥75.0 nmol/L (N=1,693)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.02 (-0.01, 0.06)*	*0.01 (-0.02, 0.04)*	***0.06 (0.03, 0.09)*[2,3]**

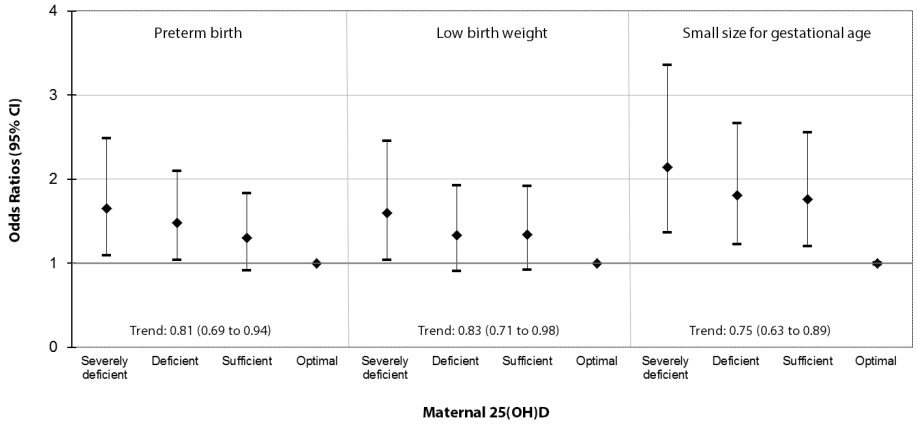
[1]Values are linear regression coefficients (95% confidence interval) and reflect the differences in fetal growth compared to the reference group. Continuous analyses reflect the differences in head circumference, femur length, estimated fetal weight during second and third trimester of pregnancy and head circumference, length and weight at birth per 1 SDS increase in maternal 25(OH)D. Multivariable model is adjusted for maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and vitamin supplements, energy, iron, calcium and zinc dietary intake during pregnancy, education, ethnicity, gestational hypertensive disorders, gestational diabetes, parity and the presence of anorexia and season when blood samples were drawn). [2]*P value* <0.05. Abbreviations: SDS standard deviation scores. [3]Also significant after applying Bonferroni correction (*P value* <0.025).

Supplementary Table 2.3.5. Associations of maternal second trimester 25(OH)D concentrations with fetal growth outcomes among Europeans only (N=4,069)[1]

	Head circumference SDS (95% Confidence Interval)		
25(OH)D concentrations	**2nd trimester**	**3rd trimester**	**Birth**
Quartile 1 (N=328)	-0.06 (-0.20, 0.08)	-0.01 (-0.14, 0.13)	-0.16 (-0.38, 0.06)
Quartile 2 (N=940)	-0.01 (-0.10, 0.08)	0.01 (-0.08, 0.09)	-0.04 (-0.17, 0.09)
Quartile 3 (N=1,260)	0.02 (-0.06, 0.10)	-0.02 (-0.09, 0.06)	-0.01 (-0.12, 0.01)
Quartile 4 (N=1,543)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.01 (-0.03, 0.05)*	*0.01 (-0.03, 0.05)*	*0.04 (-0.01, 0.10)*
	Length SDS (95% Confidence Interval)		
25(OH)D concentrations	**2nd trimester**	**3rd trimester**	**Birth**
Quartile 1 (N=328)	-0.08 (-0.22, 0.05)	-0.01 (-0.15, 0.14)	-0.17 (-0.37, 0.03)
Quartile 2 (N=940)	0.03 (-0.06, 0.11)	0.06 (-0.02, 0.15)	-0.08 (-0.21, 0.04)
Quartile 3 (N=1,260)	0.03 (-0.05, 0.10)	0.05 (-0.03, 0.13)	-0.09 (-0.19, 0.02)
Quartile 4 (N=1,543)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.01 (-0.03, 0.04)*	*-0.02 (-0.06, 0.02)*	***0.06 (0.01, 0.11)***[2]
	Weight SDS (95% Confidence Interval)		
25(OH)D concentrations	**2nd trimester**	**3rd trimester**	**Birth**
Quartile 1 (N=328)	**-0.14 (-0.28, -0.01)***	-0.08 (-0.22, 0.06)	**-0.17 (-0.32, -0.03)**[2,3]
Quartile 2 (N=940)	0.04 (-0.05, 0.13)	0.05 (-0.04, 0.14)	-0.06 (-0.14, 0.03)
Quartile 3 (N=1,260)	0.02 (-0.06, 0.10)	0.06 (-0.01, 0.14)	-0.04 (-0.11, 0.04)
Quartile 4 (N=1,543)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.01 (-0.03, 0.05)*	*0 (-0.04, 0.04)*	***0.05 (0.01, 0.10)***[2,3]

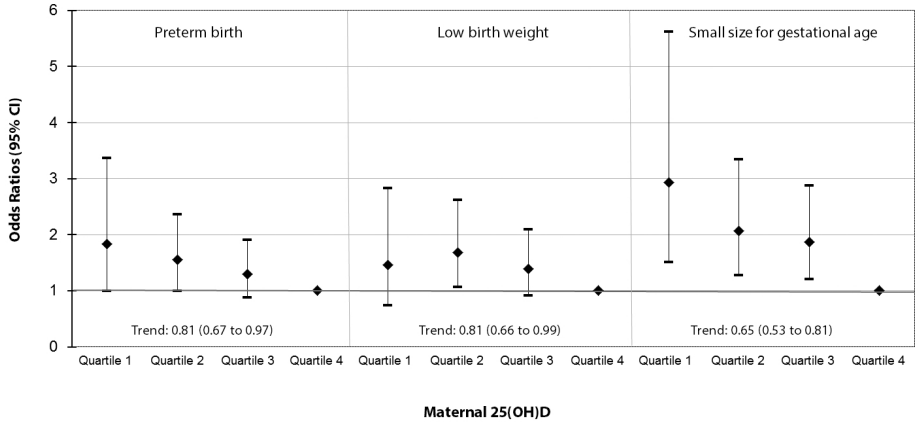
[1]Values are linear regression coefficients (95% confidence interval) and reflect the differences in fetal growth measures compared to the reference group. Continuous analyses reflect the differences in head circumference, femur length, estimated fetal weight during second and third trimester of pregnancy and head circumference, length and weight at birth per 1 SDS increase in maternal 25(OH)D. Multivariable model is adjusted for maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and vitamin supplements, energy, iron, calcium and zinc dietary intake during pregnancy, education, gestational hypertensive disorders, gestational diabetes, parity and the presence of anorexia and season when blood samples were drawn.[2]*P value* <0.05. [3]Also significant after applying Bonferroni correction (*P value* <0.025). Abbreviations: SDS standard deviation scores.

Supplementary Figure 2.3.2. Associations of maternal second trimester 25(OH)D concentrations, in cut-off groups with the risks of adverse birth outcomes (N=7,098)[1]



[1]Values are logistic regression coefficients (95% confidence intervals) and reflect the risk of adverse birth outcomes compared to the reference group. Continuous analyses reflect the risks of being preterm, having a low birth weight or being small-size for gestational age at birth per 1 SDS increase in maternal 25(OH)D. Multivariable model is adjusted for maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and vitamin supplements, energy, iron, calcium and zinc dietary intake during pregnancy, education, ethnicity, gestational hypertensive disorders, gestational diabetes, parity and the presence of anorexia and season when blood samples were drawn).

Supplementary Figure 2.3.3. Associations of maternal second trimester 25(OH) concentrations with the risks of adverse birth outcomes among Europeans only (N=4,069) [1]



[1]Values are logistic regression coefficients (95% confidence intervals) and reflect the risks of adverse birth outcomes compared to the reference group. Continuous analyses reflect the risk of being preterm, having a low birth weight or being small-size for gestational age at birth per 1 SDS increase in maternal 25(OH)D. Multivariable model is adjusted for maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and vitamin supplements, energy, iron, calcium and zinc dietary intake during pregnancy, education, gestational hypertensive disorders, gestational diabetes, parity, the presence of anorexia and season when blood samples were drawn).

Supplementary Table 2.3.6. Associations of adverse birth outcomes with cord blood 25(OH)D concentrations among Europeans only (N=2,550)[1]

Birth characteristics	N	Cord blood 25(OH)D nmol/L
Gestational age	**2,553**	
<37.0 weeks	73	0.03 (-0.25, 0.32)
37.0-41.9 weeks	2,302	Reference
≥42 weeks	178	-0.17 (-0.34, 0.01)
Trend		*-0.03 (-0.06, 0.01)*
Birth weight	**2,552**	
<2,000 grams	9	-0.60 (-1.46, 0.26)
2,000-2,499 g	45	0.22 (-0.12, 0.57)
2,500-2,999 g	318	-0.10 (-0.26, 0.05)
3,000-3,499 g	864	Reference
3,500-3,999 g	893	-0.02 (-0.13, 0.09)
4,000-4,499 g	354	-0.04 (-0.18, 0.10)
≥4,500 grams	69	-0.09 (-0.38, 0.19)
Trend		*0.03 (-0.02, 0.09)*
Birth weight for gestational age	**2,553**	
Small	79	-0.23 (-0.49, 0.04)
Normal	2,313	Reference
Large	161	-0.04 (-0.23, 0.15)
Trend		*0.03 (-0.02, 0.07)*

[1]Values are linear regression coefficients (95% confidence interval) and reflect the change in standard deviation (SDS) of cord blood 25(OH)D for each birth weight or gestational age group, compared to the reference group. Trend estimates represent the effect estimates for the continuous associations per SDS change in birth characteristic. Multivariable model is adjusted for fetal sex, maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and vitamin supplements, energy, iron, calcium and zinc dietary intake during pregnancy, education, gestational hypertensive disorders, gestational diabetes, parity and the presence of anorexia).

Chapter 2.4

Associations of maternal and fetal vitamin D status with childhood body composition and cardiovascular outcomes

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ABSTRACT

Background: Maternal vitamin D deficiency during pregnancy may have persistent adverse effects on childhood growth and development. We examined whether 25-hydroxyvitamin D (25(OH)D) concentrations during pregnancy and at birth are associated with childhood body composition and cardiovascular outcomes.

Methods: This study was embedded in a population-based prospective cohort in Rotterdam, the Netherlands among 4,903 mothers and their offspring. We measured 25(OH)D concentrations at a median gestational age of 20.4 weeks (95% range 18.5–23.4 weeks) and at birth (40.1 weeks (95% range 35.8–42.3 weeks)). We categorized 25(OH)D concentrations into severely deficient (<25.0 nmol/L); deficient (25.0 to 49.9 nmol/L); sufficient (50.0 to 74.9 nmol/L) and optimal (≥75.0 nmol/L). At 6 years, we measured childhood body mass index (BMI), fat and lean mass by Dual-energy X-ray Absorptiometry, blood pressure, and cholesterol, triglycerides and insulin concentrations.

Results: Compared to children from mothers with optimal 25(OH)D concentrations, those of severely deficient vitamin D mothers had a 0.11 standard deviation score (SDS) (95% Confidence Interval (CI) (0.02, 0.20)) higher fat mass percentage and a 0.12 SDS (95% CI (-0.21, -0.03)) lower lean mass percentage. These associations were independent of child current vitamin D status. Maternal and cord blood 25(OH)D concentrations were not associated with cardiovascular risk factors in childhood.

Conclusion: Severe maternal 25(OH)D deficiency during pregnancy is associated with an adverse childhood body fat profile, but not with childhood cardiovascular risk factors. Further studies are needed to replicate our findings, to examine the underlying mechanisms and to explore causality and the potential for public health interventions.

INTRODUCTION

Vitamin D is essential for fetal development because of its significant role during proliferation, differentiation and maturation processes of cells, including adipose tissues and muscle cells.[1–4] We have previously shown that suboptimal concentrations of maternal vitamin D are associated with low birth weight and small size for gestational age,[5] which are known to be associated with cardiovascular risk factors in the offspring.[6, 7] Studies in adults suggest that vitamin D plays an important role in cardiovascular protection and body composition profile.[8–10] Furthermore, cross-sectional studies in children reported associations of lower vitamin D status with higher adiposity measures and cardiovascular risk factors such as blood pressure, plasma lipids and insulin concentrations.[11–13] The relations of vitamin D with calcium are well known. It has previously been reported that calcium supplementation during pregnancy has long term effects on childhood blood pressure.[14] Thus far, only few studies have explored the associations of circulating fetal 25(OH)D concentrations with childhood adiposity, body composition and cardiovascular risk factors, with inconsistent results.[1, 15–18] These inconsistent findings may be due to differences between study populations. The small sample sizes of the previous studies could have limited their ability to detect associations.[1, 16, 17]

Therefore, for the current study, we hypothesized that adverse exposure to suboptimal 25(OH)D concentrations during critical periods of fetal organ development affects childhood adiposity and cardiovascular health. We explored in a population-based prospective cohort study among 4,903 mother and children pairs the associations of 25(OH)D concentrations during mid-pregnancy and in cord blood with body composition and cardiovascular risk factors at school-age. We also explored whether any association was explained by child's current 25(OH)D concentrations.

METHODS

Design and study population

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[19] All children were born between April 2002 and January 2006. Enrolment in the study was aimed at early pregnancy, but was allowed until the birth of the child. The study protocol was approved by the local Medical Ethical Committee. Written consent was obtained for all children. Mid-pregnancy 25(OH)D concentrations were measured in 7,176 mothers. For the present study, we excluded pregnancies leading to twin births (N=78) and children with congenital heart abnormalities (N=21). Among 7,077 singletons available for follow up measurements, 2,174 did not visit the research centre at the age of 6 years. Thus, the cohort for analysis comprised of 4,903 mothers and children with vitamin D measurements and childhood outcomes available. (**Supplementary Figure 2.4.1.** Flowchart).

Maternal and cord 25(OH)D blood concentrations

As previously described, maternal venous blood samples were collected in mid-pregnancy (median 20.4 weeks of gestation, 95% range 18.5–23.4 weeks), whereas umbilical cord blood samples were collected at birth (median 40.1 weeks of gestation, 95% range 35.8–42.3 weeks).[19] 25(OH)D concentrations were measured at the Eyles Laboratory at the Queensland Brain Institute, University of Queensland, Australia.[5, 20] Total 25(OH)D was calculated as the sum of $25(OH)D_2$ and $25(OH)D_3$ measured in plasma.[21] Samples were quantified using isotope dilution liquid chromatography-tandem mass spectrometry. Detailed information on 25(OH)D measurements has been described previously.[5] According to current recommendations we categorized vitamin D status into severely deficient: <25.0 nmol/L (<10.0 mg/L); deficient: 25.0 to 49.9 nmol/L (10.0 to 19.9 mg/L); sufficient: 50.0 to 74.9 nmol/L (20.0 to 29.9 mg/L); optimal ≥75.0 nmol/L (≥30.0 mg/L).[5, 22, 23]

Body composition and cardiovascular outcomes

Children's anthropometrics and body composition were measured at a median age of 6.0 years (95% range 5.7 to 8.0) by well-trained staff in our research center.[24] Height (m) was determined in standing position to the nearest millimeter without shoes using a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Weight (kg) was measured using a mechanical personal scale (SECA, Almere, The Netherlands) and body mass index (BMI) (kg/m^2) was calculated. We performed whole body Dual-energy X-ray Absorptiometry (DXA) scans (iDXA, GE-Lunar, 2008, Madison, WI, USA), that analyzed fat, and lean mass. We calculated fat mass percentage as (fat mass (kg) /weight (kg)) and lean mass percentage as (lean mass (kg) / weight (kg)).

Blood pressure was measured at the right brachial artery four times with one-minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus (Paramus, NJ, US).[25] We calculated the mean value for systolic and diastolic blood pressure using the last three blood pressure measurements of each participant. Thirty-minutes fasting blood samples were collected to measure total-cholesterol, triglycerides, and insulin concentrations, using Cobas 8000 analyzer (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.

Covariates

We used questionnaires at enrollment in the study (median 13.5 weeks of gestation) to collect information about maternal age, ethnicity, educational level, parity, and on smoking and folic acid supplementation during pregnancy.[24] Maternal energy and calcium dietary intake during pregnancy were measured at enrollment with a validated semi-quantitative food frequency questionnaire.[26] Ethnicity and educational level were defined according to the classification of Statistics Netherlands.[27, 28] Maternal height and weight were self-reported and pre-pregnancy body mass index was calculated (kg/m^2). The date of blood sampling was categorized into spring, summer, fall, and winter based on the Dutch standard seasons. Infant sex, gestational age and weight at birth were obtained from midwifes, medical records

and hospital registries. Information on breastfeeding was collected using postnatal questionnaires.[29] At the age of 6 years, child participation at sports was reported and 25(OH)D concentrations were measured in a subgroup of 3,068 subjects.[30]

Statistical analysis

We performed a non-response analysis by comparing subject characteristics between children with and without follow-up body composition and cardiovascular outcomes using T-tests, Chi-square tests and Mann-Whitney tests. We created standard deviations scores (SDS) for all outcomes to enable comparison between effect estimates. We used multivariable linear regression models to assess the associations of maternal and cord blood 25(OH)D concentrations with childhood BMI, fat mass percentage, lean mass percentage, systolic and diastolic blood pressure, and total-cholesterol, triglycerides, and insulin concentrations. We log-transformed the not-normally distributed outcomes, childhood triglycerides and insulin concentrations. Vitamin D concentrations were analyzed both continuously per standard deviation (SD)- increase and using clinical cut-offs.[5, 22, 23] The regression models were first adjusted for child's sex, child's age at outcome measurements and maternal ethnicity (basic models), and subsequently additionally for maternal age, education, pre-pregnancy body mass index, parity, smoking, folic acid supplement use and energy and dietary calcium intake during pregnancy, and season when blood samples were drawn, and for child's birth weight, gestational age at birth, breastfeeding in early life and playing sports at the age of 6 years (adjusted model). The models on cardiovascular outcomes were additionally adjusted for childhood BMI. The covariates were included in the models based on their associations with adiposity and cardiovascular outcomes in previous studies,[15, 31] or a change in effect estimates of >10%. To explore if childhood 25(OH)D status explained the associations between maternal 25(OH)D and childhood body composition measures, we performed a sensitivity analysis in which we additionally adjusted for child 25(OH)D in the subgroup of N=3,068 children with data on 25(OH)D concentrations available (childhood vitamin D model). Because of the correlations between different outcomes we did not apply adjustment for multiple testing in the main analyses. However, as sensitivity analysis we also present statistical significance after taking account for three groups of outcomes (body composition, blood pressure, blood concentrations) (*P value* <0.017 (0.05/3)). Ethnicity is strongly associated with 25(OH)D concentrations, therefore, we first adjusted the regression models for maternal ethnicity and second, we restricted the analyses to Europeans only, the largest ethnic subgroup in our cohort.[20] Since the interactions of maternal 25(OH)D with child sex were not significant, we did not stratify our analyses on child sex. To diminish potential bias associated with attrition, missing values of covariates (less than 23.5%), were multiple imputed by generating 5 independent datasets using the Markov Chain Monte Carlo (MCMC) method. The multiple imputation procedure was based on the correlation between each variable with missing values and the other subject characteristics.[32, 33] Subjects characteristics before and after imputation are shown in **Supplementary Table 2.4.1**. Statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Subject characteristics

Table 2.4.1 shows the subject characteristics. Results from the non-response analysis showed that as compared to children who did not have follow up measurements at the age of 6 years, those who did have these measurements had mothers who were on average higher educated and had higher 25(OH)D concentrations during pregnancy (**Supplementary Table 2.4.2**). The correlation coefficients of the investigated variables are given in **Supplementary Table 2.4.3**. The correlation coefficient between mid-pregnancy maternal 25(OH)D concentrations and cord blood 25(OH)D concentrations was r = 0.61.

Table 2.4.1. Subject characteristics (N=4,903)

Characteristics	
Maternal characteristics	
Age (y)	30.4 (5.03)
Pre-pregnancy body mass index (kg/m^2)	22.7 (18.1, 34.8)
Nulliparous (%)	57.8
Education level (%)	
- No higher education	54.9
- Higher education	45.1
Ethnicity (%)	
- European	60.6
- Cape Verdean	4.5
- Dutch Antillean	2.7
- Moroccan	5.9
- Turkish	8.9
- Surinamese	8.2
- Other	9.2
Smoking during pregnancy (%)	
- Never	74.2
- Until pregnancy was known	9.4
- Continued	16.4
Folic acid supplement use (%)	
- No	26.8
- Start in the first 10 weeks	31.6
- Start periconceptional	41.6
Maternal energy intake (kcal)	2,049 (490)
Maternal dietary calcium intake (mg)	1,106 (329)
Maternal blood concentrations of 25(OH)D (nmol/L)	50.4 (7.5, 122.5)
Season when blood sample was taken (%)	
- Spring	28.6
- Summer	22.6
- Autumn	25.1
- Winter	23.7

Table 2.4.1. Subject characteristics (N=4,903) (continued)

Characteristics	
Infant characteristics	
Girls (%)	50.4
Gestational age at birth (wk)	40.1 (35.8, 42.3)
Birth weight (g)	3,431 (554)
Breastfeeding in the first 4 months (%)	
- Ever	92.3
- Never	7.7
25(OH)D concentration in cord blood at birth (nmol/L)	28.8 (5.0, 81.7)
Season when cord blood sample was taken (%)	
- Spring	27.6
- Summer	26.6
- Autumn	22.5
- Winter	23.3
Child characteristics at 6 years' visit	
Age (y)	6.0 (5.7, 7.8)
Height (cm)	119.4 (6.0)
Weight (kg)	22.6 (17.6, 33.8)
Playing sports, yes (%)	43.3
Childhood blood concentrations of 25(OH)D (nmol/L)	64.0 (18.0, 133.0)
Body composition	
- Body mass index (kg/m^2)	15.9 (13.6, 21.2)
- Fat mass percentage	24.9 (5.7)
- Lean mass percentage	71.5 (5.4)
Cardiovascular risk factors	
- Systolic blood pressure (mmHg)	102.7 (8.3)
- Diastolic blood pressure (mmHg)	60.7 (6.9)
- Total-cholesterol (mmol/L)	4.2 (0.6)
- Triglycerides (mmol/L)	0.95 (0.4, 2.3)
- Insulin (pmol/L)	114.2 (17.0, 397.7)

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.

Maternal and cord 25(OH)D concentrations and childhood body composition

Table 2.4.2 shows the associations of maternal and cord blood 25(OH)D concentrations and childhood body composition measures. As compared to children of mothers who had optimal 25(OH)D concentrations, those of mothers who were severely vitamin D deficient had a 0.11 standard deviation score (SDS) (95% Confidence Interval (CI) (0.02, 0.20)) higher fat mass percentage and a 0.12 SDS (95% CI (-0.21, -0.03)) lower lean mass percentage. The associations of maternal 25(OH)D status with childhood lean mass percentage remained significant after considering multiple testing and after additional adjustment for childhood vitamin D status. When we examined the observed associations in the subgroup of children with vitamin D measurements at the age of 6 years the general pattern of findings persisted. Children of mothers who were vitamin D severely deficient had a 0.11 SDS (95% CI (0, 0.22)) higher fat

Table 2.4.2. Associations of maternal 25(OH)D concentrations and body composition measures at the age of 6 years (N=4,903)

Fetal 25(OH)D concentrations	**Difference in outcome measure (95% Confidence Interval)**				
Maternal 25(OH)D concentrations	**Body mass index (N=4,898)**	**Fat mass percentage (N=4,805)**	**Fat mass percentage (N=3,068)**	**Lean mass percentage (N=4,805)**	**Lean mass percentage (N=3,068)**
	Adjusted model	*Adjusted model*	*Childhood vitamin D model*	*Adjusted model*	*Childhood vitamin D model*
<25.0 nmol/L (N=1,142)	0.05 (-0.04, 0.15)	**0.11 (0.02, 0.20)***	0.11 (-0.01, 0.22)	**-0.12 (-0.21, -0.03)*†**	**-0.11 (-0.23, 0)***
25.0 to 49.9 nmol/L (N=1,281)	0.03 (-0.05, 0.10)	0.04 (-0.04, 0.11)	0.01 (-0.08, 0.10)	-0.04 (-0.11, 0.03)	-0.01 (-0.10, 0.08)
50.0 to 74.9 nmol/L (N=1,195)	0 (-0.07, 0.07)	0.02 (-0.05, 0.09)	0 (-0.08, 0.09)	-0.02 (-0.09, 0.05)	-0.01 (-0.09, 0.08)
≥75.0 nmol/L (N=1,285)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.01 (-0.05, 0.02)*	*-0.03 (-0.06, 0.01)*	*-0.03 (-0.07, 0.01)*	*0.03 (0, 0.06)*	*0.03 (-0.01, 0.07)*
Cord blood 25(OH)D concentrations	**Body mass index (N=3,048)**	**Fat mass percentage (N=2,998)**	**Fat mass percentage (N=1,907)**	**Lean mass percentage (N=2,998)**	**Lean mass percentage (N=1,907)**
	Adjusted model	*Adjusted model*	*Childhood vitamin D model*	*Adjusted model*	*Childhood vitamin D model*
<25.0 nmol/L (N=665)	0.09 (-0.08, 0.27)	0.02 (-0.15, 0.18)	0.11 (-0.09, 0.33)	-0.02 (-0.18, 0.14)	-0.12 (-0.33, 0.08)
25.0 to 49.9 nmol/L (N=818)	0.14 (-0.03, 0.31)	0.03 (-0.12, 0.19)	0.09 (-0.10, 0.28)	-0.03 (-0.19, 0.12)	-0.09 (-0.28, 0.11)
50.0 to 74.9 nmol/L (N=780)	0.03 (-0.14, 0.20)	-0.03 (-0.19, 0.13)	0.04 (-0.16, 0.24)	0.03 (-0.13, 0.19)	-0.04 (-0.24, 0.16)
≥75.0 nmol/L (N=790)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.03 (-0.07, 0.02)*	*-0.02 (-0.06, 0.02)*	*-0.03(-0.08, 0.02)*	*0.02 (-0.01, 0.06)*	*0.03 (-0.02, 0.08)*

Values are linear regression coefficients (95% confidence interval). Adjusted model is adjusted for maternal characteristics (age, ethnicity, body mass index before pregnancy, parity, education, smoking, folic acid supplements, dietary calcium and energy intake during pregnancy and season of blood sampling), and child characteristics (sex, birthweight, gestational age at birth, breast-feeding, and age and playing sports at the age of 6y). Childhood vitamin D model, is adjusted model additionally adjusted for child's current 25(OH)D concentrations. *p <0.05. Continuously = Maternal and cord blood vitamin D concentrations analyzed per 1 standard deviation in 25(OH)D. †Also significant after applying Bonferroni correction (p <0.017).

Table 2.4.3. Associations of maternal 25(OH)D concentrations during pregnancy with cardiovascular outcomes at the age of 6 years (N=4,903)

Fetal 25(OH)D concentrations	**Difference in outcome measure (SDS) (95% Confidence Interval)**				
Maternal 25(OH)D concentrations	**Systolic blood pressure (N=4,550)**	**Diastolic blood pressure (N=4,550)**	**Total cholesterol (N=3,281)**	**Triglycerides (N=3,270)**	**Insulin (N=3,256)**
<25.0 nmol/L (N=1,142)	0 (-0.10, 0.11)	-0.01 (-0.12, 0.10)	0.03 (-0.11, 0.16)	0 (-0.14, 0.13)	-0.08 (-0.21, 0.06)
25.0 to 49.9 nmol/L (N=1,281)	0.02 (-0.07, 0.10)	0.03 (-0.06, 0.12)	0.03 (-0.07, 0.13)	0.02 (-0.08, 0.12)	-0.01 (-0.12, 0.09)
50.0 to 74.9 nmol/L (N=1,195)	0 (-0.08, 0.08)	-0.02 (-0.10, 0.07)	0.07 (-0.03, 0.17)	0.04 (-0.06, 0.14)	**-0.10 (-0.20, 0)***
≥75.0 nmol/L (N=1,285)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0 (-0.03, 0.04)*	*0 (-0.04, 0.04)*	*0 (-0.05, 0.04)*	*-0.01 (-0.05, 0.04)*	*0.02 (-0.03, 0.06)*
Cord blood 25(OH)D concentrations	**Systolic blood pressure (N=2,847)**	**Diastolic blood pressure (N=2,847)**	**Total cholesterol (N=2,042)**	**Triglycerides (N=2,038)**	**Insulin (N=2,201)**
<25.0 nmol/L (N=665)	-0.06 (-0.25, 0.14)	-0.06 (-0.27, 0.14)	0.09 (-0.15, 0.34)	0.10 (-0.14, 0.34)	-0.02 (-0.26, 0.22)
25.0 to 49.9 nmol/L (N=818)	-0.01 (-0.20, 0.17)	-0.07 (-0.26, 0.12)	0.20 (-0.04, 0.43)	0.09 (-0.14, 0.32)	0.06 (-0.17, 0.28)
50.0 to 74.9 nmol/L (N=780)	0.01 (-0.18, 0.21)	-0.03 (-0.23, 0.17)	0.10 (-0.14, 0.34)	0.09 (-0.15, 0.32)	0.08 (-0.16, 0.31)
≥75.0 nmol/L (N=790)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.01 (-0.03, 0.06)*	*0.01 (-0.04, 0.06)*	*-0.01 (-0.07, 0.05)*	*-0.01 (-0.07, 0.05)*	*0.03 (-0.03, 0.08)*

Values are linear regression coefficients (95% confidence interval). Adjusted model is adjusted for maternal characteristics (age, ethnicity, body mass index before pregnancy, parity, education, smoking, folic acid supplements, dietary calcium and energy intake during pregnancy, and season of blood sampling), and child characteristics (sex, birthweight, gestational age at birth, breastfeeding, and age, body mass index and playing sports at the age of 6y). *p <0.05. Continuously = Maternal and cord blood vitamin D concentrations analyzed per 1 standard deviation in 25(OH)D.

mass percentage and a 0.11 SDS (95% CI (-0.23, 0)) lower lean mass percentage, as compared to children of mothers who had optimal 25(OH)D concentrations. Cord blood 25(OH)D concentrations were not associated with child body composition. **Supplementary Table 2.4.4** shows the results from the basic regression models for the associations of maternal and cord blood 25(OH)D concentrations and childhood body composition measures. We observed tendencies for similar effect estimates when we restricted the analyses to Europeans only (N=2,974), although the observed associations on fat mass percentage and lean mass percentage did not reached statistical significance due to smaller sample sizes (**Supplementary Table 2.4.5**).

Maternal and cord 25(OH)D concentrations and childhood cardiovascular risk factors

Table 2.4.3 shows that maternal and cord blood 25(OH)D concentrations were not associated with childhood blood pressure, total cholesterol, triglycerides or insulin concentrations. The results from the basic models and the analysis restricted to Europeans only (N=2,974) are given in **Supplementary Tables 2.4.6 & 2.4.7**, respectively and showed similar results.

DISCUSSION

In this population-based prospective cohort study, we observed that severely deficient maternal 25(OH)D concentrations during mid- pregnancy, but not at birth, tended to be associated with higher fat mass and lower lean mass at school-age. These associations remained similar after adjustment for childhood 25(OH)D concentrations. Maternal and cord blood 25(OH)D concentrations were not associated with childhood cardiovascular outcomes.

Interpretation and comparison with previous studies

An accumulating body of evidence suggest that suboptimal vitamin D levels are common and related to the risk of cardiovascular disease.[34, 35] Adults studies suggest that low 25(OH)D concentrations are associated with higher BMI and higher fat mass percentage.[9, 33] In line with these results, studies in animals show that vitamin D influences development and differentiation of adipocytes and muscle cells.[3, 4] Also, previous childhood studies suggest a potential role of 25(OH)D concentrations on body composition and cardiovascular outcomes.[11–13] Vitamin D is reported to be inversely associated with the development of adiposity in school-aged children.[13] Moreover, childhood vitamin D status was negatively associated with blood pressure and plasma lipids.[11, 12]

Fetal life may be a critical period for the effects of vitamin D deficiency because of the increased need and rapid fetal development. We have previously shown an association of maternal vitamin D with fetal growth patterns and birth outcomes.[5] For the current study, we hypothesized that suboptimal fetal vitamin D concentrations may increase the risk of adiposity and cardiovascular factors in the offspring. Results from previous birth cohort studies suggest a possible programming effect of maternal 25(OH)D concentrations on childhood body

composition.[16, 17] In a study among 977 women and their offspring it was observed that lower maternal 25(OH)D concentrations during pregnancy were associated with higher fat mass in 4 and 6 years old children.[16] Another study among 568 Indian women and their children, reported that low maternal 25(OH)D concentrations were associated with a lower lean mass in the offspring.[17] In line with these findings, our results suggest that low maternal 25(OH)D concentrations during mid- pregnancy are associated with a higher fat mass percentage and lower lean mass percentage in the offspring. We observed that the associations of maternal 25(OH)D concentrations during pregnancy with childhood fat and lean mass percentage were independent of birth weight and of childhood 25(OH)D concentrations. We did not observe any associations of cord blood 25(OH)D concentrations with childhood body composition. Furthermore, we observed tendencies for similar associations when we restricted the analyses to Europeans only. However, the associations were not significant, probably due to smaller numbers. It is previously shown that body composition phenotypes track and are associated with poorer outcomes in later life.[37, 38] Therefore, the observed subclinical differences in body composition in childhood may be related to adverse outcomes in later life.

Only few studies have explored the association of fetal 25(OH)D concentrations with childhood cardiovascular risk factors.[1, 15, 17] In a recent study among 4,109 mothers and children at the ages of 9.9 years and 15.4 years, a weak inverse association was observed of maternal 25(OH)D concentrations with systolic blood pressure at the age of 9 years.[15] However, this association was not present at the age of 15.4 years.[15] We did not observe any association between maternal 25(OH)D concentrations and blood pressure in 6-year-old children. Also, we did not observe any association of maternal or cord blood 25(OH)D concentrations with childhood total- cholesterol, triglycerides, and insulin concentrations. A small study in India, observed that children of vitamin D deficient mothers had higher fasting insulin resistance.[17] It may be that the different results of our findings with this study are explained by ethnic differences. When we restricted the analyses to Europeans only, we observed similar results as in the full group. Unfortunately, we did not have enough numbers in the other ethnic subgroups, to perform ethnic specific analyses. Our results do not support possible in utero effects of 25(OH)D concentrations on childhood cardiovascular risk factors.

The mechanisms by which maternal 25(OH)D concentrations during pregnancy can affect offspring body composition are poorly understood. Animal experiments relate maternal 25(OH)D concentrations to fetal muscle development. A study in rats which tracked ^{3}H-labelled vitamin D injected into pregnant rats showed that 25OHD was transferred to the fetus and stored in fetal muscle tissue.[39] 1,25-dihydroxycholecalciferol (1,25(OH)D) regulates adipocyte 11β-hydroxysteroid dehydrogenase type 1 activity, which generates active cortisol from inactive cortisone. The expression and cortisol production in human adipocytes, suggests a potential role for (1,25(OH)D) in fat mass.[40] Furthermore, vitamin D can stop the expression of an important adipogenesis regulator, peroxisome proliferator-activated receptor-gamma.[3, 41]

Although, the observed effect estimates are small and without direct individual clinical consequence, the results of this study suggest that maternal 25(OH)D concentrations are associated with offspring fat mass percentage and lean mass percentage. Suboptimal vitamin D levels in pregnant women may have persistent effects on their offspring body composition.

An adverse childhood body fat profile may increase the risk of cardiovascular and metabolic diseases in later life. With the design of our study, we are not able to establish causality for the observed associations and further studies are required. However, our findings suggest that vitamin D supplementations during pregnancy may need to be targeted at women who are vitamin D severely deficient. In the Netherlands, pregnant women are advised to use vitamin D supplements (10 µg/day). Therefore, our results support population-strategies to improve vitamin D concentrations in pregnant women to optimize offspring growth and development.

Strength and limitations

A major strength of our study the prospective design from fetal life onwards within a population-based cohort. This study is among the largest that examined the association of fetal vitamin D status with body composition and cardiovascular outcomes in a multi-ethnic sample of school-age children. We measured 25(OH)D concentrations in mid- pregnancy and cord blood at birth, assessing different critical periods. 25(OH)D concentrations are the most widely used indicator of vitamin D status.[42] Next to BMI, we also measured fat mass and lean mass using DXA.

Due to the young age of the children, it was not possible to obtain fasting blood samples, which may have led to an underestimation of the associations. Of the singleton live born children 58% participated in the follow up measurements. Mothers of the children who were lost to follow up had on average lower 25(OH)D concentrations and were on average lower educated, suggesting that our study population had a selection towards a healthier population. Finally, although we performed adjustment for many potential maternal and childhood confounders, residual confounding for the observed associations might be present.

CONCLUSION

Results from this population-based prospective cohort study suggest that severe vitamin D deficiency during mid-pregnancy may influence childhood body composition. Further studies are needed to examine the causality of these associations and to identify the long term-clinical consequences.

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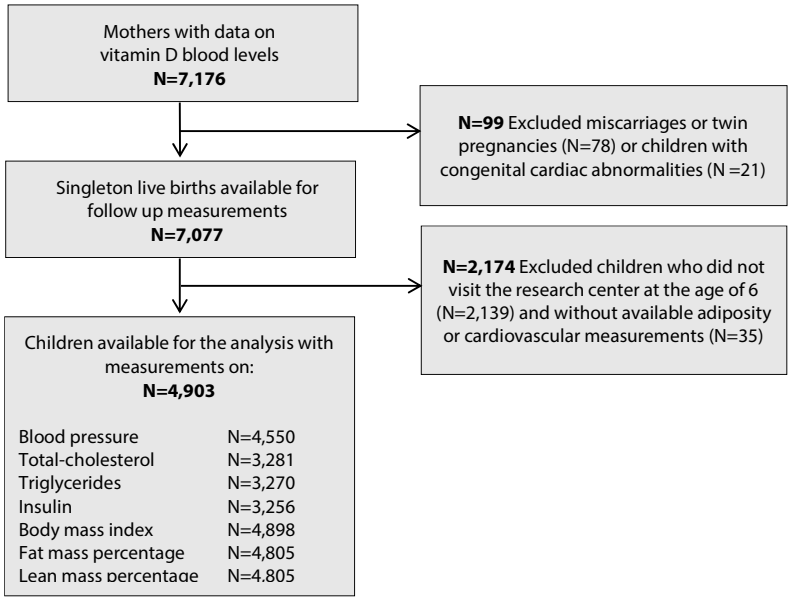
Supplementary Figure 2.4.1. Flowchart of the study participants



Supplementary Table 2.4.1. Subject characteristics (N=4,903)

Subject characteristics	Imputed	Original
Maternal characteristics		
Age (y)	30.4 (5.03)	30.4 (5.03)
Body mass index at enrolment (kg/m^2)	22.7 (18.1, 34.8)	22.7 (18.1, 34.7)
Missing (%)		*17.3*
Nulliparous (%)	57.8	57.5
Missing (%)		*0.7*
Education level (%)		
- No higher education	54.9	50.4
- Higher education	45.1	43.8
Missing (%)		*5.8*
Ethnicity (%)		
- European	60.6	60.2
- Cape Verdean	4.5	4.3
- Dutch Antillean	2.7	2.5
- Moroccan	5.9	5.5
- Turkish	8.9	8.6
- Surinamese	8.2	8.1
- Other	9.2	9.0
Missing (%)		*1.8*
Smoking during pregnancy (%)		
- Never	74.2	65.8
- Until pregnancy was known	9.4	8.2
- Continued	16.4	15.2
Missing (%)		*10.8*
Folic acid supplement use (%)		
- No	26.8	18.6
- Start in the first 10 weeks	31.6	24.5
- Start periconceptional	41.6	33.5
Missing (%)		*23.4*
Maternal energy intake (kcal)	2,049 (490)	2,051 (555)
Missing (%)		*21.5*
Maternal calcium dietary intake, (mg)	1,106 (329)	1,107 (339)
Missing (%)		*21.5*
Maternal blood concentrations of 25(OH)D (nmol/L)	50.4 (7.5, 122.5)	NI
Season when blood sample was taken (%)		
- Spring	28.6	NI
- Summer	22.6	NI
- Autumn	25.1	NI
- Winter	23.7	NI
Infant characteristics		
Girls (%)	50.4	50.4
Gestational age at birth (wk)	40.1 (35.8, 42.3)	40.1 (35.8, 42.3)
Birth weight (g)	3,431 (554)	3,432 (553)
Missing (%)		*0.2*

Supplementary Table 2.4.1. Subject characteristics (N=4,903) (continued)

Subject characteristics	Imputed	Original
Breastfeeding in the first 4 months (%)		
- Ever	92.3	73.9
- Never	7.7	5.7
Missing (%)		*20.4*
25(OH)D level in cord blood at birth (nmol/L)	28.8 (5.0, 81.7)	NI
Season when cord blood sample was taken (%)		
- Spring	28.6	NI
- Summer	22.6	NI
- Autumn	25.1	NI
- Winter	23.7	NI
Child characteristics at 6 years' visit		
Age (y)	6.0 (5.7, 7.8)	6.0 (5.7, 7.8)
Height (cm)	119.4 (6.0)	NI
Weight (kg)	22.6 (17.6, 33.8)	NI
Participation at sports, yes (%)	43.3	37.9
Missing (%)		*15.3*
Body composition		
- BMI (kg/m^2)	15.9 (13.6, 21.2)	NI
- Fat mass percentage	24.9 (5.7)	NI
- Lean mass percentage	71.5 (5.4)	NI
Cardiovascular risk factors		
- Systolic blood pressure (mmHg)	102.7 (8.3)	NI
- Diastolic blood pressure (mmHg)	60.7 (6.9)	NI
- Total-cholesterol (mmol/L)	4.2 (0.6)	NI
- Triglycerides (mmol/L)	0.95 (0.4, 2.3)	NI
- Insulin (pmol/L)	114.2 (17.0, 397.7)	NI

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. NI- not imputed

Supplementary Table 2.4.2. Subject characteristics with and without adiposity and cardiovascular measurements

	N=4,903	N=2,174	*p*-value
Maternal age (y)	30.4 (5.03)	28.4 (5.4)	**<0.001**
Maternal body mass index at enrolment (kg/m^2)	22.7 (18.8, 35.8)	22.7 (17.7, 36.0)	0.45
Nulliparous (%)	57.8	52.7	**<0.001**
Education level (%)			**<0.001**
- No higher education	54.9	68.9	
- Higher education	45.1	31.1	
Ethnicity (%)			**<0.001**
- European	60.6	45.6	
- Cape Verdean	4.5	4.9	
- Dutch Antillean	2.7	6.0	
- Moroccan	5.9	10.0	
- Turkish	8.9	10.7	
- Surinamese	8.2	11.2	
- Others	9.2	11.6	
Smoking during pregnancy (%)			**<0.001**
- Never	74.2	68.9	
- Until pregnancy was known	9.4	8.8	
- Continued	16.4	22.3	
Folic acid supplements use (%)			**<0.001**
- No	26.8	40.3	
- Start 1st to 10 weeks	31.6	30.8	
- Start periconceptional	41.6	28.9	
Maternal calories intake (kcal)	2,049 (490)	2,020 (481)	**<0.001**
Maternal calcium dietary intake, (mg)	1,106 (329)	1,068 (334)	**<0.001**
Maternal blood levels of 25(OH)D (nmol/L)	50.4 (7.5, 122.5)	38.6 (6.0, 110.7)	**<0.001**
Season when blood sample was taken (%)			**<0.001**
- Spring	28.6	31.6	
- Summer	22.6	23.5	
- Autumn	25.1	21.5	
- Winter	23.7	23.4	
Girls (%)	50.4	48.3	**<0.001**
Gestational age at birth (wk)	40.1 (35.8, 42.3)	40.0 (34.7, 42.3)	**<0.001**
Birth weight (g)	3,431 (554)	3,375 (578)	**<0.001**
Breastfeeding (%)			**<0.001**
- Ever	92.3	90.5	
- Never	7.7	9.5	
Child 25(OH)D level in cord blood at birth (nmol/L)	28.8 (5.0, 81.7)	23.9 (4.3, 79.6)	**<0.001**
Season when cord blood sample was taken (%)			**<0.001**
- Spring	27.6	23.9	
- Summer	26.6	28.9	
- Autumn	22.5	25.2	
- Winter	23.3	22.0	

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. Differences in subject characteristics comparing the groups with and without body composition and cardiovascular measurements were evaluated using T-tests for continuous normally distributed variables, Mann Whitney for non-normally distributed variables, and Chi-squared tests for categorical variables.

Supplementary Table 2.4.3. Correlation coefficients of the investigated variables

	Maternal 25(OH)D	Cord blood 25(OH)D	BMI	FMP	LMP	SBP	DBP	CHL	TGL	INSL
Maternal 25(OH)D	1.00									
Cord blood 25(OH)D	0.61**	1.00								
BMI	-0.15**	-0.12**	1,00							
FMP	-0.17**	-0.15**	0.57**	1,00						
LMP	0.17**	0.16**	-0.56**	-0.999**	1,00					
SBP	-0.07**	-0.08**	0.24**	0.19**	-0.19**	1,00				
DBP	-0.05**	-0.09**	0.08**	0.13**	-0.14**	0.62**	1,00			
CHL	-0.03	-0.01	0.09**	0.14**	-0.14**	0.08**	0.05*	1,00		
TGL	-0.01	0.03	0.05**	0.06**	-0.06**	-0.01**	-0.02	0.15**	1,00	
INSL	0.02	0.04	0.13**	0.09**	-0.09**	0.08**	-0.02	-0.03	0.19**	1.00

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed). Abbreviations: 25(OH)D- 25-hydroxyvitamin D, BMI- body mass index, FMP- fat mass percentage, LMP- lean mass percentage, SBP- systolic blood pressure, DBP- diastolic blood pressure, CHL- total cholesterol, TGL- triglycerides, INSL- insulin.

Supplementary Table 2.4.4. Crude associations of fetal 25(OH)D concentrations and body composition measures at the age of 6 years (N=4,903)

Fetal 25(OH)D concentrations	**Difference in outcome measure (95% Confidence Interval)**		
Maternal 25(OH)D concentrations	**Body mass index (N=4,898)**	**Fat mass percentage (N=4,805)**	**Lean mass percentage (N=4,805)**
<25.0 nmol/L (N=1,142)	**0.37 (0.20, 0.55)*** **	**0.26 (0.18, 0.35)*** **	**-0.27 (-0.35, -0.18)*** **
25.0 to 49.9 nmol/L (N=1,281)	**0.19 (0.05, 0.34)** **	**0.12 (0.05, 0.19)*** **	**-0.12 (-0.19, -0.05)*** **
50.0 to 74.9 nmol/L (N=1,195)	0.09 (-0.05, 0.23)	0.07 (0, 0.14)	-0.07 (-0.14, 0)
≥75.0 nmol/L (N=1,285)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	***-0.11 (-0.17, -0.05)**** **	***-0.08 (-0.11, -0.05)**** **	***0.08 (0.05, 0.11)**** **
Cord blood 25(OH)D concentrations	**Body mass index (N=3,048)**	**Fat mass percentage (N=2,998)**	**Lean mass percentage (N=2,998)**
<25.0 nmol/L (N=665)	0.14 (-0.03, 0.32)	0.09 (-0.07, 0.26)	-0.10 (-0.26, 0.07)
25.0 to 49.9 nmol/L (N=818)	0.12 (-0.05, 0.30)	0.03 (-0.13, 0.19)	-0.03 (-0.19, 0.13)
50.0 to 74.9 nmol/L (N=780)	0 (-0.18, 0.19)	-0.05 (-0.22, 0.11)	0.05 (-0.12, 0.22)
≥75.0 nmol/L (N=790)	*Reference*	*Reference*	*Reference*
Continuously (per SD)	***-0.05 (-0.09, -0.01)****	***-0.06 (-0.10, -0.03)**** **	***0.06 (0.03, 0.10)**** **

Values are linear regression coefficients (95% confidence interval). Basic model is adjusted for child's sex, age at 6-year visit and maternal ethnicity. *p <0.05, **p <0.01, ***p <0.001. Continuously = Maternal and cord blood vitamin D concentrations analyzed per 1 standard deviation in 25(OH)D.

Supplementary Table 2.4.5. Associations of maternal 25(OH)D concentrations and body composition measures at the age of 6 years, among Europeans (N=2,974)

Fetal 25(OH)D concentrations	**Difference in outcome measure (95% Confidence Interval)**				
Maternal 25(OH)D concentrations	**Body mass index (N=2,970)**	**Fat mass percentage (N=2,917)**	**Fat mass percentage (N=1,164)**	**Lean mass percentage (N=2,917)**	**Lean mass percentage (N=1,164)**
	Adjusted model	*Adjusted model*	*Childhood vitamin D model*	*Adjusted model*	*Childhood vitamin D model*
<25.0 nmol/L (N=192)	0.01 (-0.11, 0.14)	0.07 (-0.05, 0.19)	0.06 (-0.10, 0.21)	-0.08 (-0.20, 0.05)	-0.07 (-0.22, 0.09)
25.0 to 49.9 nmol/L (N=740)	0.06 (-0.02, 0.14)	0.05 (-0.03, 0.12)	0.02 (-0.07, 0.11)	-0.05 (-0.12, 0.03)	-0.02 (-0.11, 0.07)
50.0 to 74.9 nmol/L (N=909)	0.03 (-0.04, 0.10)	0.04 (-0.03, 0.11)	0.01 (-0.08, 0.09)	-0.04 (-0.11, 0.03)	-0.01 (-0.09, 0.08)
≥75.0 nmol/L (N=1,133)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.02 (-0.05, 0.02)*	*-0.02 (-0.06, 0.01)*	*-0.02 (-0.06, 0.02)*	*0.02 (0, 0.06)*	*0.02 (-0.02, 0.06)*
Cord blood 25(OH)D concentrations	**Body mass index (N=1,922)**	**Fat mass percentage (N=1,894)**	**Fat mass percentage (N=1,195)**	**Lean mass percentage (N=1,894)**	**Lean mass percentage (N=1,195)**
	Adjusted model	*Adjusted model*	*Childhood vitamin D model*	*Adjusted model*	*Childhood vitamin D model*
<25.0 nmol/L (N=486)	0.16 (-0.02, 0.31)	0.08 (-0.08, 0.24)	0.14 (-0.06, 0.33)	-0.08 (-0.24, 0.07)	-0.14 (-0.33, 0.06)
25.0 to 49.9 nmol/L (N=853)	0.11 (-0.04, 0.25)	0 (-0.14, 0.15)	0.07 (-0.10, 0.25)	0 (-0.15, 0.10)	-0.07 (-0.25, 0.10)
50.0 to 74.9 nmol/L (N=468)	0.04 (-0.11, 0.19)	-0.02 (-0.17, 0.13)	0.03 (-0.15, 0.21)	0.02 (-0.13, 0.17)	-0.03 (-0.21, 0.15)
≥75.0 nmol/L (N=119)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.04 (-0.08, 0)*	*-0.03 (-0.07, 0.01)*	*-0.04(-0.09, 0.0)1*	*0.03 (-0.01, 0.07)*	*0.04 (-0.01, 0.09)*

Values are linear regression coefficients (95% confidence interval). Adjusted model is adjusted for maternal characteristics (age, ethnicity, body mass index before pregnancy, parity, education, smoking, folic acid supplements, dietary calcium and energy intake during pregnancy and season of blood sampling), and child characteristics (sex, birthweight, gestational age at birth, breastfeeding, and age at measurements and playing sports at the age of 6y). Childhood vitamin D model, is adjusted model additionally adjusted for child's current 25(OH)D concentrations. *p <0.05. Continuously = Maternal and cord blood vitamin D concentrations analyzed per 1 standard deviation in 25(OH)D.

Supplementary Table 2.4.6. Crude associations of fetal 25(OH)D concentrations during pregnancy with cardiovascular risk factors at the age of 6 years (N=4,903)

Fetal 25(OH)D concentrations	**Difference in outcome measure (SDS) (95% Confidence Interval)**				
Maternal 25(OH)D concentrations	**Systolic blood pressure (N=4,550)**	**Diastolic blood pressure (N=4,550)**	**Total cholesterol (N=3,281)**	**Triglycerides (N=3,270)**	**Insulin (N=3,256)**
<25.0 nmol/L (N=1,142)	0.04 (-0.06, 0.14)	-0.02 (-0.12, 0.08)	0 (-0.12, 0.11)	0.01 (-0.11, 0.13)	-0.06 (-0.19, 0.06)
25.0 to 49.9 nmol/L (N=1,281)	0.02 (-0.06, 0.11)	0.01 (-0.08, 0.09)	0.02 (-0.08, 0.12)	0.03 (-0.07, 0.13)	-0.01 (-0.11, 0.09)
50.0 to 74.9 nmol/L (N=1,195)	0 (-0.09, 0.08)	-0.03 (-0.12, 0.05)	0.07 (-0.03, 0.16)	0.04 (-0.05, 0.14)	-0.09 (-0.19, 0.01)
≥75.0 nmol/L (N=1,285)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0 (-0.04, 0.03)*	*0.01 (-0.03,0.04)*	*0.01 (-0.03, 0.05)*	*-0.01 (-0.05, 0.03)*	*0.01 (-0.03, 0.06)*
Cord blood 25(OH)D concentrations	**Systolic blood pressure (N=2,847)**	**Diastolic blood pressure (N=2,847)**	**Total cholesterol (N=2,042)**	**Triglycerides (N=2,038)**	**Insulin (N=2,201)**
<25.0 nmol/L (N=665)	0.05 (-0.14, 0.24)	0.07 (-0.12, 0.27)	0.02 (-0.22, 0.25)	-0.01 (-0.24, 0.22)	0.03 (-0.20, 0.26)
25.0 to 49.9 nmol/L (N=818)	0.06 (-0.12, 0.25)	0.02 (-0.17, 0.21)	0.15 (-0.08, 0.38)	0.04 (-0.19, 0.26)	0.12 (-0.11, 0.35)
50.0 to 74.9 nmol/L (N=780)	0.03 (-0.16, 0.23)	0 (-0.20, 0.20)	0.09 (-0.16, 0.32)	0.07 (-0.17, 0.31)	0.10 (-0.14, 0.33)
≥75.0 nmol/L (N=790)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.02 (-0.06, 0.02)*	*-0.04 (-0.08,0.01)*	*0.02 (-0.03, 0.07)*	*0.03 (-0.02, 0.08)*	*0.01 (-0.04, 0.07)*

Values are linear regression coefficients (95% confidence interval). Basic model is adjusted for child's sex, age at 6-year visit and maternal ethnicity. Continuously = Maternal and cord blood vitamin D concentrations analyzed per 1 standard deviation in 25(OH)D.

Supplementary Table 2.4.7. Associations of maternal 25(OH)D concentrations during pregnancy with cardiovascular risk factors at the age of 6 years, among Europeans (N=2,974)

Fetal 25(OH)D concentrations	**Difference in outcome measure (SDS) (95% Confidence Interval)**				
Maternal 25(OH)D concentrations	**Systolic blood pressure (N=2,753)**	**Diastolic blood pressure (N=2,753)**	**Total cholesterol (N=2,203)**	**Triglycerides (N=2,000)**	**Insulin (N=1,992)**
<25.0 nmol/L (N=192)	-0.05 (-0.21, 0.11)	-0.09 (-0.25, 0.08)	0.15 (-0.06, 0.34)	0 (-0.20, 0.21)	-0.12 (-0.33, 0.08)
25.0 to 49.9 nmol/L (N=740)	0.01 (-0.08, 0.11)	0.02 (-0.08, 0.12)	0.05 (-0.07, 0.16)	0.09 (-0.03, 0.21)	0.04 (-0.08, 0.15)
50.0 to 74.9 nmol/L (N=909)	-0.02 (-0.11, 0.07)	-0.04 (-0.13, 0.05)	0.08 (-0.03, 0.18)	0.09 (-0.02, 0.20)	-0.07 (-0.18, 0.04)
≥75.0 nmol/L (N=1,133)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.01 (-0.03, 0.05)*	*0 (-0.04, 0.05)*	*-0.03 (-0.08, 0.03)*	*-0.05 (-0.10, 0.01)*	*0.01 (-0.04, 0.06)*
Cord blood 25(OH)D concentrations	**Systolic blood pressure (N=1,786)**	**Diastolic blood pressure (N=1,786)**	**Total cholesterol (N=1,294)**	**Triglycerides (N=1,292)**	**Insulin (N=1,283)**
<25.0 nmol/L (N=486)	-0.06 (-0.26, 0.14)	-0.01 (-0.22, 0.20)	0.05 (-0.21, 0.31)	0.15 (-0.10, 0.41)	0.04 (-0.22, 0.28)
25.0 to 49.9 nmol/L (N=853)	0.01 (-0.17, 0.20)	0.01 (-0.18, 0.20)	0.11 (-0.13, 0.35)	0.10 (-0.14, 0.33)	0.04 (-0.19, 0.27)
50.0 to 74.9 nmol/L (N=468)	0.01 (-0.18, 0.21)	0.05 (-0.15, 0.24)	0.06 (-0.19, 0.30)	0.11 (-0.13, 0.36)	0.03 (-0.21, 0.27)
≥75.0 nmol/L (N=119)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*0.02 (-0.03, 0.07)*	*0.01 (-0.05, 0.06)*	*-0.01 (-0.07, 0.06)*	*-0.02 (-0.08, 0.05)*	*0 (-0.06, 0.07)*

Values are linear regression coefficients (95% confidence interval). Adjusted model is adjusted for maternal characteristics (age, ethnicity, body mass index before pregnancy, parity, education, smoking, folic acid supplements, dietary calcium and energy intake during pregnancy, and season of blood sampling), and child characteristics (sex, birthweight, gestational age at birth, breastfeeding, and age at measurements, body mass index and playing sports at the age of 6y). *p <0.05. Continuously = Maternal and cord blood vitamin D concentrations analyzed per 1 standard deviation in 25(OH)D.

Chapter 2.5

Vitamin D status during fetal life and childhood kidney outcomes

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ABSTRACT

Background: Maternal vitamin D deficiency during pregnancy may influence offspring kidney health. We aimed to examine the associations of 25-hydroxyvitamin D (25(OH)D) blood levels during fetal life with kidney outcomes at school-age.

Methods: This study was embedded in a population-based prospective cohort study among 4,212 mother-child pairs. We measured maternal mid-pregnancy (18–25 weeks) and fetal cord blood (at birth) 25(OH)D levels. At a median age of 6.0 years, we measured children's combined kidney volume, glomerular filtration rate (eGFR) from creatinine and cystatin C serum levels and microalbuminuria from albumin and creatinine urine levels.

Results: Of all mothers, 21.9% had severely deficient levels (25(OH)D <25.0 nmol/L), 25.7% had deficient levels (25.0 to 49.9 nmol/L), 25% had sufficient levels (50.0 to 74.9 nmol/L), and 27.4% had optimal levels (≥75.0 nmol/L). Maternal 25(OH)D levels were not consistently associated with childhood combined kidney volume. Higher maternal 25(OH)D levels were associated with lower childhood eGFR (difference -0.94 ml/min/1.73m^2 (95% CI -1.73; -0.15) per 1 standard deviation increase in 25(OH)D). Maternal 25(OH)D levels were not associated with microalbuminuria. Cord blood 25(OH)D levels were not associated with childhood kidney outcomes. The associations of maternal 25(OH)D levels with childhood eGFR were partly explained by childhood vitamin D status.

Conclusion: Our findings suggest that maternal 25(OH)D levels during pregnancy may influence childhood kidney outcomes. These results should be considered hypothesis-generating. Further studies are needed to replicate the observations, to examine the underlying mechanisms, and to identify the long term-clinical consequences.

INTRODUCTION

Vitamin D deficiency is related to various adverse health outcomes in early and later life.[1–4] An accumulating body of evidence suggest than vitamin D status during pregnancy may persistently affect offspring health including poor weight gain, impaired development and rickets.[5, 6] Maternal vitamin D deficiency during pregnancy may also affect offspring kidney health. An experimental study in rats observed that maternal vitamin D deficiency leads to an increase in nephron number in the offspring, suggesting a stimulated nephrogenesis.[7] In line with these findings, another study in mice has shown that offspring of mothers who received a vitamin D deficient diet during pregnancy had a higher number of glomeruli and a delayed maturity of the glomeruli.[8] However, in contrast to these findings, another study in rats suggests that maternal vitamin D deficiency leads to lower kidney mass and a compromised kidney function.[9] As nephrogenesis continues until 36 weeks of gestation and largely ceases afterwards, suboptimal maternal vitamin D levels during specifically second half of pregnancy may influence kidney development.[10, 11] Smaller kidneys in early life with a reduced number of nephrons lead to glomerular hyperfiltration and sclerosis and predispose to kidney disease in adulthood.[12] To the best of our knowledge there are no studies that have observed the effects of fetal life vitamin D levels on kidney function in healthy children.

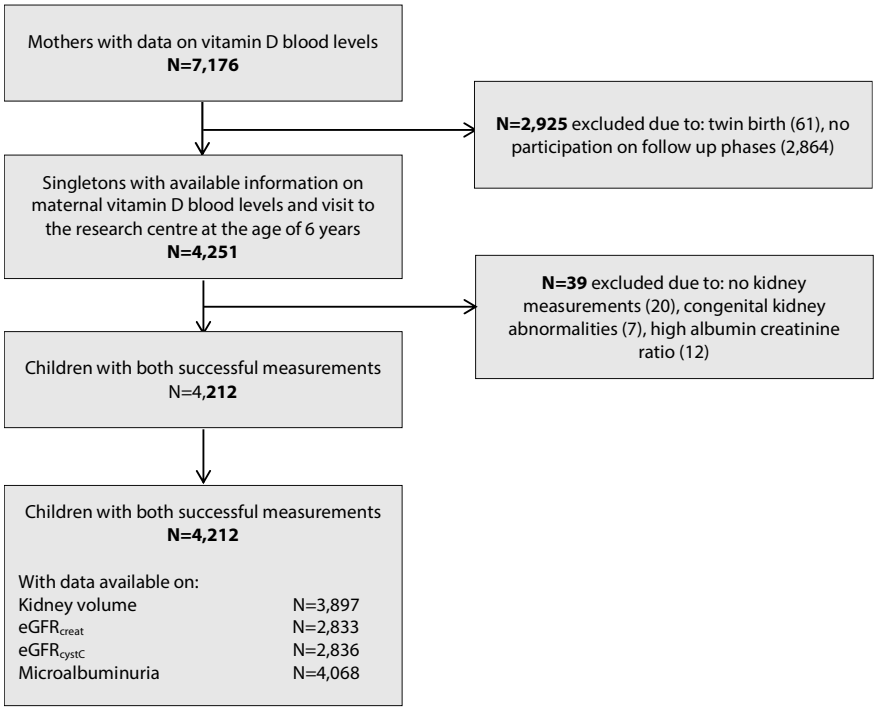
Therefore, we examined in a population-based prospective cohort study among 4,212 mother-child pairs, the associations of circulating vitamin 25-hydroxyvitamin D (25(OH)D) levels during mid-pregnancy and in cord blood at birth with childhood kidney outcomes. Additionally, we explored whether any association was explained by child's current 25(OH)D levels.

METHODS

Design and study population

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands.[13, 14] All children were born between April 2002 and January 2006. Enrolment in the study was aimed at early pregnancy, but was allowed until the birth of the child. The study was conducted according to the guidelines of the Helsinki Declaration and approved by the Medical Ethics Committee of Erasmus Medical Center, Rotterdam. Written informed consent was obtained from parents. Maternal 25(OH)D blood levels were measured in 7,176 pregnant women. In total, 4,251 of their singleton live born children participated in the kidney follow-up measurements at the age of 6 years. Children with evidence of congenital kidney abnormalities on ultrasound examination (N=7) or with abnormally high urinary albumin-creatinine ratio (>25) (N=12) were excluded from the study. Measurements on kidney ultrasound, creatinine and cystatin C from blood and albumin and creatinine from urine samples were available on 4,212 children. (**Figure 2.5.1.** Flowchart).

Figure 2.5.1. Flowchart of the study participants



Maternal and cord 25(OH)D blood levels

Maternal venous blood samples were collected in mid-pregnancy (median gestational age 20.3 weeks, range 18.5–23.3 weeks). Umbilical cord blood samples were taken immediately after delivery (median 40.1 weeks of gestation, range 35.9–42.3 weeks) by midwives and obstetricians. Measurements of 25(OH)D levels were conducted at the Eyles Laboratory at the Queensland Brain Institute, University of Queensland, Australia.

Total 25(OH)D was reported as the sum of $25(OH)D_2$ and $25(OH)D_3$ measured in plasma using a modification of a method previously described.[15] Samples were quantified using isotope dilution liquid chromatography-tandem mass spectrometry. Linearity was assessed using matrix-matched calibration standards, with R^2 values of >0.99 across the calibration range (10–125 nmol/L). Inter-assay inaccuracy and imprecision were assessed at four concentration levels for $25(OH)D_3$ (48.3, 49.4, 76.4, 139.2 nmol/L) and a single level (32.3 nmol/L) for $25(OH)D_2$ using certified reference materials and were excellent at all concentration levels tested. Inter-assay inaccuracy and imprecision were both <10% for $25(OH)D_3$ and <17% for $25(OH)D_2$, respectively. On the basis of recommendations and previous studies, we defined the following categories for vitamin D status: <25.0 nmol/L (<10.0 mg/L), severely deficient; 25 to 49.9 nmol/L (10.0 to 19.9 mg/L), deficient; 50.0 to 74.9 nmol/L (20.0 to 29.9 mg/L), sufficient; and ≥75.0 nmol/L (≥30.0 mg/L), optimal.[16–19] In children circulating 25(OH)D levels were measured at a median age of 6.0 years (range 5.6–7.4), at the Endocrine Laboratory of the VU University

Medical Center, Amsterdam as described previously in detail.[20] Child's current 25(OH)D status was available in a subgroup of 2,644 subjects.

Childhood kidney outcomes

Children's kidney outcomes were assessed at a median age of 6.0 (95% range 5.6 to 7.4) years in a dedicated research center in the Erasmus MC-Sophia Children's Hospital in Rotterdam.[21] We measured kidney volume with ultrasound. Left and right kidneys were identified in the sagittal plane along its longitudinal axis. We performed measurements of maximal bipolar kidney length, width and depth. Kidney width and depth were measured at the level of the hilum. The cross-sectional area in which the kidney appeared symmetrically round at its maximum width was used. Using the equation for a prolate ellipsoid we calculated kidney volume: volume (cm^3) = 0.523 x length (cm) x width (cm) x depth (cm).[22] Combined kidney volume was calculated by summing right and left kidney volume. Previously, we have reported good intra-observer and inter-observer correlation coefficients.[23]

We measured creatinine and cystatin C levels from non-fasting blood samples drawn by antecubital venipuncture. Creatinine levels were measured with enzymatic methods and cystatin C levels with a particle enhanced immunoturbidimetric assay (using Cobas 8000 analyzers, Roche, Almere, the Netherlands). Quality control samples demonstrated intra-assay and inter-assay coefficients of variation ranging from 0.51 to 1.37%, and 1.13 to 1.65%, respectively. Estimated glomerular filtration rate (eGFR) was calculated according to the revised Schwartz 2009 formula: $eGFR_{creat}$ = 36.5 * (height (cm)/serum creatinine (µmol/L).[24] In addition, we calculated eGFR using Zappitelli's formula based on cystatin C levels: $eGFR_{cystC}$ = 75.94/[$CysC^{1.17}$].[25] We calculated the albumin-creatinine ratio using urine creatinine (mmol/L) and urine albumin (mg/l) levels. These levels were determined with a Beckman Coulter AU analyser, creatinine levels were measured with the Jaffe reaction. In line with clinical cut-offs, microalbuminuria was defined as an albumin-creatinine ratio >2.5 mg/mmol for boys and >3.5 mg/mmol for girls.[26]

Covariates

We used questionnaires at enrollment in the study (median 13.5 weeks of gestation) to collect information about maternal age, ethnicity, educational level, and on smoking, alcohol usage and folic acid supplementation during pregnancy.[13] The presence of high cholesterol, diabetes, hypertension was available from the same questionnaires. Maternal energy intake during pregnancy was measured at enrollment with a validated semi-quantitative food frequency questionnaire.[27] Ethnicity and educational level were defined according to the classification of Statistics Netherlands.[28, 29] We measured maternal height and weight at enrollment and calculated body mass index (kg/m^2). The date of blood sampling was categorized into summer, fall, winter, and spring based on the Dutch standard seasons. Infant sex, gestational age, weight and length at birth were obtained from midwifes, medical records and hospital registries. We calculated sex- and gestational age-specific SD scores for birth weight.[30] Information on breastfeeding was collected using postnatal questionnaires as previously reported.[21] At the age of 6 years, child height was determined in standing position to the

nearest millimeter without shoes by a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Weight was measured using a mechanical personal scale (SECA, Almere, the Netherlands). We calculated body mass index (kg/m^2) and body surface area (BSA) (m^2) (using DuBois formula BSA= weight (kg)$^{0.425}$ x height (cm)$^{0.725}$ x 0.007184).

Statistical analysis

We performed a non-response analysis by comparing subject characteristics between children with and without follow-up kidney measurements. We used multivariable linear and logistic regression models to assess the associations of maternal and cord 25(OH)D levels with combined kidney volume, creatinine and cystatin C levels, $eGFR_{creat}$ and $eGFR_{cystC}$, and risk of microalbuminuria in childhood. Levels of 25(OH)D were analyzed both continuously per standard deviation (SD)- increase and using clinical cut-offs.[16–18] The regression models were first adjusted for child's sex, child's age at kidney measurements, season when blood samples were drawn and maternal ethnicity (basic models), and subsequently additionally for maternal age, education, body mass index at enrolment, smoking, alcohol use, folic acid supplement use and energy intake during pregnancy, and pre-pregnancy comorbidities, and for child's gestational age-adjusted birth weight, breastfeeding in early life, and body surface area at the age of 6 years. These covariates were included in the models based on their associations with kidney outcomes in previous studies, or a change in effect estimates of >10%. As the interactions of maternal 25(OH)D with child sex were not significant, we did not stratify our analyses on child sex. To explore if childhood 25(OH)D status explain the associations between fetal 25(OH)D and childhood kidney outcomes, we performed a sensitivity analysis in the subgroup of children with 25(OH)D levels available. To diminish potential bias associated with attrition, missing values of covariates (less than 24%), were multiple imputed by generating 10 independent datasets using the Markov Chain Monte Carlo (MCMC) method. The multiple imputation procedure was based on the correlation between each variable with missing values and the other subject characteristics.[31, 32] Statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Participant characteristics

Table 2.5.1 shows that in mothers median (95% range) 25(OH)D levels were 51.9 nmol/L (7.7, 122.1). Of all mothers, 21.9% had severely deficient levels (25(OH)D <25.0 nmol/L), 25.7% had deficient levels (25.0 to 49.9 nmol/L), 25% had sufficient levels (50.0 to 74.9 nmol/L), and 27.4% had optimal levels (≥75.0 nmol/L). In cord blood median 25(OH)D levels were 29.3 nmol/L range (5.6, 81.5). At the age of 6 years, mean (± SD) combined kidney volume was 120 cm^3 (±22.8), and mean $eGFR_{creat}$ was 119 ml/min/1.73m^2 (±16.4). Of all children, 7.6% had microalbuminuria. Subjects characteristics before and after imputation are shown in **Supplementary Table 2.5.1.** Results from the non-response analysis showed that as compared to children who did not have kidney measurements at the age of 6 years, those who did have kidney

measurements had mothers with a higher educational level and higher 25(OH)D levels during pregnancy. Children with kidney measurements also had higher 25(OH)D levels at birth (**Supplementary Table 2.5.2**).

Table 2.5.1. Subject characteristics (N=4,212)

Characteristics	
Maternal Characteristics	
Age (y)	31.3 (20.2, 39.4)
Body mass index at enrolment (kg/m^2)	22.6 (18.1, 34.3)
Education level (%)	
- No higher education	51.2
- Higher education	48.8
Ethnicity (%)	
- European	63.2
- Cape Verdean	4.1
- Dutch Antillean	2.0
- Moroccan	5.6
- Turkish	8.2
- Surinamese	7.6
- Other	9.3
Smoking during pregnancy (%)	
- Never	75
- Until pregnancy was known	9.9
- Continued	15.1
Alcohol consumption during pregnancy (%)	
- Never	42.8
- Until pregnancy was known	14.3
- Continued	42.9
Folic acid supplement use (%)	
- No	24.5
- Start in the first 10 weeks	31.2
- Start periconceptional	44.3
Maternal energy intake (kcal)	2,062 (485)
Maternal blood levels of 25(OH)D (nmol/L)	51.9 (7.7, 122.1)
Season when blood sample was taken (%)	
- Spring	27.8
- Summer	22.6
- Autumn	25.5
- Winter	24.1
Infant characteristics	
Girls (%)	50.4
Gestational age at birth (wk)	40.1 (35.9, 42.3)
Birth weight (g)	3,449 (545)
Breastfeeding in the first 4 months (%)	
- Exclusive	31.7
- Partial	61.1

Table 2.5.1. Subject characteristics (N=4,212) (continued)

Characteristics	
- Never	7.2
Child 25(OH)D level in cord blood at birth (nmol/L)	29.3 (5.2, 81.5)
Season when cord blood sample was taken (%)	
- Spring	28.2
- Summer	26.5
- Autumn	21.4
- Winter	23.9
Child characteristics at 6 years visit	
Age (y)	6.0 (5.6, 7.4)
Height (cm)	119 (5.6)
Weight (kg)	22.2 (17.4, 32.4)
Body mass index (kg/m^2)	15.8 (13.6, 20.7)
Body surface area (m^2)	0.86 (0.7, 1.1)
Childhood 25(OH)D levels (nmol/L)	64.4 (18.0, 134.0)
Combined kidney volume (cm^3)	120 (22.8)
Creatinine (µmol/L)	37.2 (5.5)
Cystatin C (µg/L)	784 (80.2)
$eGFR_{creat}$ (ml/min/1.73m^2)	119 (16.4)
$eGFR_{cystC}$ (ml/min/1.73m^2)	102.5 (14.5)
Microalbuminuria (%)	7.6

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.
Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels and $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

Maternal and cord 25(OH)D levels and childhood kidney outcomes

Table 2.5.2 gives the results from the multivariable regression models for the associations of maternal 25(OH)D levels and childhood kidney outcomes. Children of mothers who were vitamin D deficient during pregnancy had a 1.92 cm^3 (95% Confidence Interval (CI) 0.11, 3.74) larger combined kidney volume compared to children of mothers who had optimal 25(OH)D levels. However, the overall trend analysis based on continuous data for 25(OH)D was not significant. Maternal 25(OH)D levels were inversely associated with lower childhood $eGFR_{creat}$ (difference -0.94 (95% CI -1.73; -0.15) ml/min/1.73m^2 per 1 SD increase in 25(OH)D). Maternal 25(OH)D levels were not associated with $eGFR_{cystC}$ or risk of microalbuminuria. Results from the basic models of maternal and cord blood vitamin D and childhood kidney outcomes are shown in the **Supplementary Table 2.5.3** & **Supplementary Table 2.5.4**. In line with the results for $eGFR_{creat}$ and $eGFR_{cystC}$, maternal 25(OH)D levels were associated with 0.32 µmol/L (95% CI 0.07; 0.58) higher childhood creatinine levels per 1 SD increase in 25(OH)D, but not with childhood cystatin C levels (**Supplementary Table 2.5.5**). We performed a sensitivity analysis among a subgroup of 2,644 children to explore whether any association was explained by childhood 25(OH)D status. Results from this analysis are given in **Supplementary Table 2.5.6**, and suggest that the association of maternal 25(OH)D levels with childhood $eGFR_{creat}$ were at least partly explained by child 25(OH)D status.

Table 2.5.2. Associations of maternal 25(OH)D levels during pregnancy with child kidney outcomes at the age of 6 years (N=4,212)

	Difference in outcome measure (95% Confidence Interval)			
Multivariable model[£]	**Kidney volume (cm^3) N=3,897**	**$eGFR_{creat}$ ($ml/min/1.73m^2$) N=2,833**	**$eGFR_{cystC}$ ($ml/min/1.73m^2$) N=2,836**	**Microalbuminuria (odds ratio) N=4,068**
<25.0 nmol/L (N=921)	-0.76 (-3.11, 1.59)	1.52 (-0.83, 3.86)	-0.33 (-2.39, 1.72)	1.31 (0.83, 2.06)
25.0 to 49.9 nmol/L (N=1,083)	**1.92 (0.11, 3.74)***	1.39 (-0.37, 3.15)	0.66 (-0.93, 2.24)	0.91 (0.63, 1.32)
50.0 to 74.9 nmol/L (N=1,055)	0.90 (-0.82, 2.62)	1.44 (-0.24, 3.12)	0.64 (-0.87, 2.15)	1.15 (0.83, 1.59)
≥75.0 nmol/L (N=1,153)	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.55 (-1.35, 0.25)*	***-0.94 (-1.73, -0.15)****	*-0.29 (-0.99, 0.41)*	*0.93 (0.80, 1.09)*

Values are linear and logistic regression coefficients (95% confidence interval). Multivariable adjusted model [£] is adjusted for child's sex, age at 6-year visit, maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and energy intake during pregnancy, education, ethnicity, prepregnancy comorbidities, season when blood samples were drawn) and child characteristics (birthweight adjusted for gestational age, breastfeeding, and body surface area at the age of 6y). *p <0.05. Continuously = Maternal vitamin D levels analyzed per 1 standard deviation in 25(OH)D.

Abbreviations: SD standard deviation; $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

Table 2.5.3 gives the results from the multivariable regression models for the associations of cord 25(OH)D levels at birth and childhood kidney outcomes. Using the same cut-offs for vitamin D status, 42.2% of the children were categorized as severely vitamin D deficient, 36.4% as deficient, 17.3% as sufficient, and 4.1% as optimal. Cord 25(OH)D levels were not associated with any of the childhood kidney outcomes.

Table 2.5.3. Associations of 25(OH)D cord blood levels with kidney outcomes at the age of 6 years (N=2,689)

	Difference in outcome measure (95% Confidence Interval)			
Multivariable model[£]	**Kidney volume (cm^3) N=2,480**	**$eGFR_{creat}$ ($ml/min/1.73m^2$) N=1,810**	**$eGFR_{cystC}$ ($ml/min/1.73m^2$) N=1,814**	**Microalbuminuria (odds ratio) N=2,598**
<25.0 nmol/L (N=1,136)	0.80 (-3.50, 5.11)	-0.11 (-4.30, 4.09)	-0.33 (-4.23, 3.57)	0.68 (0.33, 1.41)
25.0 to 49.9 nmol/L (N=980)	0.70 (-3.41, 4.81)	-1.64 (-5.62, 2.33)	-0.14 (-3.85, 3.58)	0.61 (0.31, 1.20)
50.0 to 74.9 nmol/L (N=464)	-0.34 (-4.58, 3.80)	-0.85 (-4.97, 3.26)	-1.14 (-4.98, 2.71)	0.56 (0.27, 1.15)
≥75.0 nmol/L (N=109)	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.44 (-1.43, 0.55)*	*-0.20 (-1.18, 0.79)*	*0.17 (-0.73, 1.08)*	*0.91 (0.75, 1.11)*

Values are linear and logistic regression coefficients (95% confidence interval). Multivariable adjusted model [£] is adjusted for child's sex, age at 6-year visit, maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and energy intake during pregnancy, education, ethnicity, prepregnancy comorbidities) and child characteristics (birthweight adjusted for gestational age, breastfeeding, season when blood samples were drawn and body surface area at the age of 6y). *p <0.05. Continuously = Cord vitamin D levels analyzed per 1 standard deviation in 25(OH)D.

Abbreviations: SD standard deviation; $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels

DISCUSSION

In this population-based prospective cohort study, we observed that lower maternal, but not cord, 25(OH)D levels in blood, tended to be associated with higher eGFR and larger combined kidney volume in school-age children. The associations may be partly explained by childhood 25(OH)D levels.

Strength and limitations

A major strength of our study is the prospective design from fetal life onwards within a large population-based cohort. This study is among the first that examined the association of fetal vitamin D status with kidney health in a large multi-ethnic sample of school-age children. We used 25(OH)D levels, which are the best and most widely used indicator of vitamin D status.[33] Furthermore, we used well-established methods to measure kidney size and function.[23, 34] Kidney volume was measured by ultrasound. Kidney size is correlated with the number of glomeruli and can be used in epidemiological studies as measure of kidney development.[35] In children the estimation of GFR is challenging. Blood creatinine is most commonly used to calculate eGFR, and Schwartz formula has been validated in a pediatric population.[24] In addition to blood creatinine levels, we also calculated eGFR based on cystatin C levels using Zappitelli's formula.[25, 36] It has been suggested that blood cystatin C levels might be a better biomarker to estimate GFR because the production rate is constant, it is freely filtered, and less dependent on child weight, height and sex compared to creatinine.[37, 38] However in this study we observe that maternal 25(OH)D levels are associated with $eGFR_{creat}$ but not with $eGFR_{cystC}$. This study also has some limitations to consider. Of all children with maternal levels of 25(OH)D available, 74% had successful kidney measurements. Mothers of the children that were lost to follow up had on average lower 25(OH)D levels and were on average lower educated, suggesting that our study population had a selection towards a healthier population. Furthermore, we used the same cut-offs of 25(OH)D for pregnant woman as the documented levels for general population, while it is still not well known what optimal 25(OH)D levels are during pregnancy.[33] A limitation of our study is that we did not have information about childhood dietary data at the age of 6 years. Dietary composition can impact childhood microalbuminuria. Moreover, microalbuminuria was evaluated using urine albumin-creatinine ratio from a random urine sample, still we did not have first-morning void samples. Finally, although we performed adjustment for a large number of potential maternal and childhood confounders, residual confounding by other lifestyle factors, might still be present, as in any observational study.

We hypothesized that maternal vitamin D levels may affect offspring kidney health, by reducing the number of nephrons, which in turn lead to glomerular hyperfiltration and sclerosis, thus predisposing the individual to renal damage and subsequent development of higher blood pressure, impaired kidney function and end-stage kidney disease in adulthood.[39] The observed effect estimates in the present study are small, but important from an etiological perspective. They provide further insights into pathways leading to changes in kidney function from the earliest phase of life.

Interpretation and comparison with previous studies

In adults, vitamin D deficiency is associated with an increased risk of having microalbuminuria.[40] In a study among 15,068 individuals aged 20 years and older in the U.S., it was observed that adults who had 25(OH)D levels in the lowest quartile had an increased risk of microalbuminuria.[40] We did not observe any association between maternal or cord 25(OH)D levels and the risk of childhood microalbuminuria. It might be that differences in this clinical marker of kidney dysfunction appear at older ages. The Cardiovascular Health Study, a prospective community-based cohort among 1,705 participants aged 65 years and older in the U.S. reported that lower 25(OH)D levels were associated with a lower eGFR.[41] In contrast, we observed that higher maternal 25(OH)D levels were associated with lower eGFR in childhood. Additionally, we observed that the effect of maternal 25(OH)D levels on childhood eGFR was at least partly explained by child's current 25(OH)D levels. Childhood 25(OH)D and maternal 25(OH)D levels are correlated due to similar dietary and lifestyle factors.

Our findings are in line with animal studies. Studies in rats observed no differences in kidney volume in rats whose mothers were fed with a vitamin D deplete diet.[7] However, maternal vitamin D deficiency was associated with an increase in the number of glomeruli at 7 weeks.[7] Still, it is not known whether these additional nephrons are functional, and thus confer an advantage to renal function. Interestingly, another study comparing two generations of mice from mothers fed either standard chow or vitamin D-deficient diet, suggests that maternal vitamin D deficiency accompanies changes in the renal expression of important factors that may delay the maturation of glomeruli by extending the period of nephrogenesis.[8] In line with these observations, we observed that mothers who were 25(OH)D deficient had children with larger combined kidney volume. Also, lower maternal 25(OH)D levels were associated with an increased eGFR in school-age children. We observed no associations between cord 25(OH)D levels and childhood kidney outcomes. Our results suggest that different periods of fetal 25(OH)D exposure may have different impact on childhood kidney outcomes.

The mechanisms by which maternal vitamin D levels during pregnancy affect childhood kidney function are not known yet. Vitamin D is an important component, during cell proliferation for differentiation and maturation processes.[42] Renal proximal tubules are the major site for the conversion of the 25(OH)D to the active hormone, and thus any early changes to renal function may have consequences for later vitamin D physiology. Maka *et al*.[7] suggests that vitamin D deficient offspring may have prolonged nephrogenic proliferation, without the appropriate switch to nephron maturation. If this is the case, it is likely that the nephrons, although more numerous, may not be fully matured and may be functionally impaired.[7]

CONCLUSION

Results from this population-based prospective cohort study suggest that maternal 25(OH)D levels during pregnancy may influence childhood kidney function. Part of the observed effect may be explained by childhood 25(OH)D levels. These results should be considered as hypothesis generating. Further studies are needed to replicate the observations, to examine the underlying mechanisms and to identify the long term-clinical consequences.

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Supplementary Table 2.5.1. Subject characteristics (N=4,212)

	Imputed	Original
Maternal characteristics		
Maternal age (y)	31.3 (20.2, 39.4)	31.3 (20.2, 39.4)
Maternal body mass index at enrolment (kg/m^2)	22.6 (18.1, 34.3)	22.6 (18.1, 34.2)
Education level (%)		
- No higher education	51.2	49.7
- Higher education	48.8	50.3
Ethnicity (%)		
- European	63.2	63.8
- Cape Verdean	4.1	4.0
- Dutch Antillean	2.0	2.0
- Moroccan	5.6	5.4
- Turkish	8.2	8.2
- Surinamese	7.6	7.5
- Other	9.3	9.3
Smoking during pregnancy (%)		
- Never	75	74.9
- Until pregnancy was known	9.9	9.5
- Continued	15.1	15.6
Alcohol during pregnancy (%)		
- Never	42.8	42.2
- Until pregnancy was known	14.3	14.4
- Continued	42.9	43.5
Folic acid supplements use (%)		
- No	24.5	22.0
- Start 1st to 10 weeks	31.2	31.3
- Start periconceptional	44.3	46.7
Maternal calories intake (kcal)	2,062 (485)	2,053 (544)
Maternal blood levels of 25(OH)D (nmol/L)	51.9 (7.7, 122.1)	51.9 (7.7, 122.1)
Season when blood sample was taken (%)		
- Spring	27.8	27.8
- Summer	22.6	22.6
- Autumn	25.5	25.5
- Winter	24.1	24.1
Infant characteristics		
Girls (%)	50.4	50.5
Gestational age at birth (wk)	40.1 (35.9, 42.3)	40.1 (35.9, 42.3)
Birth weight (g)	3,449 (545)	3,448 (544)
Breastfeeding in the first 4 months (%)		
- Exclusive	31.7	25.7
- Partial	61.1	65.7
- Never	7.2	8.6
Child 25(OH)D level in cord blood at birth (nmol/L)	29.3 (5.2, 81.5)	29.3 (5.2, 81.5)

Supplementary Table 2.5.1. Subject characteristics (N=4,212) (continued)

	Imputed	Original
Season when cord blood sample was taken (%)		
- Spring	28.2	28.2
- Summer	26.5	26.5
- Autumn	21.4	21.4
- Winter	23.9	23.9
Child characteristics at 6y visit		
Age (y)	NI	6.0 (5.6, 7.4)
Height (cm)	119 (5.6)	119 (5.6)
Weight (kg)	22.2 (17.4, 32.4)	22.2 (17.4, 32.4)
Body mass index (kg/m^2)	15.8 (13.6, 20.7)	15.8 (13.6, 20.7)
Body surface area (m^2)	0.86 (0.7, 1.1)	0.86 (0.7, 1.1)
Kidney volume (cm^3)	NI	120 (22.8)
Creatinine (µmol/L)	NI	37.2 (5.5)
Cystatin C (µg/L)	NI	784 (80.2)
$eGFR_{creat}$ (ml/min/1.73m^2)	NI	119 (16.4)
$eGFR_{cystC}$ (ml/min/1.73m^2)	NI	102.5 (14.5)
Microalbuminuria (%)	NI	7.6

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.
Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels and $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels; NI, not imputed

Supplementary Table 2.5.2. Subject characteristics with and without kidney measurements

	N=4,212	N=1,509	p-value
Maternal characteristics			
Maternal age (y)	31.3 (20.2, 39.4)	29.4 (19.0, 38.4)	**0.001**
Maternal body mass index at enrolment (kg/m^2)	22.6 (18.1, 34.3)	22.4 (17.6, 35.6)	0.25
Education level (%)			**0.001**
- No higher education	51.2	62.0	
- Higher education	48.8	38.0	
Ethnicity (%)			**0.001**
- European	63.2	50.6	
- Cape Verdean	4.1	4.5	
- Dutch Antillean	2.0	4.3	
- Moroccan	5.6	8.4	
- Turkish	8.2	11.6	
- Surinamese	7.6	10.4	
- Others	9.3	10.2	
Smoking during pregnancy (%)			**0.001**
- Never	75	70.7	
- Until pregnancy was known	9.9	7.9	
- Continued	15.1	21.4	

Supplementary Table 2.5.2. Subject characteristics with and without kidney measurements (continued)

	N=4,212	N=1,509	p-value
Alcohol during pregnancy (%)			**0.001**
- Never	42.8	54.2	
- Until pregnancy was known	14.3	12.7	
- Continued	42.9	33.1	
Folic acid supplements use (%)			**0.001**
- No	24.5	33.8	
- Start 1st to 10 weeks	31.2	31.5	
- Start periconceptional	44.3	34.7	
Maternal calories intake (kcal)	2,062 (485)	2,028 (581)	0.09
Maternal blood levels of 25(OH)D (nmol/L)	51.9 (7.7, 122.1)	43.3 (6.7, 111.6)	**0.001**
Season when blood sample was taken (%)			**0.03**
- Spring	27.8	30.5	
- Summer	22.6	24.1	
- Autumn	25.5	22.1	
- Winter	24.1	23.3	
Infant characteristics			
Girls (%)	50.4	48.0	0.1
Gestational age at birth (wk)	40.1 (35.9, 42.3)	40.1 (35.6, 42.3)	**0.01**
Birth weight (g)	3,449 (545)	3,396 (565)	**0.002**
Breastfeeding (%)			**0.001**
- Exclusive ≥4 months	31.7	20.0	
- Partial ≥4 months	61.1	67.2	
- Never or ≤4 months	6.2	12.8	
Child 25(OH)D level in cord blood at birth (nmol/L)	29.3 (5.1, 81.5)	25.7 (4.5, 80.5)	**0.001**
Season when cord blood sample was taken (%)			0.09
- Spring	28.2	24.4	
- Summer	26.5	29.6	
- Autumn	21.4	22.6	
- Winter	23.9	23.4	

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. Differences in subject characteristics comparing the groups with and without kidney measurements were evaluated using T-tests for continuous normally distributed variables, Mann Whitney for non-normally distributed variables, and Chi-squared tests for categorical variables.
Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels and $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

Supplementary Table 2.5.3. Associations of maternal 25(OH)D levels during pregnancy with kidney outcomes at the age of 6 years (N=4,212)

	Difference in outcome measure (95% Confidence Interval)			
	Kidney volume (cm^3) N=3,897	**$eGFR_{creat}$ ($ml/min/1.73m^2$) N=2,833**	**$eGFR_{cystC}$ ($ml/min/1.73m^2$) N=2,836**	**Microalbuminuria (odds ratio) N=4,068**
Basic Model#				
<25.0 nmol/L (N=921)	-0.56 (-3.12, 2.00)	1.20 (-1.02, 3.41)	-0.49 (-2.46, 1.48)	1.19 (0.78, 1.82)
25.0 to 49.9 nmol/L (N=1,083)	**2.23 (0.18, 4.27)***	1.28 (-0.46, 3.02)	0.63 (-0.92, 2.19)	0.87 (0.61, 1.25)
50.0 to 74.9 nmol/L (N=1,055)	1.31 (-0.64, 3.27)	1.53 (-0.15, 3.21)	0.62 (-0.88, 2.12)	1.15 (0.84, 1.58)
≥75.0 nmol/L (N=1,153)	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.44 (-1.32, 0.44)*	***-0.82(-1.57, -0.06)****	*-0.21 (-0.89, 0,46)*	*0.96 (0.82, 1.11)*

Values are linear and logistic regression coefficients (95% confidence interval). Basic model# is adjusted for child's sex, age at 6-year visit, season when blood samples were drawn and maternal ethnicity. *p <0.05. Continuously = Maternal vitamin D levels analyzed per 1 standard deviation in 25(OH)D.

Abbreviations: SD standard deviation; $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

Supplementary Table 2.5.4. Associations of 25(OH)D cord blood levels with kidney outcomes at the age of 6 years (N=2,689)

	Difference in outcome measure (95% Confidence Interval)			
	Kidney volume (cm^3) N=2,480	**$eGFR_{creat}$ ($ml/min/1.73m^2$) N=1,810**	**$eGFR_{cystC}$ ($ml/min/1.73m^2$) N=1,814**	**Microalbuminuria (odds ratio) N=2,598**
Basic Model#				
<25.0 nmol/L (N=1,136)	0.71 (-4.14, 5.55)	-0.46 (-4.61, 3.69)	-0.83 (-4.69, 3.04)	0.88 (0.52, 1.50)
25.0 to 49.9 nmol/L (N=980)	1.84 (-2.80, 6.48)	-1.29 (-5.26, 2.69)	-0.17 (-3.88, 3.53)	0.72 (0.46, 1.12)
50.0 to 74.9 nmol/L (N=464)	-0.23 (-4.91, 4.45)	-0.73 (-4.85, 3.39)	-1.16 (-4.99, 2.67)	1.09 (0.73, 1.62)
≥75.0 nmol/L (N=109)	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.14 (-1.23, 0.96)*	*-0.03 (-0.91, 0.97)*	*0.41 (-0.46, 1.29)*	*0.97 (0.80, 1.16)*

Values are linear and logistic regression coefficients (95% confidence interval). Basic model# *is* adjusted for child's sex and age at 6-year visit, season when blood samples were drawn and maternal ethnicity. *p <0.05. Continuously = Cord vitamin D levels analyzed per 1 standard deviation in 25(OH)D.

Abbreviations: SD standard deviation; $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

Supplementary Table 2.5.5. Associations of 25(OH)D blood levels during pregnancy and in cord blood with creatinine and cystatin C at the age of 6 years

	25(OH)D during pregnancy (N=2,838)			25(OH)D in cord blood (N=1,815)	
	Creatinine (umol/L) N=2,837	Cystatin C (ug/L) N=2,836		Creatinine (umol/L) N=1,814	Cystatin C (ug/L) N=1,814
Basic Model[#]			**Basic Model[#]**		
<25.0 nmol/L (N=614)	-0.38 (-1.09, 0.34)	3.50 (-7.40, 14.40)	<25.0 nmol/L (N=771)	0.32 (-1.04, 1.68)	2.82 (-17.88, 23.53)
25.0 to 49.9 nmol/L (N=741)	-0.43 (-0.99, 0.14)	-5.33 (-13.93, 3.27)	25.0 to 49.9 nmol/L (N=660)	0.75 (-0.56, 2.05)	1.69 (-18.14, 21.51)
50.0 to 74.9 nmol/L (N=724)	-0.52 (-1.07, 0.02)	-1.97 (-10.27, 6.33)	50.0 to 74.9 nmol/L (N=312)	0.21 (-1.14, 1.56)	6.81 (-13.72, 27.35)
≥75.0 nmol/L (N=759)	*Reference*	*Reference*	≥75.0 nmol/L (N=72)	*Reference*	*Reference*
Continuously (per SD)	***0.29 (0.04, 0.53)****	*1.89 (-1.84, 5.63)*	*Continuously (per SD)*	*-0.04 (-0.35, 0.26)*	*-1.41 (-6.09, 3.28)*
Multivariable model[£]			**Multivariable model[£]**		
<25.0 nmol/L (N=614)	-0.53 (-1.28, 0.23)	2.68(-8.70, 14.05)	<25.0 nmol/L (N=771)	0.05 (-1.30, 1.41)	0.07(-20.86, 20.99)
25.0 to 49.9 nmol/L (N=741)	-0.47 (-1.04, 0.10)	-5.34 (-14.09, 3.40)	25.0 to 49.9 nmol/L (N=660)	0.65 (-0.64, 1.93)	1.47 (-18.41, 21.35)
50.0 to 74.9 nmol/L (N=724)	-0.50 (-1.04, 0.05)	-1.95 (-10.30, 6.41)	50.0 to 74.9 nmol/L (N=312)	0.26 (-1.07, 1.59)	7.02 (-13.56, 27.60)
≥75.0 nmol/L (N=759)	*Reference*	*Reference*	≥75.0 nmol/L (N=72)	*Reference*	*Reference*
Continuously (per SD)	***0.32 (0.07, 0.58)****	*2.32 (-1.55, 6.19)*	*Continuously (per SD)*	*0.20 (-0.09, 0.49)*	*2.51 (-1.97, 6.98)*

Values are linear regression coefficients (95% confidence interval). Basic model[#] *is* adjusted for child's sex, age at 6-year visit, season when blood samples were drawn and maternal ethnicity. Multivariable adjusted model[£] is additionally adjusted for maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and energy intake during pregnancy, education, prepregnancy comorbidities) and child characteristics (birthweight adjusted for gestational age, breastfeeding and body surface area at the age of 6y). *p <0.05. Continuously = Levels of vitamin D analyzed per 1 standard deviation in 25(OH)D.

Supplementary Table 2.5.6. Associations of maternal 25(OH)D levels during pregnancy with child kidney outcomes additionally adjusted for child 25(OH)D levels at the age of 6 years (N=2,644)

	Difference in outcome measure (95% Confidence Interval)			
	Kidney volume (cm^3) N=2,472	**$eGFR_{creat}$ ($ml/min/1.73m^2$) N=2,627**	**$eGFR_{cystC}$ ($ml/min/1.73m^2$) N=2,630**	**Microalbuminuria (odds ratio) N=2,578**
Multivariable model[£]				
<25.0 nmol/L (N=571)	0.18 (-2.80, 3.17)	0.45 (-1.99, 2.88)	-1.05 (-3.21, 1.12)	1.09 (0.61, 1.94)
25.0 to 49.9 nmol/L (N=688)	**2.57 (0.30, 4.84)***	1.12 (-0.71, 2.94)	0.55 (-1.11, 2.20)	0.97 (0.62, 1.51)
50.0 to 74.9 nmol/L (N=683)	**2.17 (0.02, 4.33)***	1.08 (-0.65, 2.82)	0.48 (-1.13, 2.03)	1.01 (0.67, 1.53)
≥75.0 nmol/L (N=702)	*Reference*	*Reference*	*Reference*	*Reference*
Continuously (per SD)	*-0.87 (-1.88, 0.15)*	*-0.70 (-1.52, 0.15)*	*-0.10 (-0.84, 0.63)*	*0.96 (0.73, 1.17)*

Values are linear and logistic regression coefficients (95% confidence interval). Multivariable adjusted model[£] is adjusted for child's sex, age at 6 year visit, maternal characteristics (age, body mass index at intake, alcohol consumption, smoking during pregnancy, folic acid and energy intake during pregnancy, education, ethnicity, prepregnancy comorbidities, season when blood samples were drawn) and child characteristics (birthweight adjusted for gestational age, breastfeeding, and body surface area and 25(OH)D levels at the age of 6y). *p <0.05. Continuously = Maternal vitamin D levels analyzed per 1 standard deviation in 25(OH)D.

Abbreviations: SD standard deviation; $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

Chapter 2.6

Associations of maternal and paternal blood pressure patterns and hypertensive disorders during pregnancy, with childhood blood pressure

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ABSTRACT

Objective: Hypertensive disorders in pregnancy may affect offspring cardiovascular risk. We examined the associations of maternal blood pressure throughout pregnancy and hypertensive disorders in pregnancy with childhood blood pressure. Specific focus was on the comparison with paternal blood pressure effects, the identification of critical periods and the role of birth outcomes and childhood body mass index in the observed associations.

Methods: This study was embedded in a population-based prospective cohort study among 5,310 mothers, fathers and their children. We measured maternal blood pressure in each trimester of pregnancy and paternal blood pressure once. Information about hypertensive disorders in pregnancy was obtained from medical records. We measured childhood blood pressure at the median age of 6.0 years (95% range 5.7, 8.0).

Results: Both maternal and paternal blood pressure were positively associated with childhood blood pressure (all *p*-values <0.05), with similar effect estimates. Conditional regression analyses showed that early, mid- and late pregnancy maternal blood pressure were all independent and positively associated with childhood blood pressure, with the strongest effect estimates for early pregnancy. As compared to children from mothers without hypertensive disorders in pregnancy, those from mothers with hypertensive disorders in pregnancy had 0.13 SDS (95% Confidence Interval (CI) 0.05 to 0.21) higher diastolic blood pressure. The observed associations were not materially affected by birth outcomes and childhood body mass index.

Conclusions: Both maternal and paternal blood pressure affect childhood blood pressure, independent of fetal and childhood growth measures, with the strongest effect for maternal blood pressure in early pregnancy.

INTRODUCTION

Gestational hypertension and preeclampsia affect up to 8% of all pregnant women worldwide and are associated with both maternal and offspring cardiovascular health and disease.[1–4] It has been suggested that these associations are explained by maternal vasculotoxic factors in pregnancies with hypertensive disorders, which affect vascular development.[5, 6] Moreover, early placental and fetal microvasculature maladaptations may lead to a higher blood pressure in both pregnant women and their offspring.[7] In addition to hypertensive disorders in pregnancy, higher blood pressure within the normal range during pregnancy may be associated with higher offspring blood pressure.[8–12] It is not known if the associations of maternal blood pressure with offspring blood pressure are explained by direct maternal or intra-uterine mechanisms or rather reflect shared family-based lifestyle-related or genetic factors. Comparing maternal and paternal blood pressure effects may help to disentangle the direct maternal or intra-uterine mechanisms.[13] It is also unknown which period of pregnancy is most critical for the effects of maternal blood pressure on the offspring's blood pressure. Finally, the associations of hypertensive disorders in pregnancy, with childhood blood pressure may be explained in part by lower offspring birth weight and higher body mass index (BMI).[8]

In a population-based prospective cohort study from early pregnancy onwards among 5,310 mothers, fathers and children, we examined the associations of maternal blood pressure in different periods of pregnancy and hypertensive disorders in pregnancy with blood pressure in school-aged children. The specific focus was on the comparison with paternal blood pressure effects, the identification of critical periods and the role of birth outcomes and childhood body mass index in the observed associations.

METHODS

Design and study population

This study was embedded in the Generation R Study, a population-based prospective cohort study from early pregnancy onwards in Rotterdam, the Netherlands.[14, 15] The study has been approved by the local Medical Ethics Committee. Written informed consent was obtained from the parents. All pregnant women were enrolled between 2001 and 2005. Of all eligible children in the study area, 61% participated at birth in the study. In total 8,713 initially normotensive mothers had information about blood pressure measurements available, and of those, 8,475 gave birth to singleton live-born children. In total, 5,810 (69%) of these children participated in detailed follow-up studies at the age of 6 years. We excluded children with missing blood pressure measurements (N=477) or with congenital cardiac abnormalities (N=23) leading to a population for analysis of 5,310 mothers and their children (Flowchart is given in **Supplementary Figure 2.6.1**).

Maternal and paternal blood pressure

We measured maternal and paternal blood pressure using the Omron 907 automated digital oscillometric spygmanometer (OMRON Healthcare Europe, Hoofddorp, the Netherlands).[16] As described previously, all participants were seated in an upright position with back support and were asked to relax for 5 minutes.[17] 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. For participants with an upper arm circumference exceeding 33cm, a larger cuff (32–42 cm) was used. We used the mean value of 2 blood pressure readings over a 60-second interval. Blood pressure was measured in 4,098 mothers in early pregnancy (gestational age median 13.4 (95% range 9.8–17.5) weeks), 5,006 mothers in mid-pregnancy (gestational age median 20.5 (95% range 18.5–23.5) weeks) and 5,104 mothers in late pregnancy (gestational age median 30.2 (95% range, 28.5–32.9) weeks). Three, two, and one blood pressure measurements were available for 3,842; 1,214 and 254 mothers, respectively. Of the population for analysis, blood pressure was measured during mid-pregnancy in 3,805 fathers.

Hypertensive disorders in pregnancy

Information on hypertensive disorders in pregnancy, including gestational hypertension and preeclampsia, was obtained through medical records.[18] Mothers suspected of any hypertensive disorder in pregnancy based on the records were crosschecked with original charts by a trained medical record abstractor.[18] The following criteria were used to identify women with gestational hypertension: development of systolic blood pressure of ≥140 mmHg and/or diastolic blood pressure of ≥90 mmHg after 20 weeks of gestation in previously normotensive women. These criteria and the presence of proteinuria (defined as 2 or more dipstick readings of 2 or greater, or 1 catheter sample reading of 1 or greater, or a 24-hour urine collection containing at least 300 mg of protein) were used to identify women with preeclampsia.[19]

Childhood blood pressure

Childhood 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).[20] A cuff was selected with a cuff width approximately 40% of the arm circumference and long enough to cover 90% of the arm circumference.[20] We used the mean systolic and diastolic blood pressure values using the last three blood pressure readings. Using normative values from the "Fourth report on the diagnosis, evaluation and treatment of high blood pressure in children and adolescents" from the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents,[21, 22] we calculated the standard deviation scores (SDS) for individual systolic and diastolic blood pressure values. Subsequently, we used these individual SDS to categorize children in blood pressure tertiles. Children whose average systolic and/or diastolic blood pressure based on three readings was ≥95th percentile for age, sex and height were classified as hypertensive.

Covariates

We assessed maternal and paternal age at enrollment in the study. Information on maternal and paternal ethnicity and educational level, maternal parity, folic acid supplement use, smoking and alcohol consumption was obtained by questionnaires.[14] At enrollment, we measured maternal and paternal height and weight without shoes and heavy clothing and calculated body mass index. Information on infant sex, gestational age at birth and birth weight was obtained from medical records. At the age of 6 years, we measured child height and weight and calculated body mass index.

Statistical analysis

First, we performed a non-response analysis by comparing subject characteristics between children with and without follow-up blood pressure measurements by using T-tests, Chi-square tests and Mann-Whitney tests. Second, we examined maternal longitudinal blood pressure patterns during pregnancy for mothers in tertiles of childhood blood pressure. For these analyses, we used mixed effects regression models. These regression models enable analyses on repeatedly measured outcomes, accounting for the correlation between repeated measurements within the same participant, and allowing for incomplete outcome data.[23] Details of the mixed effects regression models are given in the **Supplementary Methods**. We also examined the associations of maternal blood pressure in different periods of pregnancy and paternal blood pressure with childhood blood pressure in three linear regression models; 1) a confounder model, which included covariates selected based on their associations with the outcome of interest based on previous studies or a change in effect estimate of >10%; 2) a birth model, which included gestational age and weight at birth in addition to the confounder model; 3) a childhood model, which included child current body mass index in addition to the confounder model. We used similar multiple regression models to examine the associations of hypertensive disorders in pregnancy with childhood blood pressure. Third, we used similar linear and logistic regression models to explore the combined effects of maternal blood pressure in early and late pregnancy and the combined effects of maternal blood pressure and paternal blood pressure on childhood blood pressure and risk of hypertension. For these analyses, we created tertiles of both maternal and paternal blood pressure. Fourth, we performed conditional regression analyses to identify the independent associations of maternal blood pressure measurements in early, mid- and late pregnancy, taking into account their correlations, with childhood blood pressure and risk of hypertension.[24] We constructed blood pressure values for each trimester, which are statistically independent from blood pressure values for other trimesters, by using standardized residuals obtained from regression of blood pressure values at a specific time point (dependent variable) on blood pressure values obtained at a previous time point.[24–26] This approach enabled identification of critical periods for maternal blood pressure during pregnancy that, independent of other periods during pregnancy influenced childhood blood pressure. Details of these conditional regression models are given in the **Supplementary Methods**. To reduce potential bias associated with missing data, missing values of covariates (maternal and paternal ethnicity, educational level and body mass index, paternal age, maternal parity, folic acid supplement

use, smoking and alcohol consumption, infant birth weight and child body mass index), were multiple imputed (n=5 imputations), according to the Fully Conditional Specification method (predictive mean matching), assuming no monotone missing pattern. We report the pooled effect estimates after the multiple imputation procedure [27] Participant characteristics before and after imputation and the percentages of missing values are given in the **Supplementary Table 2.6.1**.

The multiple imputation procedure was performed using Statistical Package for the Social Sciences version 21.0. Statistical analyses were performed using the Statistical Package for the Social Sciences version 21.0 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). The mixed effects regression analyses were performed with the Statistical Analysis System, Prox Mixed module (version 9.3; SAS Institute Inc, Cary NC).

RESULTS

Subject characteristics

Table 2.6.1 shows the participant characteristics. In our cohort, 410 children (7.7%) were classified as hypertensive. Results from the non-response analysis showed that as compared to children with blood pressure follow-up measurements, those without these measurements had a lower birth weight and gestational age. Mothers of children with blood pressure measurements were older, used less alcohol, but smoked more frequently as compared to

Table 2.6.1. Subject characteristics (N=5,310)

Characteristics	
Maternal Characteristics	
Age, y	30.9 (19.7, 39.3)
Height, cm	167.5 (7.5)
Weight, kg	69.3 (12.7)
Body mass index, kg/m^2	23.5 (3.8)
Parity, n (%)	
- 0	3,008 (56.6)
- ≥1	2,302 (43.4)
Educational level mother, n (%)	
- Primary or secondary	2,854 (53.7)
- Higher	2,456 (46.3)
Ethnicity, n (%)	
- European	3,167 (59.6)
- Non-European	2,143 (40.4)
Smoking during pregnancy, n (%)	
- No	3,792 (71.4)
- Yes	1,518 (28.6)
Alcohol using during pregnancy, n (%)	
- No	2,478 (46.7)
- Yes	2,832 (53.3)
Folic acid supplements during pregnancy, n (%)	
- No	1,695 (31.9)

Table 2.6.1. Subject characteristics (N=5,310) (continued)

Characteristics	
- Yes	3,615 (68.1)
Blood pressure	
Early pregnancy	
- Gestational age, weeks	13.4 (9.8, 17.5)
- Systolic blood pressure, mmHg	115.5 (12.0)
- Diastolic blood pressure, mmHg	68.1 (9.3)
Mid- pregnancy	
- Gestational age, weeks	20.5 (18.5, 23.5)
- Systolic blood pressure, mmHg	116.8 (11.9)
- Diastolic blood pressure, mmHg	67.2 (9.3)
Late pregnancy	
- Gestational age, weeks	30.2 (28.5, 32.9)
- Systolic blood pressure, mmHg	118.4 (11.9)
- Diastolic blood pressure, mmHg	69.1 (9.2)
Hypertensive disorders in pregnancy, n (%)	
Any	308 (5.8)
Gestational hypertension	215 (4.0)
Preeclampsia	93 (1.8)
Paternal characteristics	
Age, y	33.0 (21.7, 45.2)
Height, cm	181.9 (7.7)
Weight, kg	83.7 (11.6)
Body mass index, kg/m^2	25.3 (3.2)
Ethnicity, n (%)	
- European	3,274 (61.7)
- Non-European	2,036 (38.3)
Educational level, n (%)	
- Primary or secondary	2,896 (54.5)
- Higher	2,414 (45.5)
Systolic blood pressure, mmHg	130.2 (13.5)
Diastolic blood pressure, mmHg	73.4 (10.6)
Birth characteristics	
Female, n (%)	2,656 (50.0)
Gestational age, weeks	40.1 (35.9, 42.3)
Birth weight, g	3,430 (548)
Childhood characteristics	
Age, y	6.0 (5.7, 8.0)
Height, cm	119.5 (6.1)
Weight, kg	23.3 (4.3)
Body mass index, kg/m^2	16.2 (1.9)
Systolic blood pressure, mmHg	102.7 (8.2)
Diastolic blood pressure, mmHg	60.7 (6.9)
*Z score Systolic blood pressure	0.53 (0.7)
*Z score Diastolic blood pressure	0.34 (0.6)
# Blood pressure ≥95th percentile, n (%)	410 (7.7)

Values represent means (SD), medians (95% range), or numbers (%). *Z scores of systolic and diastolic blood pressure are calculated using normative values from the "Fourth report on the diagnosis, evaluation and treatment of high blood pressure in children and adolescents" from the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents.

#Blood pressure ≥95th percentile (systolic and/or diastolic blood pressure ≥95th percentile) for age, sex and height on three measurements. Participant characteristics before and after imputation are shown **Supplementary Table 2.6.1**.

mothers of children who were lost to follow up. Moreover, maternal systolic blood pressure throughout pregnancy was lower for the children without follow up blood pressure measurements (**Supplementary Table 2.6.2**).

Maternal and paternal blood pressure and childhood blood pressure

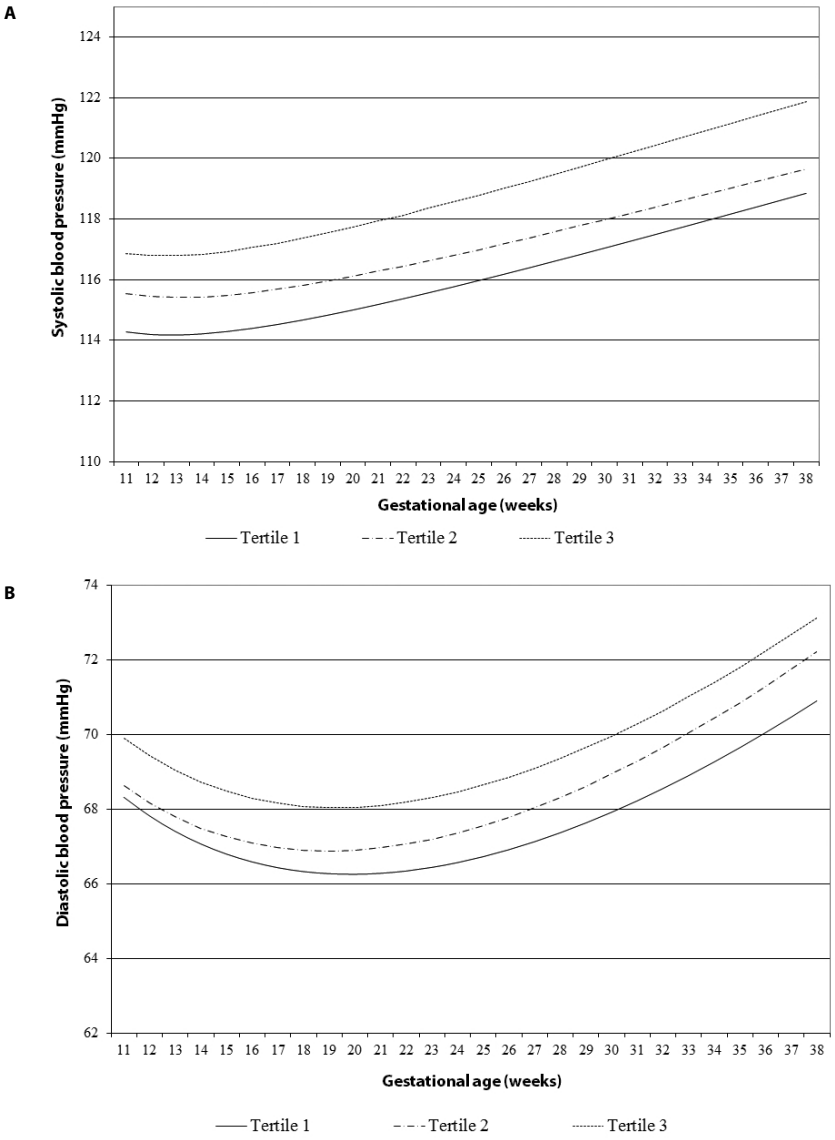
Figure 2.6.1 shows that children in the highest tertile of systolic blood pressure had mothers with higher systolic blood pressure throughout pregnancy than children in the lowest tertile of systolic blood pressure. For each tertile of childhood blood pressure, maternal blood pressure increased with advanced gestational age. There was no significant difference in the slope of maternal systolic blood pressure between the tertiles of their children blood pressure. For all childhood diastolic blood pressure tertiles, maternal diastolic blood pressure had a mid-pregnancy dip, with an increase thereafter. Diastolic blood pressure was highest throughout pregnancy for mothers of children in the highest tertile. The exact corresponding regression coefficients for gestational age-independent (intercept) and gestational age dependent differences (interaction childhood blood pressure and gestational age) are given in **Supplementary Table 2.6.3**. Additional analyses showed that higher maternal blood pressure in early, mid- and late pregnancy and paternal blood pressure were all separately associated with higher childhood blood pressure (all *p*-values <0.05). The effect estimates for mother and father were similar and not affected by birth outcomes or childhood body mass index (**Supplementary Table 2.6.4**).

Figure 2.6.2A shows the combined associations of maternal blood pressure during early and late pregnancy. Compared with children from mothers with a blood pressure in the lowest tertiles during both early and late pregnancy, those with a blood pressure in the highest tertiles during both early and late pregnancy had 0.24 SDS (95% Confidence Interval (CI) 0.16, 0.31) and 0.18 SDS (95% CI 0.11, 0.24) higher systolic and diastolic blood pressure, respectively. In addition, within each tertile of maternal early pregnancy blood pressure, maternal late pregnancy blood pressure was associated with a higher childhood blood pressure with the strongest effect estimates in early pregnancy. **Figure 2.6.2B** shows the combined associations of maternal early pregnancy and paternal blood pressure. Compared with children of mothers and fathers with a blood pressure in the lowest tertiles, those from mothers and fathers with a blood pressure in the highest tertiles had 0.26 SDS (95% CI 0.17, 0.37) and 0.19 SDS (95% CI 0.11, 0.28) higher systolic and diastolic blood pressure, respectively. Results from the confounder and birth models for these stratified analyses are given in **Supplementary Figure 2.6.2(A-D)**. None of the statistical interactions terms were significant.

Maternal and paternal blood pressure and childhood hypertension

Figure 2.6.3A shows the results of the combined associations of maternal blood pressure during early and late pregnancy with the risk of childhood hypertension. Children of mothers with a systolic and diastolic blood pressure in the highest tertiles during both early and late pregnancy had a higher risk of hypertension: Odds Ratio (OR) 2.66 (95% CI 1.71, 4.13) and 1.63 (95% CI 1.09, 2.46), respectively, as compared to children from mothers with a systolic and diastolic blood pressure in the lowest tertiles during both early and late pregnancy. **Fig-**

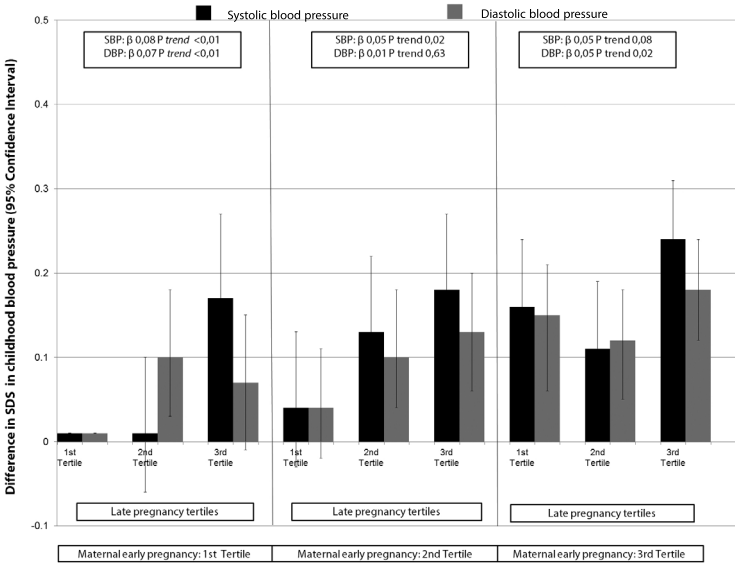
Figure 2.6.1. Maternal blood pressure patterns from children in different blood pressure tertiles (N=5,310)



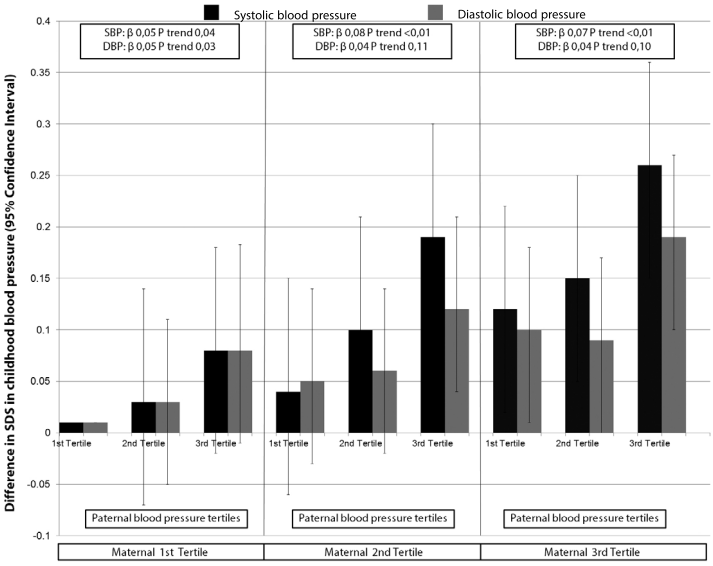
Maternal blood pressure pattern per childhood blood pressure tertile. (A) Systolic blood pressure. Difference in maternal systolic blood pressure (mmHg) between childhood systolic blood pressure tertiles based on mixed effects regression models. Model: Maternal systolic blood pressure = $\beta_0 + \beta_1$* child systolic blood pressure tertile + β_2 * gestational age + β_3 * gestational age^{-2} + β_4 * child systolic blood pressure tertile* gestational age. (B) Diastolic blood pressure. Difference in maternal diastolic blood pressure (mmHg) for childhood diastolic blood pressure tertiles based on mixed effects regression analysis. Model: Maternal diastolic blood pressure = $\beta_0 + \beta_1$* child diastolic blood pressure tertile + β_2 * gestational age + β_3 * gestational age$^{0.5}$ + β_4 * child diastolic blood pressure tertile * gestational age. Effect estimates (95% confidence intrevals) are given in **Supplementary Table 2.6.3.**

Figure 2.6.2. Combined associations of maternal and paternal blood pressure with childhood blood pressure (N=5,310)

A. **Maternal blood pressure in early and late pregnancy**



B. **Maternal and paternal blood pressure**

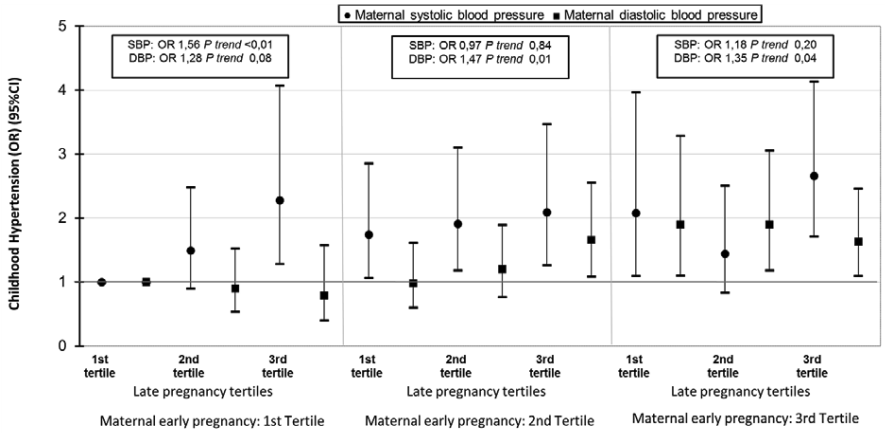


Values are regression coefficients (95% confidence intervals) from multiple linear regression models. Estimates are based on multiple imputed data. Estimates regarding childhood systolic blood pressure are assessed by combining parental systolic blood pressure tertiles. Estimates regarding childhood diastolic blood pressure are assessed by combining parental diastolic blood pressure tertiles. The interaction term of maternal late and early pregnancy blood pressure and the interaction term of maternal and paternal blood pressure were not statistically significant.

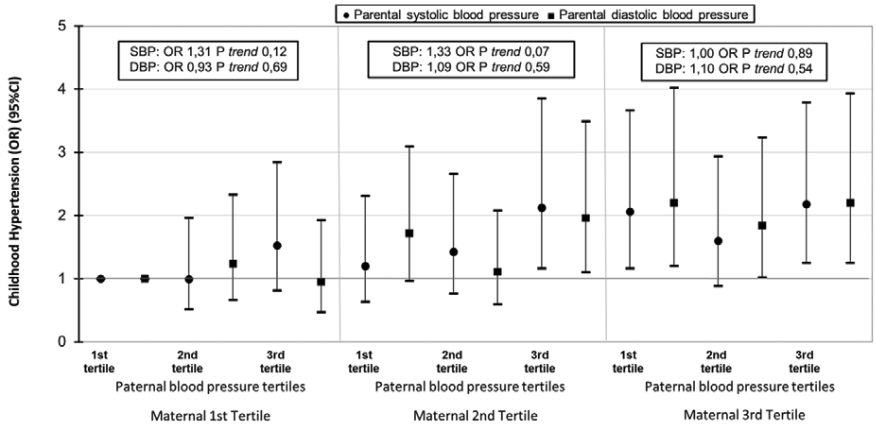
ure 2.6.3B shows the combined associations of maternal early pregnancy and paternal blood pressure with the risk of childhood hypertension. Children of mothers and fathers with blood pressure in the highest tertiles had a higher risk of having hypertension OR 2.18 (95% CI 1.25, 3.79) and 2.20 (95% CI 1.25, 3.93), respectively for systolic and diastolic blood pressure, as

Figure 2.6.3. Combined associations of maternal and paternal blood pressure with childhood hypertension (N=5,310)

A. **Maternal blood pressure in early and late pregnancy**



B. **Maternal and paternal blood pressure**



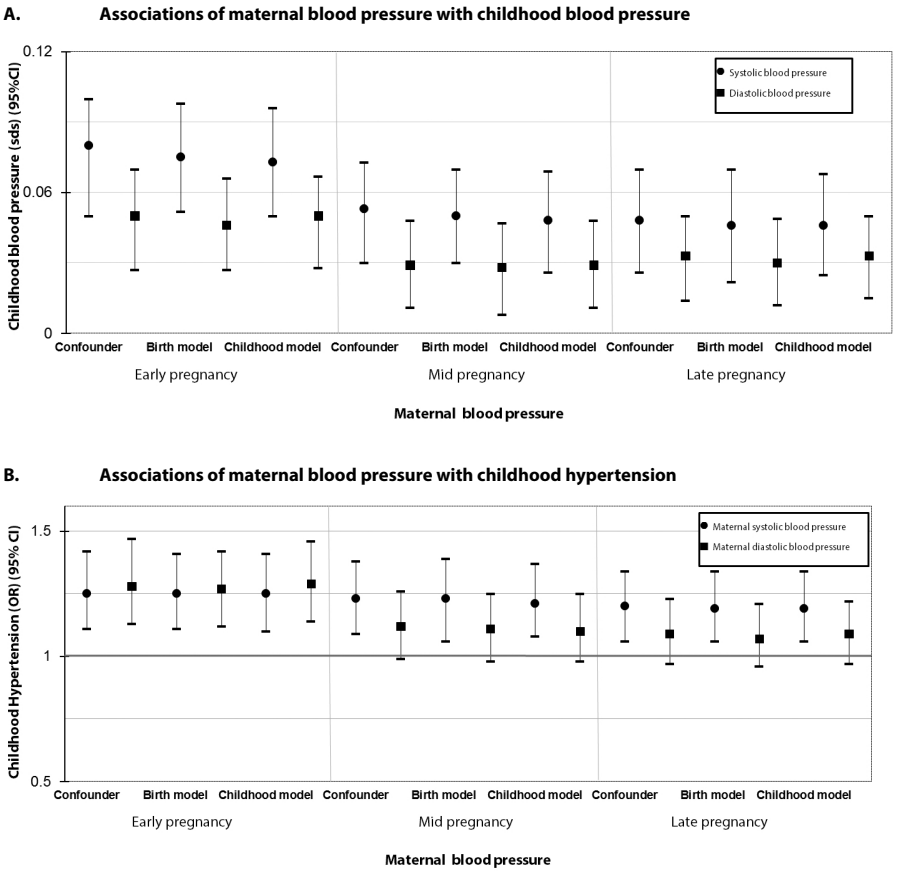
Values are regression coefficients (95% confidence intervals) from logistic regression models. Estimates are based on multiple imputed data. Estimates regarding childhood hypertension are assessed by combining parental systolic and diastolic blood pressure tertiles, respectively.

compared to children from mothers and fathers with a systolic and diastolic blood pressure in the lowest tertiles.

Critical periods of maternal blood pressure for childhood blood pressure and hypertension

Figure 2.6.4A shows that maternal blood pressure in early, mid- and late pregnancy were all independently associated with childhood blood pressure (all *p*-values <0.05). The strongest effect estimates were observed for early pregnancy maternal blood pressure (differences in

Figure 2.6.4. Associations of maternal blood pressure with childhood blood pressure and hypertension from conditional regression models (N=5,310)



Values are linear (A) and logistic (B) regression coefficients (95% confidence intervals) that reflect the difference in childhood systolic and diastolic blood pressure per standardized residual for maternal blood pressure during each trimester of pregnancy independent from previous trimesters blood pressure measurements. Confounder models are adjusted for maternal age, pre-pregnancy body mass index, ethnicity, parity, educational level, smoking during pregnancy, alcohol consumption and folic acid supplement intake. Birth models are confounder models additionally adjusted for birth weight and gestational age. Childhood models are confounder models additionally adjusted for child current body mass index.

childhood systolic and diastolic blood pressure 0.08 SDS (95% CI 0.05, 0.10) and 0.05 SDS (95% CI 0.03, 0.07) per standardized residual increase in maternal systolic and diastolic blood pressure).

Figure 2.6.4B shows that maternal systolic blood pressure, but not diastolic, in early, mid- and late pregnancy were all independently associated with the risk of childhood hypertension (all *p*-values <0.05). The strongest effect estimates were observed for early pregnancy maternal blood pressure (OR in childhood risk of hypertension 1.25 (95% CI 1.11, 1.42) per standardized residual increase in maternal systolic blood pressure).

Hypertensive disorders in pregnancy and childhood blood pressure

Table 2.6.2 shows that as compared to children from mothers without hypertensive disorders in pregnancy, those from mothers with hypertensive disorders in pregnancy had a higher diastolic, but not systolic, blood pressure. These associations were mainly driven by gestational hypertension (difference in diastolic blood pressure 0.13 SDS (95% Confidence Interval (CI) 0.05, 0.21) between children from mothers with and without gestational hypertension). Preeclampsia was not associated with childhood blood pressure.

Table 2.6.2. Associations of hypertensive disorders in pregnancy with childhood blood pressure (N=5,310)

	Childhood blood pressure (SDS)		
	Confounder model	**Birth model**	**Childhood model**
None (N=4,888)	Reference	Reference	Reference
Any complications (N=308)			
Childhood systolic blood pressure	0.07 (-0.02, 0.15)	0.03 (-0.05, 0.12)	0.06 (-0.02, 0.14)
Childhood diastolic blood pressure	**0.10 (0.02, 0.17)****	**0.08 (0.01, 0.15)***	**0.10 (0.02, 0.17)****
Gestational hypertension (N=215)			
Childhood systolic blood pressure	0.06 (-0.04, 0.15)	0.04 (-0.06, 0.13)	0.06 (-0.04, 0.15)
Childhood diastolic blood pressure	**0.13 (0.05, 0.21)****	**0.11 (0.03, 0.19)****	**0.13 (0.05, 0.21)****
Preeclampsia (N=93)			
Childhood systolic blood pressure	0.14 (-0.01, 0.28)	0.06 (-0.08, 0.21)	0.14 (-0.01, 0.28)
Childhood diastolic blood pressure	0.03 (-0.09, 0.15)	-0.01 (-0.13, 0.11)	0.03 (-0.09, 0.15)

Values are regression coefficients (95% confidence intervals) based on multiple linear regression models. Estimates are based on multiple imputed data.
Pregnancies without gestational hypertension or preeclampsia were taken as reference category. Confounder models are adjusted for maternal age, pre-pregnancy body mass index, ethnicity, parity, educational level, smoking during pregnancy, alcohol consumption, and folic acid supplement intake.
Birth models are confounder models additionally adjusted for gestational age at birth and birthweight.
Childhood models are confounders models additionally adjusted for childhood current body mass index. *P <0.05 **P <0.01

DISCUSSION

In this population-based prospective cohort study, we observed that both higher maternal blood pressure throughout pregnancy and paternal blood pressure are associated with higher childhood blood pressure. Early, mid- and late pregnancy maternal blood pressure

were all independently associated with childhood blood pressure, with the strongest effect estimates for early pregnancy. Gestational hypertension was associated with higher childhood diastolic blood pressure. The observed associations were largely independent from fetal and childhood growth measures.

Methodological considerations

A major strength of our study is the prospective design from early pregnancy onwards within a large population-based cohort. Furthermore, we measured maternal blood pressure in different pregnancy periods. Not all mothers had blood pressure measurements in each trimester of pregnancy. Restricting our analyses to mothers who had blood pressure measurements in all three trimesters (N=3,842), revealed similar results as in the full group. Of all children from mothers with information about blood pressure and pregnancy complications 65% participated in the follow-up measurements at the age of 6 years and had blood pressure information available. Compared to children with blood pressure follow-up measurements, those without follow-up measurements had mothers with lower systolic blood pressure throughout pregnancy and had lower weight at birth and younger gestational age at birth. A selective loss to follow-up may have reduced variation in blood pressure development and therefore reduced the power to detect differences. Moreover, loss to follow-up would lead to selection bias if the associations of maternal blood pressure with childhood blood pressure would be different between those included and those not included in the final analyses. Although we do not expect this likely, selection bias cannot be excluded. Blood pressure has a large within-participant variation and is liable to measurement error. This measurement error may have led to an underestimation of the observed effect estimates.[17] Furthermore, the number of cases with hypertensive disorders in pregnancy was relatively small, which might have led to lack of power for the associations of hypertensive disorders in pregnancy with childhood blood pressure. Family history of hypertension may also influence childhood blood pressure. Unfortunately, information about family history of hypertension was available for only a small subset of our cohort. Finally, although we performed adjustment for a large number of potential maternal and paternal confounders, residual confounding by other socioeconomic or lifestyle related factors might still be present, as in any observational study.

Interpretation of main findings

We hypothesized that higher maternal blood pressure within the normal range during pregnancy and hypertensive disorders in pregnancy influence blood pressure development in childhood. This hypothesis is based on previous studies suggesting that hypertensive disorders in pregnancy are associated with higher offspring blood pressure.[2, 8, 9, 28] A study among 6,343 mother-children pairs in the United Kingdom, showed that gestational hypertension, but not preeclampsia, was associated with a higher blood pressure in children aged 9 years.[8] A systemic review and meta-analyses, with data from 18 studies, showed that children of mothers with preeclampsia had a higher blood pressure in childhood and early adulthood.[28] A recent study from the United Kingdom suggested that children of mothers with hypertensive disorders in pregnancy had higher blood pressure at the ages of 7 to 18

years.[12] In line with the results of these previous studies, we observed that higher maternal blood pressure during pregnancy was associated with a higher blood pressure in children aged 6 years. Children from mothers with hypertensive disorders in pregnancy had a higher diastolic blood pressure, compared with children of mothers without hypertensive disorders in pregnancy. These associations were driven mainly by gestational hypertension, and not present for preeclampsia. Consequently, results from both previous and our study suggest that maternal blood pressure during pregnancy affect childhood blood pressure. Nevertheless, not much is known about the specific maternal and paternal effects, critical periods, and role of fetal and childhood growth in the associations.

In the current study, we observed that both, maternal and paternal blood pressure were associated with childhood blood pressure and risk of hypertension. In addition, within each tertile of maternal blood pressure, higher paternal blood pressure was associated with childhood blood pressure. Only a few previous studies have explored the effect of maternal and paternal blood pressure on childhood blood pressure and risk of hypertension.[29–31] These studies suggest that both higher maternal and paternal blood pressure levels are associated with an increased risk for higher childhood blood pressure in offspring.[29, 31] The presence of hypertension in both parents has additive effects on childhood blood pressure levels.[29, 32]A recent study suggested that children from hypertensive parents had a higher risk of hypertension.[32] Similar associations for maternal and paternal blood pressure suggest that genetic or shared family based factors, rather than direct intra-uterine programming, may explain the associations of maternal blood pressure with childhood blood pressure.[13] Our results suggest that both maternal and paternal blood pressure are important, at similar magnitude, for childhood blood pressure.

We aimed to identify critical periods during pregnancy that impact childhood blood pressure. Our results suggest that early, mid- and late pregnancy are all independently associated with childhood blood pressure. Differences between early, mid- and late pregnancy were small, but slightly stronger effect estimates were observed for early pregnancy. A recent study from a prospective cohort in the United Kingdom, also showed that early pregnancy appeared to be the period during pregnancy with the most influence on childhood blood pressure.[12] Some mechanisms have been hypothesized to underlie the association of maternal blood pressure levels during early pregnancy with blood pressure levels in offspring.[7] Higher maternal blood pressure in early pregnancy may be a marker of maternal and placental vascular maladaptation's,[33] leading to fetal growth restriction and abnormal fetal vascular development,[34] that may subsequently affect childhood blood pressure.[35] In addition, higher maternal blood pressure in early pregnancy may be predictors of hypertensive disorders in pregnancy that, in turn, may be predictors of maternal and offspring cardiovascular diseases later in life. Consequently, although blood pressure in each period of pregnancy seems to be independently associated with childhood blood pressure, early pregnancy in particular may be critical for childhood blood pressure.

Consistent evidence suggests that preterm birth and low birth weight are associated with childhood blood pressure, although the effects seem to be small.[36, 37] Moreover, BMI is one of the strongest predictors of blood pressure in childhood.[26] Consequently, associations of

maternal blood pressure with childhood pressure may be partly explained by preterm birth, low birth weight and high BMI. We observed, however, that the effect estimates of parental blood pressure or hypertensive disorders in pregnancy with childhood blood pressure did not materially change after additional adjustment for birth outcomes or childhood BMI. We also explored whether including size at birth for gestational age, instead of birth weight, would affect the results, but this was not the case. These findings are in line with the large study from the United Kingdom, showing that the effects of hypertensive disorders in pregnancy on childhood blood pressure were largely independent from maternal and childhood obesity.[2] Current results suggest that the associations of parental blood pressure and hypertensive disorders in pregnancy with childhood blood pressure are not explained by fetal and childhood growth measures.

The prevalence of hypertension in children and adolescents in the Western countries has been reported at 1% to 5%.[38] In addition to the already known childhood risk factors (eg, BMI) for developing primary hypertension, other parental factors should be considered in screening guidelines.[38] Young offspring of mothers, who had high blood pressure in early pregnancy or gestational hypertension may compose specific groups at risk for having a high blood pressure from childhood onwards. Whether these findings can be translated to primary prevention strategies for primary hypertension in children and adolescents should be further studied.

CONCLUSION

In summary, our results suggest that both higher maternal blood pressure throughout pregnancy and paternal blood pressure influence childhood blood pressure. Early, mid- and late pregnancy maternal blood pressure were all independently associated with childhood blood pressure, with the strongest effect estimates for early pregnancy. The observed associations were largely independent of fetal and childhood growth measures. Further follow-up studies are needed to investigate whether parental blood pressure and hypertensive disorders in pregnancy affect cardiovascular risk at older ages.

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Supplementary Methods. Statistical analyses

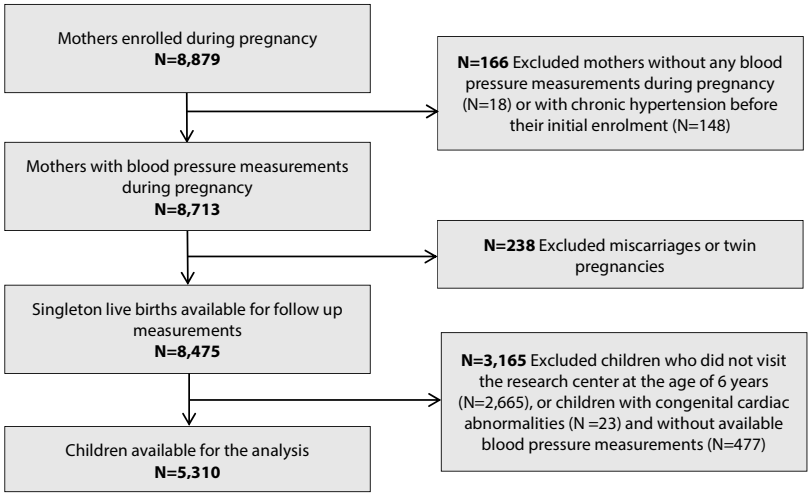
Mixed effects regression models. We used unbalanced repeated measurement regression models to examine maternal longitudinal blood pressure patterns in tertiles of childhood blood pressure. These models take the correlation between repeated measurements within the same subject into account by modelling the correlated errors of these measurements.[1] Both gestational age-independent (difference constant over time) and gestational age-dependent (difference not-constant over time) effects were assessed. We constructed best-fitting models for maternal blood pressure patterns. We started with a linear model and examined whether adding second-degree fractional polynomial of gestational age improved the models by comparing the deviances and goodness of fit. Since adding fractional polynomials of gestational age to the model improved the model fit, we included these fractional polynomials in the final models. We used a compound symmetry covariance structure. Childhood blood pressure in tertiles were included in these models as intercept and as an interaction term with gestational age. The final models can be written as:

Maternal systolic blood pressure. Difference in maternal systolic blood pressure (mmHg) between childhood systolic blood pressure tertiles based on repeated measurement regression analysis = $\beta_o + \beta_1$* child systolic blood pressure tertile + β_2 * gestational age + β_3 * gestational age^{-2} + β_4 * child systolic blood pressure tertile* gestational age.

Maternal diastolic blood pressure. Difference in maternal diastolic blood pressure (mmHg) for childhood diastolic blood pressure tertiles based on repeated measurement analysis = β_o + β_1* child diastolic blood pressure tertile + β_2 * gestational age + β_3 * gestational age$^{0.5}$ + β_4 * child diastolic blood pressure tertile * gestational age.

Conditional regression analyses. We performed conditional regression analyses to identify the independent associations of first, second and third trimester maternal blood pressure, taking into account their correlations, with childhood blood pressure.[2] We constructed blood pressure values for each trimester, which are statistically independent from blood pressure values for other trimesters, by using standardized residuals obtained from regression of blood pressure values at a specific time point (dependent variable) on blood pressure values obtained at a previous time point.[2–4] These standardized residuals, which are assumed to be independent of the estimated regression line (and thus from the previous blood pressure), were taken forward to the regression models as independent variable with childhood blood pressure (dependent variable). As conditional blood pressure measurements are statistically independent of each other, this approach allows inclusion of blood pressure values from different trimesters simultaneously in one linear regression model when continuous childhood blood pressure was the outcome, or in one logistic regression model when childhood hypertension was the outcome. For the conditional analyses, we imputed maternal blood pressure measures. Results from these datasets were pooled and presented in the conditional results.

Supplementary Figure 2.6.1. Flowchart of the study participants



Supplementary Table 2.6.1. Subject characteristics in the original and imputed dataset (N=5,310)

	Observed	Imputed
Maternal characteristics		
Age, y	30.9 (19.7, 39.3)	30.9 (19.7, 39.3)
Height, cm	167.5 (7.4)	167.5 (7.5)
Missing, n (%)	19 (3.6)	
Weight, kg	69.3 (12.8)	69.3 (12.7)
Missing, n (%)	18 (3.4)	
Body mass index, kg/m^2	24.7 (4.3)	24.7 (4.3)
Missing, n (%)	36 (6.8)	
Parity, n (%)		
- 0	2,990 (56.3)	3,008 (56.6)
- ≥1	2,280 (42.9)	2,302 (43.4)
Missing, n (%)	40 (0.8)	
Educational level mother, n (%)		
- Primary or secondary	2,673 (50.3)	2,854 (53.7)
- Higher	2,295 (43.2)	2,456 (46.3)
Missing, n (%)	342 (6.5)	
Ethnicity, n (%)		
- European	3,135 (59.0)	3,167 (59.6)
- Non-European	2,059 (38.8)	2,143 (40.4)
Missing, n (%)	116 (2.2)	
Smoking during pregnancy, n (%)		
- No	3,476 (65.4)	3,792 (71.4)
- Yes	1,224 (23.1)	1,518 (28.6)
Missing, n (%)	610 (11.5)	
Alcohol using during pregnancy, n (%)		
- No	2,138 (40.3)	2,478 (46.7)
- Yes	2,512 (47.3)	2.832 (53.3)
Missing, n (%)	660 (12.4)	
Folic acid supplements during pregnancy, n (%)		
- No	1,017 (19.2)	1,695 (31.9)
- Yes	3,023 (56.9)	3,615 (68.1)
Missing, n (%)	1,270 (23.9)	
Blood pressure		
Early pregnancy		
- Gestational age, weeks	13.2 (9.8, 17.4)	13.4 (9.8, 17.5)
- Systolic blood pressure, mmHg	115.5 (12.0)	115.5 (12.0)
- Diastolic blood pressure, mmHg	68.1 (9.3)	68.1 (9.3)
Mid- pregnancy		
- Gestational age, weeks	20.5 (18.5, 23.5)	20.5 (18.5, 23.5)
- Systolic blood pressure, mmHg	116.8 (11.9)	116.8 (11.9)
- Diastolic blood pressure, mmHg	67.2 (9.3)	67.2 (9.3)
Late pregnancy		
- Gestational age, weeks	30.2 (28.5, 32.9)	30.2 (28.5, 32.9)
- Systolic blood pressure, mmHg	118.4 (11.9)	118.4 (11.9)
- Diastolic blood pressure, mmHg	69.1 (9.2)	69.1 (9.2)
Hypertensive disorders in pregnancy, n (%)		
Any	308 (5.8)	308 (5.8)

Supplementary Table 2.6.1. Subject characteristics in the original and imputed dataset (N=5,310) (continued)

	Observed	Imputed
Gestational hypertension	215 (4.0)	215 (4.0)
Preeclampsia	93 (1.8)	93 (1.8)
Paternal characteristics		
Age, y	33.0 (22.3, 45.8)	33.0 (21.7, 45.2)
Missing, n (%)	1,284 (24.2)	
Height, cm	182.1 (7.9)	181.9 (7.7)
Missing, n (%)	1,289 (24.3)	
Weight, kg	83.9 (13.0)	83.7 (11.6)
Missing, n (%)	1,286 (24.2)	
Body Mass Index, m/kg^2	25.3 (3.4)	25.3 (3.2)
Missing, n (%)	1,293 (24.4)	
Ethnicity, n (%)		
- European	2,736 (51.5)	3,274 (61.7)
- Non-European	1,155 (21.8)	2,036 (38.3)
Missing, n (%)	1,419 (26.7)	
Educational level father, n (%)		
- Primary or secondary	1,661 (31.3)	2,896 (54.5)
- Higher	1,841 (34.7)	2,414 (45.5)
Missing, n (%)	1,808 (34.0)	
Systolic blood pressure, mmHg	130.2 (13.5)	130.2 (13.5)
Diastolic blood pressure, mmHg	73.4 (10.6)	73.4 (10.6)
Birth characteristics		
Female, n (%)	2,656 (50.0)	2,656 (50.0)
Gestational age, weeks	40.1 (35.9, 42.3)	40.1 (35.9, 42.3)
Birth weight, g	3,431 (548)	3,430 (548)
Missing, n (%)	8 (0.1)	
Small size for gestational age, n (%)	267 (5.0)	267 (5.0)
Appropriate size for gestational age, n (%)	4,777 (89.9)	4,777 (89.9)
Childhood characteristics		
Age, y	6.0 (5.7, 8.0)	6.0 (5.7, 8.0)
Height, cm	119.5 (6.0)	119.5 (6.1)
Missing, n (%)	7 (0.1)	
Weight, kg	23.3 (4.3)	23.3 (4.3)
Missing, n (%)	7 (0.1)	
Body mass index, kg/m^2	16.2 (1.9)	16.2 (1.9)
Missing, n (%)	7 (0.1)	
Systolic blood pressure, mmHg	102.7 (8.2)	102.7 (8.2)
Diastolic blood pressure, mmHg	60.7 (6.9)	60.7 (6.9)
*Z score Systolic blood pressure	0.53 (0.7)	0.53 (0.7)
*Z score Diastolic blood pressure	0.34 (0.6)	0.34 (0.6)
[#] Blood pressure ≥95th percentile, n (%)	410 (7.7)	410 (7.7)

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution.

*Z scores of systolic and diastolic blood pressure are calculated using normative values from the "Fourth report on the diagnosis, evaluation and treatment of high blood pressure in children and adolescents" from the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents.

[#]Blood pressure ≥95th percentile (systolic and/or diastolic blood pressure ≥95th percentile) for age, height and gender on repeated measurement.

Supplementary Table 2.6.2. Subject characteristics in children with and without follow-up blood pressure measurements (N=8,452)

	N=5,310	N=3,142	*p*-value
Maternal characteristics			
Age, y	30.9 (19.7, 39.3)	28.9 (18.5, 39.0)	**<0.01**
Height, cm	167.5 (7.4)	166.4 (7.4)	**<0.01**
Weight, kg	69.3 (12.8)	69.1 (13.6)	0.55
Body mass index, kg/m^2	23.5 (4.1)	23.6 (4.5)	0.55
Parity (%)			
- 0	2,990 (56.3)	1,663 (54)	**0.02**
- ≥1	2,280 (42.9)	1,414 (46)	
Educational level mother, n (%)			**<0.01**
- Primary or secondary	2,673 (50.3)	1,781 (56.7)	
- Higher	2,295 (43.2)	936 (39.8)	
Ethnicity, n (%)			**<0.01**
- European	3,135 (59.0)	1,407 (44.8)	
- Non-European	2,059 (38.8)	1,386 (44.1)	
Smoking during pregnancy, n (%)			**<0.01**
- No	3,476 (65.4)	1,887 (60.1)	
- Yes	1,224 (23.1)	783 (24.9)	
Alcohol using during pregnancy, n (%)			**<0.01**
- No	2,138 (40.3)	1,471 (46.8)	
- Yes	2,512 (47.3)	1,152 (36.7)	
Folic acid supplements during pregnancy, n (%)			**<0.01**
- No	1,017 (19.2)	823 (26.2)	
- Yes	3,023 (56.9)	1,393 (44.3)	
Blood pressure			
Early pregnancy			
Gestational age, weeks	13.2 (9.8, 17.4)	13.4 (9.5, 17.6)	**<0.01**
Systolic blood pressure, mmHg	115.5 (12.0)	114.7 (12.1)	**<0.01**
Diastolic blood pressure, mmHg	68.1 (9.3)	67.7 (9.3)	0.08
Mid- pregnancy			
Gestational age, weeks	20.5 (18.5, 23.5)	20.4 (18.5, 23.9)	0.07
Systolic blood pressure, mmHg	116.8 (11.9)	115.8 (11.8)	**<0.01**
Diastolic blood pressure, mmHg	67.2 (9.3)	66.6 (9.0)	**0.01**
Late pregnancy			
Gestational age, weeks	30.2 (28.5, 32.9)	30.3 (28.4, 32.9)	**0.02**
Systolic blood pressure, mmHg	118.4 (11.9)	117.4 (12.0)	**<0.01**
Diastolic blood pressure, mmHg	69.1 (9.2)	68.5 (9.2)	**<0.01**
Hypertensive disorders in pregnancy, n (%)			
Any	305 (5.8)	160 (5.1)	0.58
Gestational hypertension	215 (4.0)	95 (3.0)	**0.03**
Preeclampsia	93 (1.8)	65 (2.1)	**0.02**
Paternal characteristics			
Age, y	33.0 (22.3, 45.8)	31.8 (21.0, 44.5)	**<0.01**
Height, cm	182.1 (7.9)	181.8 (7.7)	**<0.01**

Supplementary Table 2.6.2. Subject characteristics in children with and without follow-up blood pressure measurements (N=8,452) (continued)

	N=5,310	N=3,142	*p*-value
Weight, kg	83.9 (13.0)	83.6 (11.6)	**<0.01**
Body Mass Index, m/kg^2	25.3 (3.4)	25.3 (3.6)	0.59
Ethnicity, n (%)			**<0.01**
- European	2,736 (51.5)	1,150 (36.6)	
- Non-European	1,155 (21.8)	697 (22.2)	
Educational level father, n (%)			**<0.01**
- Primary or secondary	1,661 (31.3)	818 (26.0)	
- Higher	1,841 (34.7)	722 (23.0)	
Systolic blood pressure, mmHg	130.2 (13.5)	129.8 (13.6)	0.25
Diastolic blood pressure, mmHg	73.4 (10.6)	72.9 (10.9)	0.12
Birth characteristics			
Female, n (%)	2,656 (50.0)	1,534 (48.9)	0.30
Gestational age, weeks	40.1 (35.9, 42.3)	40.0 (35.0, 42.4)	**<0.01**
Birth weight, g	3,431 (548)	3,387 (576)	**<0.01**

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. Differences in subject characteristics comparing the groups with and without blood pressure measurements were evaluated using T-tests for continuous normally distributed variables, Mann Whitney for non-normally distributed variables, and Chi-squared tests for categorical variables.

Supplementary Table 2.6.3. Effect estimates from the longitudinally measured maternal blood pressure and childhood blood pressure

	Difference in systolic blood pressure			
Childhood blood pressure	**Intercept** (mmHg)	***P*-value**[b]	**Slope** (mmHg/week of gestation)	***P*-value**[b]
Tertile 1	109.6	*P* <0.001	-0.02	*P* = 0.51
Tertile 2	111.1	*P* = 0.15	-0.03	*P* = 0.17
Tertile 3	112.0	*P* <0.001	*Reference*	
	Difference in diastolic blood pressure			
	Intercept (mmHg)	***P*-value**[b]	**Slope** (mmHg/week of gestation)	***P*-value**[b]
Tertile 1	97.7	*P* <0.001	-0.02	*P* = 0.22
Tertile 2	97.8	*P* <0.001	0.01	*P* = 0.48
Tertile 3	99.1	*P* <0.001	*Reference*	

[a] Values are based on mixed effects regression models and reflect the change in maternal blood pressure during pregnancy in mmHg per tertile of childhood blood pressure compared to the reference group of children in the highest tertile.
[b] P–value reflects the significance level of the estimate.

Supplementary Table 2.6.4. Associations of maternal and paternal blood pressure during pregnancy with childhood blood pressure (N=5,310)

	Childhood blood pressure (SDS)		
	Confounder model	**Birth model**	**Childhood model**
Maternal blood pressure (SDS)			
Early pregnancy (N=4,098)			
Systolic blood pressure	0.07 (0.05, 0.09)**	0.07 (0.05, 0.09)**	0.07 (0.04, 0.09)**
Diastolic blood pressure	0.04 (0.02, 0.06)**	0.04 (0.02, 0.06)**	0.04 (0.02, 0.06)**
Mid- pregnancy (N=5,006)			
Systolic blood pressure	0.08 (0.06, 0.10)**	0.08 (0.06, 0.10)**	0.07 (0.05, 0.09)**
Diastolic blood pressure	0.05 (0.04, 0.07)**	0.05 (0.03, 0.07)**	0.05 (0.04, 0.07)**
Late pregnancy (N=5,104)			
Systolic blood pressure	0.08 (0.06, 0.10)**	0.08 (0.06, 0.10)**	0.08 (0.06, 0.10)**
Diastolic blood pressure	0.06 (0.04, 0.08)**	0.06 (0.04, 0.08)**	0.06 (0.04, 0.08)**
Paternal blood pressure *(N=3,805)*			
Systolic blood pressure	0.05 (0.03, 0.08)**	0.05 (0.03, 0.08)**	0.06 (0.04, 0.08)**
Diastolic blood pressure	0.06 (0.04, 0.08)**	0.05 (0.03, 0.07)**	0.06 (0.04, 0.08)**

Values are regression coefficients (95% confidence intervals) based from multiple linear regression models. Estimates are based on multiple imputed data.

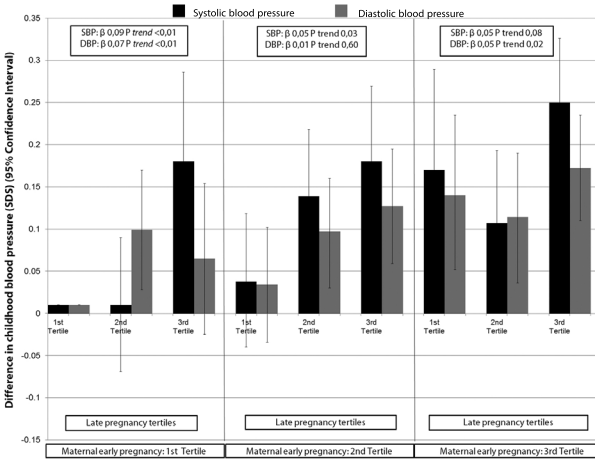
Confounder model is focused on maternal blood pressure are adjusted for maternal age, gestational age at measurement, pre-pregnancy body mass index, parity, ethnicity, educational level, smoking and alcohol consumption during pregnancy, and folic acid intake; Models focused on paternal blood pressure are adjusted for paternal age, body mass index, ethnicity, and educational level.

Birth models are confounder models additionally adjusted for gestational age at birth and birth weight.

Childhood models are confounders models additionally adjusted for childhood current body mass index. *P <0.05 **P <0.01

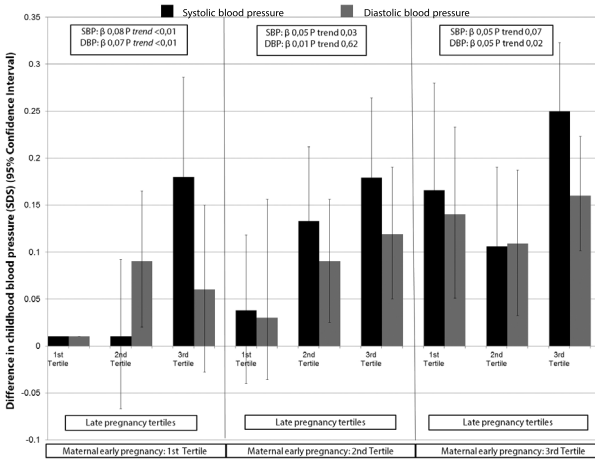
Supplementary Figure 2.6.2. Combined associations of maternal and paternal blood pressure with childhood blood pressure, confounder and birth models (N=5,310)

A. Maternal blood pressure in early and late pregnancy, confounder model



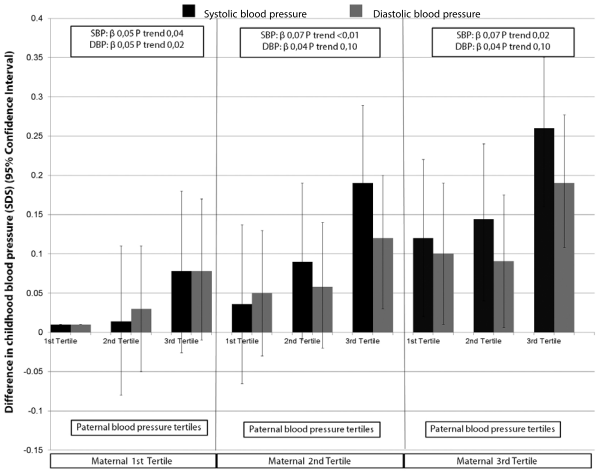
Values are regression coefficients (95% confidence intervals) from multiple linear regression models. Estimates are based on multiple imputed data. Models are adjusted for maternal age, gestational age at measurement, pre-pregnancy body mass index, parity, ethnicity, educational level, smoking and alcohol consumption during pregnancy, folic acid supplement intake. Estimates regarding childhood systolic blood pressure are assessed by combining maternal early pregnancy with late pregnancy systolic blood pressure tertiles. Estimates regarding childhood diastolic blood pressure are assessed by combining maternal early with late pregnancy diastolic blood pressure tertiles.

B. Maternal blood pressure in early and late pregnancy, birth model



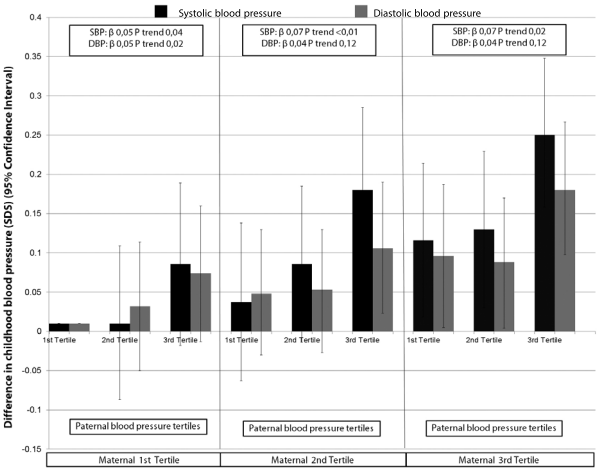
Values are regression coefficients (95% confidence intervals) from multiple linear regression models. Estimates are based on multiple imputed data. Models are adjusted for maternal age, gestational age at measurement, pre-pregnancy body mass index, parity, ethnicity, educational level, smoking and alcohol consumption during pregnancy, folic acid supplement intake, birthweight and gestational age at birth. Estimates regarding childhood systolic blood pressure are assessed by combining maternal early pregnancy with late pregnancy systolic blood pressure tertiles. Estimates regarding childhood diastolic blood pressure are assessed by combining maternal early with late pregnancy diastolic blood pressure tertiles.

C. Maternal and paternal blood pressure, confounder model



Values are regression coefficients (95% confidence intervals) based from multiple linear regression models. Estimates are based on multiple imputed data. Models focused on maternal blood pressure are adjusted for maternal and paternal age, ethnicity, educational level and (pre-pregnancy) body mass index; gestational age at measurement, maternal smoking and alcohol consumption during pregnancy, folic acid supplement intake. Estimates regarding childhood systolic blood pressure are assessed by combining maternal early pregnancy systolic blood pressure tertiles with paternal systolic blood pressure tertiles. Estimates regarding childhood diastolic blood pressure are assessed by combining maternal early pregnancy diastolic blood pressure tertiles with paternal diastolic blood pressure tertiles.

D. Maternal and paternal blood pressure, birth model



Values are regression coefficients (95% confidence intervals) based from multiple linear regression models. Estimates are based on multiple imputed data. Models focused on maternal blood pressure are adjusted for maternal and paternal age, ethnicity, educational level and (pre-pregnancy) body mass index; gestational age at measurement, maternal smoking and alcohol consumption during pregnancy, folic acid supplement intake, birthweight and gestational age at birth. Estimates regarding childhood systolic blood pressure are assessed by combining maternal early pregnancy systolic blood pressure tertiles with paternal systolic blood pressure tertiles. Estimates regarding childhood diastolic blood pressure are assessed by combining maternal early pregnancy diastolic blood pressure tertiles with paternal diastolic blood pressure tertiles.

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Chapter 3

Genetic and childhood factors

Chapter 3.1

Influence of common genetic variants on childhood kidney outcomes

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ABSTRACT

Background: Kidney measures in early life are associated with kidney disease in later life. We hypothesized that these associations are partly explained by common genetic variants that lead to both smaller kidneys with lower kidney function in early childhood and kidney disease in adulthood.

Methods: We examined in a population-based prospective cohort study among 4,119 children the associations of a weighted genetic risk score combining 20 previously identified common genetic variants related to adult $eGFR_{creat}$ with kidney outcomes in children aged 6.0 years (95% range 5.7–7.8). Childhood kidney outcomes included combined kidney volume, glomerular filtration rate (eGFR) based on creatinine levels, and microalbuminuria based on albumin and creatinine urine levels.

Results: We observed that the genetic risk score based on variants related to impaired kidney function in adults was associated with a smaller combined kidney volume (*P*-value 3.0×10^{-3}) and with a lower eGFR (*P*-value 4.0×10^{-4}) in children. The genetic risk score was not associated with microalbuminuria.

Conclusion: Common genetic variants related to impaired kidney function in adults already lead to subclinical changes in childhood kidney outcomes. The well-known associations of kidney measures in early life with kidney disease in later life may at least be partly explained by common genetic variants.

INTRODUCTION

End stage kidney disease seems to partly originate in early life.[1, 2] Smaller kidneys in early life with a reduced number of nephrons lead to glomerular hyperfiltration and sclerosis and predispose to kidney disease in adulthood.[3, 4] Also, risk factors for impaired kidney function track from childhood to adulthood.[5, 6] Early kidney growth and development is a complex developmental process and is influenced by many environmental factors.[7, 8] Various nutritional and environmental exposures have been suggested to affect early kidney development and may subsequently influence the risk of kidney disease in later life.[9, 10]

However, the associations between early life kidney characteristics and kidney disease in later life may also be explained by common genetic variants. These variants may affect early kidney development and function and thereby predispose individuals to kidney disease in later life. Several genes are known to be involved in kidney development.[8] Rare mutations in these genes can cause severe anomalies, such as agenesis or dysgenesis of the kidney.[7] These mutations do not explain variation in kidney development within normal range. However, large genome wide association studies (GWAS) in adults have identified 30 common genetic variants associated with impaired kidney function.[11–13] For this study, we hypothesized that these genetic variants also affect kidney growth and function in early childhood, and thereby partly explain the well-known associations of early life kidney measures with kidney disease in later life.

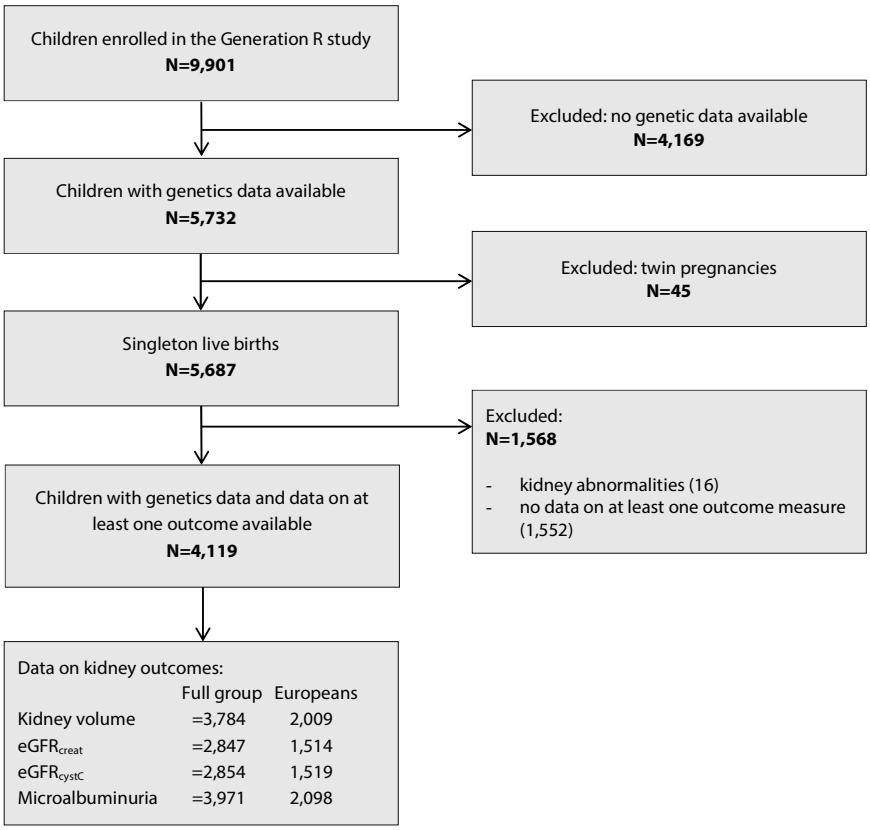
To test this hypothesis, we examined, in a population-based prospective cohort study among 4,119 children, the associations of genetic risk scores combining previously identified common genetic variants related to impaired kidney function in adults with kidney outcomes in school-age children.

METHODS

Design and study population

This study was embedded in the Generation R Study, a multiethnic population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands, which has been described in detail previously.[14] The study has been approved by Medical Ethical Committee of Erasmus MC, University Medical Center Rotterdam. All children were born between April 2002 and January 2006 and form a largely prenatally enrolled birth cohort that is currently being followed until young adulthood. Written consent was obtained for every participant. A genome wide association screen was available in 5,732 children (see below for details). The present analyses were performed among 4,119 singleton live births for whom we have detailed information on kidney outcomes at the median age of 6 years (95% range 5.7, 7.8). The flowchart of study participants is given in the **Figure 3.1.1**.

Figure 3.1.1. Flowchart of the study participants



Genetic variants

DNA was isolated from cord blood samples. If DNA samples from cord blood were missing (in 6.3% of the participants), DNA was isolated from blood samples at follow-up measurements. Genome-wide association arrays were run using the Illumina 610 Quad and 660 platforms.[15] A stringent process of quality control was applied. Individuals with low sample call rates or sex mismatches were excluded. Before imputation, Single Nucleotide Polymorphisms (SNPs) were excluded in case of high levels of missing data (SNP call rate <98%), highly significant departures from Hardy-Weinberg equilibrium (P <1*10^{-6}), or low minor allele frequencies (<1%).[14] MACH software was used to impute genotypes to the cosmopolitan panel of HapMap II (release 22).[16, 17] Three SNPs (rs6431731, rs2453580, and rs881858) had imputation qualities between 0.68 and 0.9, indicating reasonable imputation. All other SNPs had imputation qualities above 0.9, indicating good imputation. Based on previous studies, we identified 30 SNPs robustly associated with adult kidney function: 29 for adult $eGFR_{creat}$ and 1 for adult microalbuminuria.[11–13] Of these 30 SNPs, information on 26 was available in the GWAS. Information on rs11078903, rs7805747, rs2279463, and rs7422339, all previously associated with

adult $eGFR_{creat}$, was not available. However, rs3798156 was used as a perfect proxy ($R^2 = 1$) for rs2279463 and rs11078902 was used as a perfect proxy for rs11078903. No perfect proxies were available in the GWAS dataset for the other two SNPs. As the focus of this study was to examine SNPs related to estimated glomerular filtration rate and not to creatinine metabolism, 20 out of these 28 SNPs were used for the genetic risk score analyses. The individual SNPs used are shown in **Supplementary Table 3.1.2**.

Childhood kidney outcomes

Kidney outcomes were assessed at a dedicated research center in the Erasmus MC-Sophia Children's Hospital in Rotterdam by well-trained staff.[18]. We measured kidney volume with ultrasound, using an ATL-Philips HDI 5000 instrument (Seattle, WA), with a 2.0–5.0 MHz curved array transducer. We identified the left and right kidneys in the sagittal plane along its longitudinal axis. Also, we performed measurements of maximal bipolar kidney length, width and depth. Kidney width and depth were measured at the level of the hilum. The cross-sectional area in which the kidney appeared symmetrically round at its maximum width was used. Kidney volume was calculated using the equation for a prolate ellipsoid: volume (cm^3) = 0.523 x length (cm) x width (cm) x depth (cm).[19] Combined kidney volume was calculated by summing right and left kidney volume. We previously reported good intra-observer and inter-observer correlation coefficients.[20]

Non-fasting blood samples were drawn by antecubital venipuncture. Creatinine concentrations were measured with enzymatic methods and cystatin C levels with a particle enhanced immunoturbidimetric assay (using Cobas 8000 analyzers, Roche, Almere, the Netherlands). Quality control samples demonstrated intra-assay and inter-assay coefficients of variation of 0.51% for creatinine and 1.65% for cystatin C, and 1.37% for creatinine and 1.13% for cystatin C, respectively. We estimated glomerular filtration rate (eGFR) according to the revised Schwartz 2009 formula: $eGFR_{creat}$ = 36.5 * (height (cm)/ serum creatinine (µmol/l)).[21] Additionally, we estimated the glomerular filtration rate using Zappitelli's formula based on cystatin C levels: $eGFR_{cystC} = 75.94/[CysC.^{1.17}]$.[22]

Urine creatinine (mmol/l) and urine albumin (mg/l) levels were determined with a Beckman Coulter AU analyser, creatinine levels were measured with the Jaffe reaction. We also calculated the albumin-creatinine ratio. In line with clinical cut-offs, microalbuminuria was defined as an albumin-creatinine ratio >2.5 mg/mmol for boys and >3.5 mg/mmol for girls.[23]

Statistical analysis

First, we performed multiple linear and logistic regression analyses, adjusted for sex, age at measurements and the first four principal components, to examine the associations of the 21 SNPs (20 SNPs for kidney function per se and 1 for microalbuminuria) individually with combined kidney volume, creatinine and cystatin C levels, $eGFR_{creat}$ and $eGFR_{cystC}$ and the risk of microalbuminuria in childhood, assuming additive genetic effects. To adjust for multiple testing in the analyses of the individual SNPs, we applied Bonferroni correction for the number of SNPs tested. A *P value* of $<2.4 \times 10^{-3}$ (0.05/21) was considered statistically significant. Second, for each outcome we calculated the percentage of variance explained by all SNPs combined by deducting the unadjusted R^2 of the model including only the covariates from the unadjusted R^2 of the

full model including all SNPs and the covariates, which were sex, age at measurements, and the first four principal components from our genome wide data. Third, we combined the 20 SNPs related to adult $eGFR_{creat}$ in a genetic risk score that summed the number of $eGFR_{creat}$-decreasing alleles weighted by their previously reported effect sizes in adults. As for every SNP, the number of effect alleles can be 0, 1, or 2, the weighted risk score was rescaled to a score ranging from 0 to 40, the maximum number of effect alleles, and rounded to the nearest integer. Additionally, we computed an unweighted genetic risk score based on the 20 adult $eGFR_{creat}$ SNPs to see whether the results were independent of the weight based on adults findings, by adding the number of risk alleles for the 20 SNPs. Linear and logistic regression analyses were performed to examine the association of these risk scores with combined kidney volume, creatinine and cystatin C levels, $eGFR_{creat}$, $eGFR_{cystC}$ and the risk of microalbuminuria in childhood, adjusted for the same covariates as mentioned above. All analyses were performed first in the full group and subsequently repeated as sensitivity analyses in the subgroup of children of European ethnicity, the largest ethnic subgroup in our population. A child was classified as European if he/she was within four standard deviations from the HapMap CEU panel mean value for all first four principal components, based on the genetic data. To assess whether the associations were different by child sex we evaluated the statistical interaction by adding the product term of the child sex and individual SNPs to the models. However, no significant interactions were observed. We also aimed to explore whether child height affected the associations of the genetic risk score with combined kidney volume. We performed a sensitivity analysis to explore whether adding 7 SNPs related to creatinine metabolism (rs10774021, rs10794720, rs491567, rs6465825, rs9895661, rs2453533, rs3798156) to the genetic risk score would affect the results.[11] In addition, we explored the association of a genetic risk score including only 11 of the 20 SNPs, which may have a role related to kidney developmental outcomes (nephrogenesis (rs881858, VEGFA; rs626277, DACH1); glomerular filtration barrier and podocyte function (rs11959928, DAB2; rs3925584, MPPED2; rs13538, NAT8), metabolic function of the kidney (rs1260326, GCKR; rs10109414, STC1), solute transport (rs12460876, SLC7A9; rs6420094, SLC34A1) and renal cell structure development (rs12917707, UMOD; rs17319721, SHROOM3)).[13, 24–26] Furthermore, we explored the effect of 2 SNPs known to be associated with $eGFR_{cystC,}$ by adding them to the unweighted risk score. All analyses were performed using the Statistical Package for the Social Sciences version 21.0 for Windows (SPSS IBM, Chicago, IL).

RESULTS

Participant characteristics

The flowchart of study participants is given in the **Figure 3.1.1**. **Table 3.1.1.** shows the characteristics of the participants. In the full group the mean combined kidney volume and $eGFR_{creat}$ were 120 cm^3 (SD ±23.3) and 119 ml/min/1.73m^2 (SD ±16), respectively. Of all the children, 7.9% had microalbuminuria and 0.07% had an eGFR <60 ml/min/1.73 m^2. The characteristics of the subgroup of European children only were similar to those of the full group (**Supplementary Table 3.1.1**).

Table 3.1.1. Subjects characteristics (N=4,119)

Subjects characteristics	
Girls (%)	49.7
Gestational age at birth (weeks)	40.1 (36.4-42.3)
Birth weight (g)	3,464 (515)
Age at kidney measurements (y)	6.0 (5.7-7.8)
Height at kidney measurements (m)	1.20 (0.06)
Weight at kidney measurements (kg)	23.3 (4.2)
Combined kidney volume (cm^3)	120.0 (23.3)
Creatinine (µmol/l)	37.4 (5.7)
Cystatin C (µg/l)	783 (83)
$eGFR_{creat}$ ($ml/min/1.73m^2$)	119 (16)
$eGFR_{cystC}$ ($ml/min/1.73m^2$)	103 (15)
Microalbuminuria (%)	7.9

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

Individual genetic variants and childhood kidney outcomes

Results for the associations of the individual SNPs with childhood kidney outcomes are given in the Supplemental Material. Of the 20 available SNPs known to be associated with $eGFR_{creat}$ and the single SNP associated with microalbuminuria in adults, none were individually significantly associated with kidney volume, $eGFR_{creat}$, $eGFR_{cystC}$, and microalbuminuria, in the full group (**Supplementary Table 3.1.2**). In both the full group and the European ancestry subgroup, rs3925584, located upstream of MPPED2, was associated with childhood creatinine blood levels (P values 1.8×10^{-3} and 5.6×10^{-4}, respectively). In addition, in the European ancestry subgroup, rs11078902 in CDK12 was associated with childhood creatinine blood levels and with $eGFR_{creat}$ (P values 1.1×10^{-3} and 2.3×10^{-3}, respectively) (**Supplementary Tables 3.1.3, 3.1.4, and 3.1.5**). The 20 adult kidney function SNPs together explained 1.3%, 1.4%, and 0.6% of the variance in combined kidney volume, $eGFR_{creat}$ and $eGFR_{cystC}$ in childhood, respectively.

Genetic risk score and childhood kidney outcomes

Table 3.1.2 presents the associations of the weighted genetic risk score based on 20 SNPs known to be associated with impaired adult $eGFR_{creat}$ with childhood kidney outcomes. The weighted risk score ranged from 13 to 33 with a mean of 22.8 (SD 2.8) and was significantly associated with childhood combined kidney volume (*P value* 3.0×10^{-3}). For each additional average risk allele, combined kidney volume was -0.39 cm^3 (95% Confidence Interval (CI) -0.64, -0.13) smaller. The difference in combined kidney volume between the lowest and highest risk categories (≤22 and ≥33 risk alleles, respectively) was 2.62 cm^3 (**Figure 3.1.2**, **panel a**), which corresponds with a 2.2% difference. A higher weighted genetic risk score was also associated with a lower $eGFR_{creat}$ (*P value* 4.0×10^{-4}). For each additional average risk allele, $eGFR_{creat}$ was -0.38 $ml/min/1.73m^2$ (95% CI -0.60; -0.17) lower. The difference in mean $eGFR_{creat}$

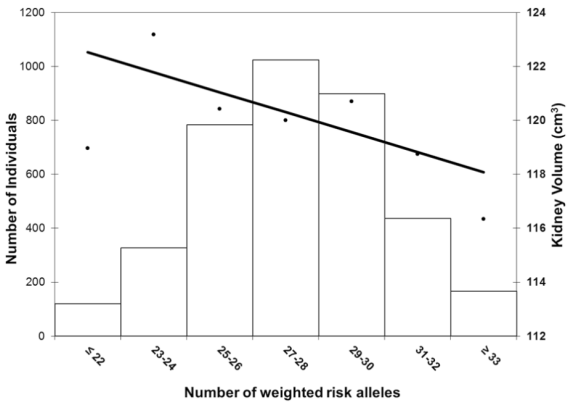
Table 3.1.2. Associations of the genetic risk score based on adult kidney function with childhood kidney outcomes (N=4,119)[a]

	Genetic risk score					
	Weighted			Unweighted		
	Difference (95% Confidence Interval)	*P value*	% of explained variance	Difference (95% Confidence Interval)	*P value*	% of explained variance
Childhood kidney outcomes (N)						
Kidney volume (cm^3)[b] (3,784)	-0.39 (-0.64; -0.13)	**$3.0*10^{-3}$**	0.3	-0.38 (-0.63; -0.13)	**$3.2*10^{-3}$**	0.3
$eGFR_{creat}$ (ml/min/1.73m^2)[b] (2,847)	-0.38 (-0.60; -0.17)	**$4.0*10^{-4}$**	0.4	-0.40 (-0.61; -0.20)	**$1.7*10^{-4}$**	0.7
$eGFR_{cystC}$ (ml/min/1.73m^2)[b] (2,854)	-0.27 (-0.47; -0.07)	**$8.0*10^{-3}$**	0.2	-0.28 (-0.48; -0.09)	**$5.0*10^{-3}$**	0.3

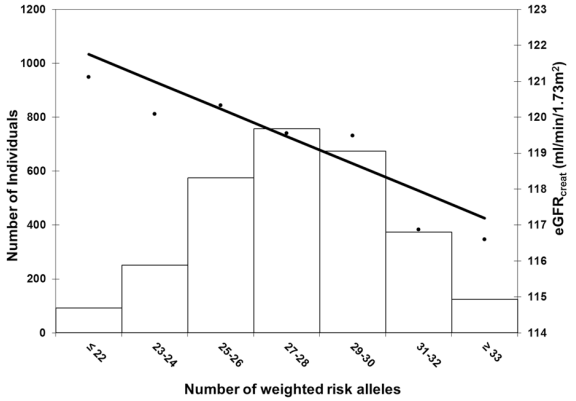
[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates. [b] Values are beta coefficients from linear regression models adjusted for child age, sex and the first four principal components from the genetic data. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels. *P value* for the significant associations **<0.05**. The percentage of variance explained by the genetic risk score, was calculated by comparing the unadjusted R^2 between a model including the risk score and the covariates and a model including only the covariates, which were sex, age at measurements, and the first four principal components.

between the lowest and highest risk categories was 4.46 ml/min/1.73m^2 (**Figure 3.1.2**, **panel b**), which corresponds with a 3.7% difference. Similarly, per additional average risk allele of the adult risk score, childhood $eGFR_{cystC}$ was -0.27 ml/min/1.73m^2 (95% CI -0.47; -0.07) lower. The difference in mean $eGFR_{cystC}$ between the lowest and highest risk categories was 3.38 ml/

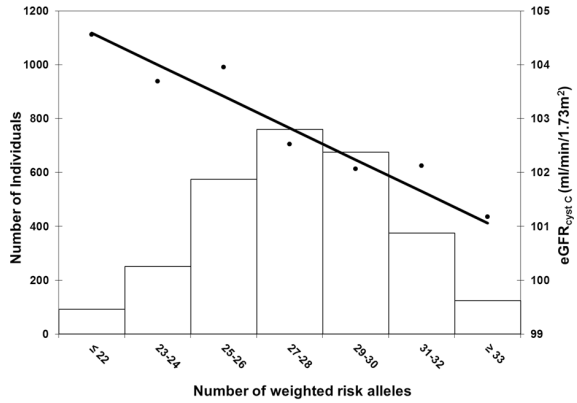
Figure 3.1.2, panels a-c Effect of the weighted adult $eGFR_{creat}$ genetic risk score on childhood kidney outcomes (N=4,119)



a. Effect of adult eGFR creatinine-based genetic risk score on childhood combined kidney volume:
The *x* axis gives the categories of the risk score (overall sum of risk alleles, weighted by previously reported effect sizes, rescaled and rounded to the nearest integer). The left y axis gives the number of individuals in each risk-score category, whereas the right y axis gives the mean combined kidney volume. The histogram reflects the observed number of individuals in each risk-score category. The dots and regression line reflect the estimated mean combined kidney volume for each risk-score category. *Values* based on the continuous risk score, as presented in Table 2. ***P value* $3.0x10^{-3}$**



b. Effect of adult eGFR creatinine-based genetic risk score on childhood eGFR creatinine-based:
The *x* axis gives the categories of the risk score (overall sum of risk alleles, weighted by previously reported effect sizes, rescaled and rounded to the nearest integer). The left y axis gives the number of individuals in each risk-score category, whereas the right y axis gives the mean $eGFR_{creat}$. The histogram reflects the observed number of individuals in each risk-score category. The dots and regression line reflect the estimated mean $eGFR_{creat}$ for each risk-score category. *P value* is based on the continuous risk score, as presented in Table 2. *****P value* 4.0x10^{-4}**



c. Effect of adult eGFR creatinine-based genetic risk score on childhood eGFR cystatin C-based:
The *x* axis gives the categories of the risk score (overall sum of risk alleles, weighted by previously reported effect sizes, rescaled and rounded to the nearest integer). The left y axis gives the number of individuals in each risk-score category, whereas the right y axis gives the mean $eGFR_{cystC}$. The histogram reflects the observed number of individuals in each risk-score category. The dots and regression line reflect the estimated mean $eGFR_{cystC}$ for each risk-score category. *P value* is based on the continuous risk score, as presented in Table 2. *****P value* 8.0x10^{-3}**

min/1.73m^2 (**Figure 3.1.2**, **panel c**). We did not observe an association of the weighted risk score with the risk of childhood microalbuminuria (data not shown).

Results from the unweighted risk score (mean 21.8, SD 2.8) were similar to those of the weighted score (**Table 3.1.2**). **Supplementary Table 3.1.6** shows that higher genetic risk scores were also associated with higher childhood creatinine blood levels and cystatin C blood levels (*P values* <0.05). Results in the subgroup of only European children tended to be

similar to those in the full group, although the association of both risk scores with $eGFR_{cystC}$ lost its significance and the unweighted risk score lost its significance with combined kidney volume in this group (**Supplementary Table 3.1.7**). The effect of the genetic risk score on combined kidney volume did not change after adjusting our model additionally for child height (**Supplementary Table 3.1.8**).

The genetic risk scores including 27 SNPs, with 7 additional SNPs related to creatinine metabolism in adults, and the genetic risk score including 11 SNPs which may have a known function related to kidney development, revealed similar results as our main risk score. Results are shown in **Supplementary Tables 3.1.9** and **3.1.10**. In the **Supplementary Table 3.1.11**, is shown the effect of the unweighted risk score which includes the additional 2 SNPs related to $eGFR_{cystC}$. The results did not change, but the effect estimates on $eGFR_{cystC}$ and cystatin C were larger.

DISCUSSION

We observed in a large population-based prospective cohort study, that a higher genetic risk score combining previously identified common genetic variants related to lower kidney function in adults was also associated with a smaller combined kidney volume and lower $eGFR_{creat}$ and $eGFR_{cystC}$ in childhood.

Previous GWAS have identified many common genetic variants related to impaired kidney function in adults.[11–13] Of the 20 available SNPs known to be associated with adult $eGFR_{creat}$ and the single SNP associated with adult microalbuminuria, only 2 were individually associated with childhood kidney outcomes. First, we observed an association of rs3925584, located upstream of *MPPED2,* with creatinine blood levels both in the full group and in the subgroup of European children only. In zebrafish, knockdown of *MPPED2* caused abnormal podocyte anatomy.[11] Rs3925584 has also been associated with magnesium levels in a large GWAS.[27] Rs3925584 is also located close to *DCDC5*, which has been associated with bone mineral density.[28] Second, rs11078902 in *CDK12* was associated with $eGFR_{creat}$ and creatinine blood levels in the subgroup of European children only.[11] *CDK12*, cyclin-dependent kinase 12, regulates the expression of genes involved in DNA repair and is required for the maintenance of genomic stability. *CDK* inhibitors have been described to play a role in human glomerular disease.[29] All these associations were directionally consistent with results reported in previous GWAS among adults.[11, 13] None of the included common genetic variants were associated with childhood microalbuminuria. The results for the associations of individual SNPs with childhood kidney outcomes should be interpreted carefully. The original GWAS meta-analysis discovery studies in adults were performed in much larger samples than the current study and our negative results may have occurred due to a lack of power.

In the current study, the weighted genetic risk score related to impaired kidney function in adults was associated with smaller combined kidney volume and lower eGFR in childhood. For an average child in our population, the observed effect estimates correspond with a 0.3% smaller combined kidney volume, a 0.3% lower $eGFR_{creat}$ and a 0.3% lower $eGFR_{cystC}$ per each

additional risk allele. We did not observe an association of the genetic risk score with microalbuminuria. We found similar results for the weighted and unweighted risk genetic scores, suggesting that the adult weights used in this score did not strongly influence the observed associations. Adding to the risk score 7 SNPs previously reported to play a role in creatinine production and secretion revealed similar results. Restricting the genetic risk score to the 11 SNPs with a likely or known function related to kidney development revealed similar results with slightly stronger effect estimates. This restricted genetic risk score may be more specific to our outcomes because of the young age range. Including in the unweighted risk score two additional SNPs known to be associated with $eGFR_{cystC}$,[13, 24] showed similar associations, with stronger effect estimates on $eGFR_{cystC}$ and cystatin C, suggesting a strong influence of the 2 added SNPs on these 2 markers. Ideally, the weights in the genetic risk score would be child-specific, as these best reflect the relative importance of the variants in children. For this, we would need results from large-scale GWAS with independent replication samples on childhood kidney function outcomes. However, a GWAS focused on kidney function among healthy children is not available yet. A recent paper described a GWAS to identify common genetic variants influencing renal function in children with CKD. Although no genome-wide significant associations were found, a number of potentially interesting subthreshold SNPs were identified.[30] This underlines the need for large-scale GWAS efforts to discover the genetic background of kidney function in children, both with and without CKD.

We hypothesized that common genetic variants may partly explain the associations of early life kidney measures with kidney function in later life. The observed associations of the genetic risk scores related to kidney function in adults with kidney outcomes in childhood support this hypothesis. However, confirmation of our findings in other children cohorts and further functional studies are required to establish the causal genes and the mechanisms underlying the associations of these variants with kidney function in childhood and disease in adulthood. In the same cohort as the current study, we have reported associations of smaller kidneys with lower kidney function.[31] To explore if the associations of the genetic risks score with kidney function outcomes were explained by kidney volume, we additionally adjusted the kidney function measures for combined kidney volume. However, the results did not change. Also, adding childhood height to the models associating the genetic risk score with combined kidney volume did not materially change the results. The observed effect estimates in the present study are small, but important from an etiological perspective. They provide further insights into pathways leading to changes in kidney function from the earliest phase of life. Whether common genetic variants related to kidney outcomes in childhood and disease in adulthood are clinically useful for identification of groups at risk needs further study.

Some methodological issues need to be considered. Major strengths of the current study are the detailed phenotypes and the relatively large number of subjects. Still, our sample size was too small to identify many of the associations of individual common genetic variants with childhood kidney outcomes, assuming similar effect sizes as in adults. It has been shown that kidney function and the prevalence of chronic kidney disease vary across ethnic groups in adults.[32] Our study population was multi-ethnic, but a sensitivity analysis

in European children only, as the largest ethnic subgroup, revealed similar results as in the full group. The other ethnic subgroups were too small to analyze individually. Of all children with genetic information, data on kidney outcomes was available in 72%. As compared to children with kidney follow-up measurements, those without these measurements had a lower birth weight (*P value* <0.05) (data not shown). A selective loss to follow-up may have reduced variation in birth weight and early kidney size and therefore reduced the power to detect differences. We performed detailed measurements of childhood kidney outcomes. Kidney size correlates with the number of glomeruli and can be used as a measure of kidney development.[33] In children the estimation of GFR is challenging. Blood creatinine is most commonly used to calculate eGFR. In addition to blood creatinine levels, we also calculated eGFR based on cystatin C levels using Zappitelli's formula.[22, 34] It has been suggested that blood cystatin C levels might be a better biomarker to estimate GFR because the production rate is constant, it is freely filtered, and less dependent on child weight, height and sex compared to creatinine.[35, 36] Finally, in our study we have captured the vast majority of, but not all, known SNPs related to kidney function, as not all SNPs were available in the GWAS dataset.

CONCLUSION

Our results suggest that common genetic variants related to kidney function in adults could influence kidney structure and function from early childhood onwards. The previously observed associations of early life kidney measures with kidney disease in later life seem to be partly explained by common genetic variants.

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Supplementary Table 3.1.1. European children characteristics (N=2,186)

Subjects characteristics	
Girls (%)	49.4
Gestational age at birth (wk)	40.3 (36.4-42.3)
Birth weight (g)	3,449 (516)
Age at kidney measurements (y)	6.0 (5.6-7.1)
Height at kidney measurements (m)	1.20 (0.06)
Weight at kidney measurements (kg)	22.8 (3.4)
Combined kidney volume (cm^3)	120.0 (22.4)
Creatinine (µmol/l)	37.0 (5.5)
Cystatin C (µg/l)	784 (85)
$eGFR_{creat}$ (ml/min/1.73m^2)	120 (16)
$eGFR_{cystC}$ (ml/min/1.73m^2)	103 (16)
Microalbuminuria (%)	7.3

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

Supplementary Table 3.1.2. Associations of SNPs with kidney outcomes in the full group (N=4,119)[a]

SNP	Nearest Gene	Chr	Position	EA/OA	EAF	Kidney volume Beta (SE)[b]	Kidney volume *P value*	$eGFR_{creat}$ Beta (SE)[b]	$eGFR_{creat}$ *P value*	$eGFR_{cystC}$ Beta (SE)[b]	$eGFR_{cystC}$ *P value*	Microalbuminuria OR (CI)[c]	Microalbuminuria *P value*
Previously identified in $eGFR_{creat}$													
rs3925584	MPPED2	11	30,716,911	T/C	0.60	-0.65 (0.54)	0.23	-1.04 (0.45)	0.02	-0.55 (0.42)	0.19	0.92 (0.78; 1.10)	0.37
rs6431731	DDX1	2	15,780,453	T/C	0.96	-2.93 (1.48)	0.05	-0.71 (1.27)	0.58	-1.19 (1.18)	0.32	0.74 (0.48; 1.14)	0.17
rs12124078	DNAJC16	1	15,742,486	G/A	0.30	1.21 (0.56)	0.03	0.12 (0.47)	0.80	-0.06 (0.43)	0.90	0.92 (0.77; 1.11)	0.40
rs2453580	SLC47A1	17	19,378,913	C/T	0.41	-1.14 (0.60)	0.06	-0.87 (0.50)	0.09	-0.60 (0.47)	0.20	0.95 (0.78; 1.15)	0.61
rs2928148	INO80	15	39,188,842	G/A	0.52	-0.33 (0.53)	0.53	-0.78 (0.44)	0.08	-0.14 (0.41)	0.73	1.07 (0.90; 1.27)	0.43
rs10109414	STC1	8	23,807,096	T/C	0.36	-1.59 (0.54)	$3.3*10^{-3}$	-0.55 (0.46)	0.23	-0.16 (0.42)	0.71	1.08 (0.91; 1.29)	0.37
rs11959928	DAB2	5	39,432,889	A/T	0.43	-0.43 (0.53)	0.42	-0.83 (0.44)	0.06	-0.11 (0.41)	0.78	1.24 (1.05; 1.47)	0.01
rs12460876	SLC7A9	19	38,048,731	T/C	0.62	-0.42 (0.53)	0.42	0.15 (0.44)	0.74	0.28 (0.41)	0.49	1.08 (0.92; 1.29)	0.35
rs1260326	GCKR	2	27,584,444	C/T	0.62	-0.10 (0.53)	0.85	-0.06 (0.45)	0.90	-0.77 (0.42)	0.06	1.10 (0.93; 1.31)	0.27
rs12917707	UMOD	16	20,275,191	G/T	0.86	-0.23 (0.76)	0.76	0.31 (0.64)	0.62	0.31 (0.60)	0.61	1.28 (0.98; 1.66)	0.07
rs13538	NAT8	2	73,721,836	A/G	0.75	-0.35 (0.63)	0.58	-0.69 (0.53)	0.19	-1.02 (0.49)	0.04	0.95 (0.77; 1.15)	0.58
rs1394125	UBE2Q2	15	73,946,038	A/G	0.35	0.34 (0.56)	0.54	-0.70 (0.47)	0.14	-0.25 (0.44)	0.57	1.08 (0.91; 1.30)	0.37
rs17319721	SHROOM3	4	77,587,871	A/G	0.36	-1.42 (0.54)	$8.4*10^{-3}$	-0.68 (0.45)	0.13	-0.40 (0.42)	0.35	1.10 (0.92; 1.30)	0.30
rs267734	ANXA9	1	149,218,101	T/C	0.83	-1.65 (0.67)	0.01	-1.03 (0.56)	0.07	-0.30 (0.52)	0.57	1.22 (0.97; 1.53)	0.09
rs347685	TFDP2	3	143,289,827	A/C	0.74	-1.63 (0.58)	$4.9*10^{-3}$	-0.004 (0.49)	0.99	-0.08 (0.45)	0.86	0.84 (0.70; 1.00)	0.05
rs4744712	PIP5K1B	9	70,624,527	A/C	0.41	0.15 (0.52)	0.77	-1.13 (0.44)	0.01	-0.42 (0.41)	0.30	1.14 (0.96; 1.34)	0.13
rs626277	DACH1	13	71,245,697	A/C	0.53	0.25 (0.52)	0.63	0.15 (0.44)	0.74	-0.15 (0.41)	0.72	1.06 (0.90; 1.26)	0.48
rs6420094	SLC34A1	5	176,750,242	G/A	0.31	0.12 (0.55)	0.82	-0.60 (0.46)	0.19	-0.34 (0.43)	0.42	1.08 (0.91; 1.29)	0.36
rs881858	VEGFA	6	43,914,587	A/G	0.92	-0.56 (0.64)	0.38	-0.55 (0.53)	0.30	-0.41 (0.49)	0.40	0.80 (0.66; .98)	0.03
rs11078902	CDK12	17	34,885,409	C/G	0.31	1.05 (0.59)	0.07	1.23 (0.49)	0.01	-0.11 (0.45)	0.81	0.76 (0.62; .92)	$5.0*10^{-3}$
Previously identified in microalbuminuria													
rs1801239	CUBN	10	16,959,058	C/T	0.10	2.18 (0.87)	0.01	1.69 (0.73)	0.02	-0.09 (0.68)	0.89	0.88 (0.66; 1.18)	0.40

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates. [b]Values are beta coefficients from linear regression models adjusted for age, gender and the first four principal components. [c]Values are OR from logistic regression coefficients for models adjusted for age at measurements, gender and the first four principal components. Bonferroni adjusted *P value* cutoff for the associations **$<2.4 \times 10^{-3}$**.

Chr chromosome; CI 95% confidence interval; EA/OA effect allele/other allele; EAF effect allele frequency; OR odds ratio; SE standard error

Supplementary Table 3.1.3. Associations of SNPs with kidney outcomes in the European children (N=2,186)[a]

SNP	Nearest Gene	Chr	Position	EA/OA	EAF	Kidney Volume Beta (SE)[b]	Kidney Volume *P value*	$eGFR_{creat}$ Beta (SE)[b]	$eGFR_{creat}$ *P value*	$eGFR_{cystC}$ Beta (SE)[b]	$eGFR_{cystC}$ P value	Microalbuminuria OR (CI)[c]	Microalbuminuria *P value*
Previously identified in $eGFR_{creat}$													
rs3925584	MPPED2	11	30,716,911	T/C	0.60	0.13 (0.71)	0.86	-1.65 (0.59)	$4.8*10^{-3}$	-1.10 (0.59)	0.06	0.93 (0.74; 1.18)	0.57
rs6431731	DDX1	2	15,780,453	T/C	0.96	-1.79 (1.7)	0.29	0.41 (1.47)	0.78	-1.73 (1.46)	0.24	0.65 (0.40; 1.08)	0.10
rs12124078	DNAJC16	1	15,742,486	G/A	0.30	1.63 (0.74)	0.03	-0.004 (0.62)	0.99	0.26 (0.62)	0.68	0.97 (0.75; 1.24)	0.78
rs2453580	SLC47A1	17	19,378,913	C/T	0.41	-1.17 (0.79)	0.14	-0.62 (0.66)	0.34	-1.01 (0.66)	0.12	0.85 (0.65; 1.11)	0.24
rs2928148	INO80	15	39,188,842	G/A	0.52	-0.18 (0.69)	0.80	-0.09 (0.58)	0.88	-0.04 (0.58)	0.94	0.99 (0.78; 1.25)	0.92
rs10109414	STC1	8	23,807,096	T/C	0.36	-2.01 (0.71)	$4.6*10^{-3}$	-0.38 (0.59)	0.53	0.08 (0.59)	0.89	1.06 (0.83; 1.35)	0.64
rs11959928	DAB2	5	39,432,889	A/T	0.43	-0.69 (0.70)	0.32	-1.43 (0.59)	0.02	-0.38 (0.59)	0.52	1.11 (0.88; 1.41)	0.37
rs12460876	SLC7A9	19	38,048,731	T/C	0.62	-0.47 (0.71)	0.51	-0.56 (0.59)	0.34	0.14 (0.59)	0.81	1.06 (0.84; 1.36)	0.62
rs1260326	GCKR	2	27,584,444	C/T	0.62	-1.03 (0.71)	0.15	-0.21 (0.60)	0.73	-0.67 (0.60)	0.26	1.09 (0.86; 1.39)	0.49
rs12917707	UMOD	16	20,275,191	G/T	0.86	-0.26 (0.97)	0.79	-0.10 (0.83)	0.91	-0.60 (0.83)	0.47	1.29 (0.90; 1.84)	0.17
rs13538	NAT8	2	73,721,836	A/G	0.75	-0.14 (0.86)	0.87	-1.55 (0.72)	0.03	-1.44 (0.71)	0.05	1.03 (0.76; 1.39)	0.85
rs1394125	UBE2Q2	15	73,946,038	A/G	0.35	1.04 (0.74)	0.16	-1.01 (0.63)	0.11	-0.27 (0.63)	0.67	1.09 (0.85; 1.40)	0.48
rs17319721	SHROOM3	4	77,587,871	A/G	0.36	-1.41 (0.70)	0.04	-0.40 (0.58)	0.49	0.17 (0.58)	0.78	1.14 (0.90; 1.44)	0.28
rs267734	ANXA9	1	149,218,101	T/C	0.83	-1.39 (0.84)	0.10	-1.27 (0.70)	0.07	-0.43 (0.70)	0.54	1.17 (0.87; 1.57)	0.31
rs347685	TFDP2	3	143,289,827	A/C	0.74	-1.31 (0.76)	0.09	0.30 (0.65)	0.64	-0.16 (0.65)	0.80	0.81 (0.64; 1.04)	0.11
rs4744712	PIP5K1B	9	70,624,527	A/C	0.41	-0.22 (0.70)	0.76	-1.20 (0.59)	0.04	0.18 (0.59)	0.76	1.03 (0.81; 1.31)	0.82
rs626277	DACH1	13	71,245,697	A/C	0.53	0.80 (0.70)	0.25	0.23 (0.58)	0.69	0.06 (0.58)	0.93	1.07 (0.84; 1.35)	0.58
rs6420094	SLC34A1	5	176,750,242	G/A	0.31	-0.67 (0.73)	0.37	-0.10 (0.61)	0.88	-0.42 (0.61)	0.49	1.16 (0.91; 1.48)	0.23
rs881858	VEGFA	6	43,914,587	A/G	0.92	0.002 (0.84)	0.99	0.48 (0.70)	0.49	0.18 (0.70)	0.80	0.83 (0.63; 1.09)	0.18
rs11078902	CDK12	17	34,885,409	C/G	0.31	1.66 (0.79)	0.04	1.96 (0.64)	**$2.3*10^{-3}$**	-0.17 (0.65)	0.80	1.06 (0.73; 1.54)	0.75
Previously identified in microalbuminuria													
rs1801239	CUBN	10	16,959,058	C/T	0.10	2.56 (1.12)	0.02	0.58 (0.95)	0.54	0.30 (0.95)	0.75	1.07 (0.74; 1.56)	0.72

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates. [b]Values are beta coefficients from linear regression models adjusted for age, gender and the first four principal components. [c]Values are OR from logistic regression coefficients for models adjusted for age at measurements, gender and the first four principal components. Bonferroni adjusted *P value* cutoff for the associations **$<2.4 \times 10^{-3}$**.

Chr chromosome; CI 95% confidence interval; EA/OA effect allele/other allele; EAF effect allele frequency; OR odds ratio; SE standard error

Supplementary Table 3.1.4. Associations of SNPs with creatinine and cystatin C in the full group (N=2,854)[a]

SNP	Nearest Gene	Chr.	Position	EA/OA	EAF	Cystatin C Beta (SE)[b]	Cystatin C *P value*	Creatinine Beta (SE)[b]	Creatinine *P value*
Previously identified in $eGFR_{creat}$									
rs3925584	MPPED2	**11**	30,716,911	T/C	0.60	3.57 (2.29)	0.12	0.46 (0.15)	**$1.8*10^{-3}$**
rs6431731	DDX1	2	15,780,453	T/C	0.96	6.87 (6.45)	0.29	0.29 (0.42)	0.49
rs12124078	DNAJC16	1	15,742,486	G/A	0.30	0.45 (2.38)	0.85	0.02 (0.15)	0.91
rs2453580	SLC47A1	17	19,378,913	C/T	0.41	4.92 (2.57)	0.06	0.31 (0.17)	0.06
rs2928148	INO80	15	39,188,842	G/A	0.52	0.62 (2.25)	0.79	0.25 (0.15)	0.09
rs10109414	STC1	8	23,807,096	T/C	0.36	1.76 (2.32)	0.45	0.21 (0.15)	0.17
rs11959928	DAB2	5	39,432,889	A/T	0.43	1.59 (2.25)	0.48	0.32 (0.15)	0.03
rs12460876	SLC7A9	19	38,048,731	T/C	0.62	-0.96 (2.24)	0.67	-0.05 (0.14)	0.71
rs1260326	GCKR	2	27,584,444	C/T	0.62	4.76 (2.27)	0.04	0.02 (0.15)	0.88
rs12917707	UMOD	16	20,275,191	G/T	0.86	-1.70 (3.27)	0.60	-0.12 (0.21)	0.57
rs13538	NAT8	2	73,721,836	A/G	0.75	4.93 (2.70)	0.07	0.13 (0.17)	0.45
rs1394125	UBE2Q2	15	73,946,038	A/G	0.35	1.82 (2.41)	0.45	0.16 (0.16)	0.31
rs17319721	SHROOM3	4	77,587,871	A/G	0.36	2.38 (2.30)	0.30	0.26 (0.15)	0.08
rs267734	ANXA9	1	149,218,101	T/C	0.83	2.23 (2.86)	0.44	0.32 (0.18)	0.09
rs347685	TFDP2	3	143,289,827	A/C	0.74	-0.07 (2.48)	0.98	-0.09 (0.16)	0.59
rs4744712	PIP5K1B	9	70,624,527	A/C	0.41	3.10 (2.22)	0.16	0.31 (0.14)	0.03
rs626277	DACH1	13	71,245,697	A/C	0.53	1.93 (2.22)	0.39	-0.003 (0.14)	0.98
rs6420094	SLC34A1	5	176,750,242	G/A	0.31	1.87 (2.34)	0.43	0.16 (0.15)	0.29
rs881858	VEGFA	6	43,914,587	A/G	0.92	2.97 (2.70)	0.27	0.18 (0.17)	0.31
rs11078902	CDK12	17	34,885,409	C/G	0.31	-0.17 (2.48)	0.94	-0.45 (0.16)	$4.9*10^{-3}$
Previously identified in microalbuminuria									
rs1801239	CUBN	10	16,959,058	C/T	0.10	0.38 (3.71)	0.92	-0.35 (0.24)	0.15

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates.
[b]Values are beta coefficients from linear regression models adjusted for age, gender and the first four principal components. Bonferroni adjusted *P value* cutoff for the associations **$<2.4 \times 10^{-3}$**.
Chr chromosome; EA/OA effect allele/other allele; EAF effect allele frequency; SE standard error

Supplementary Table 3.1.5. Associations of SNPs with creatinine and cystatin C in the European children (N=1,519)[a]

SNP	Nearest Gene	Chr.	Position	EA/ OA	EAF	Cystatin C Beta (SE)[b]	Cystatin C *P value*	Creatinine Beta (SE)[b]	Creatinine *P value*
Previously identified in $eGFR_{creat}$									
rs3925584	MPPED2	11	30,716,911	T/C	0.60	6.97 (3.13)	0.03	0.67 (0.20)	**$5.6*10^{-4}$**
rs6431731	DDX1	2	15,780,453	T/C	0.96	10.20 (7.80)	0.19	0.01 (0.49)	0.99
rs12124078	DNAJC16	1	15,742,486	G/A	0.30	-0.78 (3.30)	0.81	0.06 (0.21)	0.78
rs2453580	SLC47A1	17	19,378,913	C/T	0.41	7.95 (3.51)	0.02	0.32 (0.22)	0.14
rs2928148	INO80	15	39,188,842	G/A	0.52	0.60 (3.11)	0.85	0.04 (0.19)	0.85
rs10109414	STC1	8	23,807,096	T/C	0.36	0.37 (3.16)	0.91	0.21 (0.20)	0.30
rs11959928	DAB2	5	39,432,889	A/T	0.43	4.08 (3.14)	0.19	0.51 (0.20)	0.01
rs12460876	SLC7A9	19	38,048,731	T/C	0.62	0.81 (3.14)	0.80	0.16 (0.20)	0.41
rs1260326	GCKR	2	27,584,444	C/T	0.62	4.08 (3.18)	0.20	0.08 (0.20)	0.69
rs12917707	UMOD	16	20,275,191	G/T	0.86	2.97 (4.42)	0.50	0.01 (0.28)	0.97
rs13538	NAT8	2	73,721,836	A/G	0.75	6.92 (3.82)	0.07	0.28 (0.24)	0.25
rs1394125	UBE2Q2	15	73,946,038	A/G	0.35	1.00 (3.37)	0.77	0.17 (0.21)	0.41
rs17319721	SHROOM3	4	77,587,871	A/G	0.36	-0.71 (3.12)	0.82	0.09 (0.19)	0.63
rs267734	ANXA9	1	149,218,101	T/C	0.83	3.47 (3.74)	0.36	0.47 (0.23)	0.04
rs347685	TFDP2	3	143,289,827	A/C	0.74	0.14 (3.45)	0.97	-0.14 (0.22)	0.53
rs4744712	PIP5K1B	9	70,624,527	A/C	0.41	0.74 (3.17)	0.82	0.38 (0.20)	0.05
rs626277	DACH1	13	71,245,697	A/C	0.53	0.78 (3.11)	0.80	-0.02 (0.19)	0.91
rs6420094	SLC34A1	5	176,750,242	G/A	0.31	1.93 (3.27)	0.56	-0.08 (0.20)	0.69
rs881858	VEGFA	6	43,914,587	A/G	0.92	-0.90 (3.72)	0.81	-0.12 (0.23)	0.60
rs11078902	CDK12	17	34,885,409	C/G	0.31	-0.60 (3.45)	0.86	-0.70 (0.22)	**$1.1*10^{-3}$**
Previously identified in microalbuminuria									
rs1801239	CUBN	10	16,959,058	C/T	0.10	-.200 (5.06)	0.69	-0.03 (0.32)	0.94

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates.
[b]Values are beta coefficients from linear regression models adjusted for age, gender and the first four principal components. Bonferroni adjusted *P value* cutoff for the associations **$<2.4 \times 10^{-3}$**.
Chr chromosome; EA/OA effect allele/other allele; EAF effect allele frequency; SE standard error

Supplementary Table 3.1.6. Associations of genetic risk scores with creatinine and cystatin C in the full group (N=2,854)[a]

	Genetic risk score					
	Weighted			Unweighted		
	Difference (95% Confidence Interval)	*P value*	% of explained variance	Difference (95% Confidence Interval)	*P value*	% of explained variance
Childhood kidney outcomes (N)						
Cystatin C (µg/l)[b] (2,854)	1.84 (0.75; 2.92)	**$1.0*10^{-3}$**	0.4	1.93 (0.86; 3.00)	**$4.2*10^{-4}$**	0.4
Creatinine (µmol/l)[b] (2,854)	0.13 (0.06; 0.19)	**$4.7*10^{-4}$**	0.5	0.13 (0.06; 0.20)	**$1.9*10^{-4}$**	0.5

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates
[b]Values are beta coefficients from linear regression models adjusted for age, gender and the first four principal components. *P value* for the associations **<0.05**. The percentage of variance explained by the genetic risk score, was calculated by comparing the unadjusted R^2 between a model including the risk score and the covariates and a model including only the covariates, which were sex, age at measurements, and the first four principal components.

Supplementary Table 3.1.7. Associations of genetic risk scores with kidney measures in European children (N=2,186)[a]

	Genetic risk score					
	Weighted			Unweighted		
	Difference (95% Confidence Interval)	*P value*	% of explained variance	Difference (95% Confidence Interval)	*P value*	% of explained variance
Childhood kidney outcomes (N)						
Kidney volume (cm^3)[b] (N=2,009)	-0.34 (-0.68; -0.06)	**0.046**	0.2	-0.31 (-0.64; 0.02)	0.06	0.2
$eGFR_{creat}$ (ml/min/1.73m^2)[b] (N=1,514)	-0.40 (-0.68; -0.12)	**$5.0*10^{-3}$**	0.5	-0.40 (-0.67; -0.12)	**$5.3*10^{-3}$**	0.5
$eGFR_{cystC}$ (ml/min/1.73m^2)[b] (N=1,519)	-0.27 (-0.55; 0.01)	0.06	0.2	-0.27 (-0.55; 0.003)	0.05	0.2
Creatinine (µmol/l)[b] (N=1,519)	0.12 (0.03; 0.22)	**$9.0*10^{-3}$**	0.6	0.13 (0.04; 0.22)	**$6.0*10^{-3}$**	0.5
Cystatin C (µg/l)[b] (N=1,519)	1.95 (0.46; 3.44)	**0.01**	0.4	2.02 (0.55; 3.50)	**$7.0*10^{-3}$**	0.5

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates
[b]Values are beta coefficients from linear regression models adjusted for age, gender and the first four principal components. *P* value for the associations **<0.05**; $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystc}$ estimated glomerular filtration rate calculated based on cystatin C blood levels. The percentage of variance explained by the genetic risk score, was calculated by comparing the unadjusted R^2 between a model including the risk score and the covariates and a model including only the covariates, which were sex, age at measurements, and the first four principal components.

Supplementary Table 3.1.8. Associations of genetic risk score with childhood kidney outcomes additionally adjusting for combined kidney volume (N=4,119)[a]

	Genetic risk score	
	Weighted	
	Difference (95% Confidence Interval)	*P value*
Childhood kidney outcomes (N)		
$eGFR_{creat}$ (ml/min/1.73m^2)[b] (2,847)	-0.35 (-0.56; -0.13)	**$1.0*10^{-3}$**
$eGFR_{cystC}$ (ml/min/1.73m^2)[b] (2,854)	-0.32 (-0.52; -0.11)	**$2.0*10^{-3}$**

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates. [b]Values are beta coefficients from linear regression models adjusted for child age, sex, kidney volume and the first four principal components from the genetic data. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels. P value for the associations **<0.05**.

Supplementary Table 3.1.9. Associations of the genetic risk score based on adult $eGFR_{creat}$ and creatinine metabolism SNPs with childhood kidney outcomes (N=4,119)[a]

	Genetic risk score					
	Weighted			Unweighted		
	Difference (95% Confidence Interval)	*P value*	% of explained variance	Difference (95% Confidence Interval)	*P value*	% of explained variance
Childhood kidney outcomes (N)						
Kidney volume (cm^3)[b] (3,784)	-0.43 (-0.66; -0.21)	**$2.0*10^{-4}$**	0.3	-0.43 (-0.66; -0.21)	**$1.6*10^{-4}$**	0.3
$eGFR_{creat}$ (ml/min/1.73m^2)[b] (2,847)	-0.42 (-0.61; -0.23)	**$1.3*10^{-5}$**	0.6	-0.45 (-0.63; -0.26)	**$3.0*10^{-6}$**	0.7
$eGFR_{cystC}$ (ml/min/1.73m^2)[b] (2,854)	-0.23 (-0.41; -0.06)	**$1.0*10^{-2}$**	0.2	-0.24 (-0.42; -0.07)	**$6.2*10^{-3}$**	0.3

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates. [b]Values are beta coefficients from linear regression models adjusted for child age, sex and the first four principal components from the genetic data. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels. P value for the associations **<0.05**. The percentage of variance explained by the genetic risk score, was calculated by comparing the unadjusted R^2 between a model including the risk score and the covariates and a model including only the covariates, which were sex, age at measurements, and the first four principal components.

Supplemental Table 3.1.10. Associations of the genetic risk score based on 11 SNPs with a likely function on kidney development and childhood kidney outcomes (N=4,119)[a]

	Genetic risk score			
	Weighted		Unweighted	
	Difference (95% Confidence Interval)	*P value*	Difference (95% Confidence Interval)	*P value*
Childhood kidney outcomes (N)				
Kidney volume (cm^3)[b] (3,784)	-0.50 (-0.84; -0.16)	**$3.9*10^{-3}$**	-0.50 (-0.84; -0.17)	**$3.0*10^{-3}$**
$eGFR_{creat}$ ($ml/min/1.73m^2$)[b] (2,847)	-0.40 (-0.69; -0.12)	**$5.6*10^{-3}$**	-0.42 (-0.70; -0.14)	**$3.0*10^{-3}$**
$eGFR_{cystC}$ ($ml/min/1.73m^2$)[b] (2,854)	-0.29 (-0.56; -0.03)	**$3.0*10^{-2}$**	-0.31 (-0.57; -0.05)	**$2.0*10^{-2}$**

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates. [b]Values are beta coefficients from linear regression models adjusted for child age, sex and the first four principal components from the genetic data. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels. P value for the associations **<0.05**. The percentage of variance explained by the genetic risk score, was calculated by comparing the unadjusted R^2 between a model including the risk score and the covariates and a model including only the covariates, which were sex, age at measurements, and the first four principal components.

Supplemental Table 3.1.11. Associations of the genetic risk score including 2 $eGFR_{cystC}$ SNPs with kidney measures in the full group (N=4,119)[a]

	Genetic risk score	
	Unweighted	
	Difference (95% Confidence Interval)	*P value*
Childhood kidney outcomes (N)		
Kidney volume (cm^3)[b] (N=3,784)	-0.41 (-0.66; -0.17)	**$1.0*10^{-3}$**
$eGFR_{creat}$ ($ml/min/1.73m^2$)[b] (N=2,847)	-0.44 (-0.64; -0.23)	**$2.9*10^{-5}$**
$eGFR_{cystC}$ ($ml/min/1.73m^2$)[b] (N=2,854)	-0.52 (-0.71; -0.33)	**$7.4*10^{-8}$**
Creatinine (µmol/l)[b] (N=2,854)	0.14 (0.07; 0.20)	**$7.0*10^{-5}$**
Cystatin C (µg/l)[b] (N=2,854)	3.28 (2.25; 4.31)	**$5.6*10^{-10}$**

[a]Analyses were performed in children with complete data on genetics, at least one outcome under study, and covariates [b]Values are beta coefficients from linear regression models adjusted for age, gender and the first four principal components. *P* value for the associations **<0.05**; $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels.

Chapter 3.2

Infant breastfeeding and kidney function in school-aged children

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ABSTRACT

Background: Early life factors may influence kidney growth and function throughout the life course. We examined the associations of breastfeeding duration, exclusivity and age at introduction of solid foods with kidney outcomes at school-age.
Study Design: Prospective cohort study from fetal life onward.
Settings & participants: 5,043 children in the Netherlands.
Predictors: Infant feeding was assessed prospectively using questionnaires.
Outcomes & measurements: At a median age of 6.0 years, we measured kidney volume with ultrasound, glomerular filtration rate (eGFR) from serum creatinine levels, and microalbuminuria from urinary albumin and creatinine levels.
Results: 92% of all children were ever breastfed, of whom 27% were breastfed for more than 6 months, and 21% were breastfed exclusively for at least 4 months. Compared with ever breastfed children, never breastfed children had smaller combined kidney volume (-2.69 cm^3 (95% Confidence Interval -4.83, -0.56)) and lower eGFR (-2.42 ml/min/1.73m^2 (95% CI -4.56, -0.28)) at school-age. Among breastfed children, shorter duration of breastfeeding was associated with lower kidney volume and lower microalbuminuria risk (*p*-values <0.05). Compared to exclusive breastfeeding for 4 months, non-exclusive breastfeeding in the first 4 months was associated with smaller combined kidney volume and lower eGFR (*p*-values <0.05). Associations with eGFR were largely explained by kidney volume. Age at introduction of solid foods was not associated with any of the kidney outcomes.
Limitations: Observational study, so causality cannot be established. Follow-up measurements were available in 76% of children.
Conclusion: These results suggest that breastfeeding is associated with subclinical changes in kidney outcomes in childhood. Further studies are needed to explore whether early life nutrition also affects the risk of kidney disease in adulthood.

INTRODUCTION

Adverse exposures during sensitive periods of early development may persistently affect kidney growth and function.[1] The third trimester of fetal life and early infancy are thought to be particularly critical periods for kidney development because nephron development is largely completed in this period.[2, 3] Suboptimal nephron development, may lead to glomerular hyperfiltration and subsequently to impaired kidney function in later life.[3] Animal studies have shown that although nephrogenesis is largely completed after 36 weeks of gestation, it continues until early infancy.[4] The importance of early life for later kidney outcomes is illustrated by studies in humans showing associations of preterm birth and low birth weight with risk of kidney disease in adulthood.[5] Also, children born preterm and with small size for gestational age at birth have subclinical changes in kidney function in adolescence.[6] We have recently shown that both lower fetal and early infant weight growth are associated with lower kidney function in childhood,[7] supporting the hypothesis that exposures in both fetal life and infancy are critical for kidney development. Little is known about specific early life exposures that persistently affect kidney function. Infant diet is a key factor for early growth and development and may influence kidney function in infants.[8, 9] One previous study examined the associations of breastfeeding versus formula feeding and kidney outcomes and observed that infants who were exclusively formula fed had increased kidney growth during the first 3months, but this difference was no longer apparent at the age of 18 months.[9] Infant feeding may influence kidney outcomes through differences in protein content and load.[8]

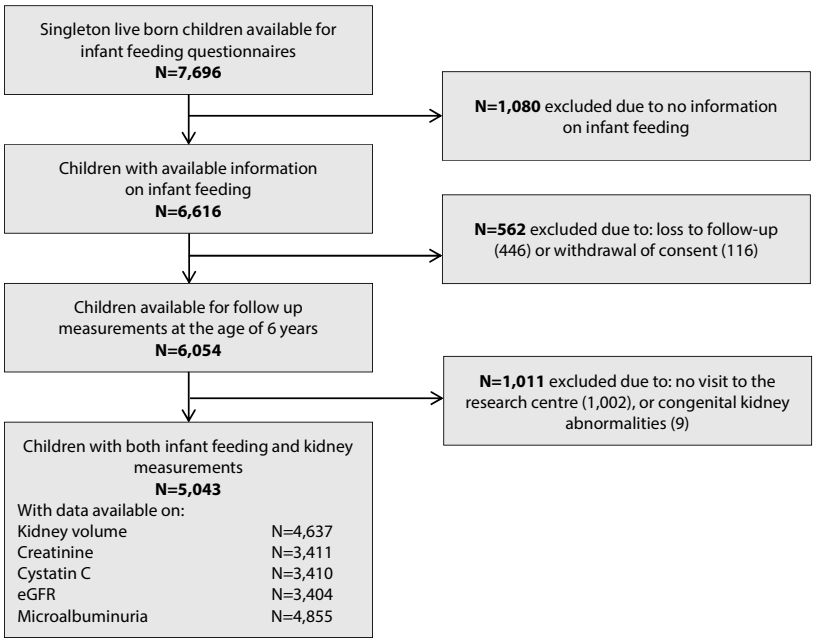
In a population-based prospective cohort study of 5,043 children, we examined the associations of breastfeeding duration and exclusiveness and age at introduction of solid foods with kidney outcomes in children at the age of 6 years. Subclinical differences in kidney outcomes in childhood do not have short-term consequences but may predispose individuals to kidney disease in later life.[5]

METHODS

Design and study population

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 April 2002 and January 2006. The study protocol was approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center, Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained from all parents. For the present study, we included only singleton live births (N=7,696) who had information on breastfeeding available (N=6,616 [86%]). Of these children, 6,054 participated in the follow-up measurements at the age of 6 years. Children with evidence of congenital kidney abnormalities on ultrasound examination were excluded from the study (N=9). At a median age of 6.0 years (95% range 5.6, 7.4), kidney ultrasound and blood and urine samples were available on 5,043 children (**Figure 3.2.1.** Flowchart).

Figure 3.2.1. Flowchart of study participants included for the main analysis



Infant feeding assessment

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. We described this approach previously in detail.[11] Briefly, mothers were asked whether they ever breastfed their child (yes/ no) and at what age they stopped 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 (FFQ). 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 in the first 4 months; and 2) exclusive for at least 4 months. 'Non-exclusive in the first 4 months' indicates infants who received both breastfeeding and formula feeding or solids during the first 4 months. 'Exclusive for at least 4 months' indicates infants who have been breastfed, without any other milk, solids or fluids during the first 4 months. These categories were available in the questionnaires and similar to those used in previous studies.[11, 12] 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 FFQ. Age at introduction of solid foods was categorized as: 1) <4 months; 2) 4–4.9 months; and 3) ≥5 months.

Childhood kidney outcomes

Childhood kidney dimensions

Children's kidney outcomes were assessed in a dedicated research center in the Erasmus MC-Sophia Children's Hospital in Rotterdam by well-trained staff.[13] Kidney volume was measured with ultrasound, using an ATL-Philips HDI 5000 instrument (Seattle, WA, USA), equipped with a 2.0–5.0 MHz curved array transducer. We identified the left and right kidney in the sagittal plane along its longitudinal axis. We performed measurements of maximal bipolar kidney length, width and depth. Kidney width and depth were measured at the level of the hilum. The cross-sectional area in which the kidney appeared symmetrically round at its maximum width was used. Kidney volume was calculated using the equation for a prolate ellipsoid: volume (cm^3) = 0.523 x length (cm) x width (cm) x depth (cm).[14] Combined kidney volume was calculated by summing right and left kidney volumes. We previously reported good intra- and inter-observer correlation coefficients.[15]

Childhood kidney function

Non-fasting blood samples were drawn by antecubital venipuncture. Creatinine was measured with enzymatic methods and cystatin C levels with a particle enhanced immunoturbidimetric assay (using Cobas 8000 analyzers; Roche). Quality control samples demonstrated intra- and inter-assay coefficients of variation ranging from 0.51 to 1.37%, and 1.13 to 1.65%, respectively. Estimated glomerular filtration rate (eGFR) was calculated according to the revised Schwartz 2009 formula: eGFR = 36.5 * (height (cm)/ serum creatinine (µmol/l).[16] Urine creatinine (mmol/l) and urine albumin (mg/l) levels were determined with a Beckman Coulter AU analyser; creatinine was measured with the Jaffe reaction. We calculated the albumin-creatinine ratio. In line with clinical cut-offs, microalbuminuria was defined as an albumin-creatinine ratio >2.5 mg/mmol for boys and >3.5 mg/mmol for girls.[17] Because our main interest was in normal variation, we excluded children with an albumin-creatinine ratio >25 for the microalbuminuria analysis given that higher albumin-creatinine ratio may reflect underlying kidney disease. With this cut-off, we excluded 14 children. When we included these children in our analysis, the effect estimates did not change.

Covariates

Information on maternal age, ethnicity, educational level and income were obtained with questionnaires at enrollment. Information about maternal smoking, alcohol use, and folic acid supplementation also were obtained by questionnaires.[10] Information on maternal smoking and alcohol use during pregnancy was categorized as follows: never; until pregnancy was known; or continued during pregnancy. Maternal protein intake during pregnancy was measured at enrollment (median 13.5 weeks of gestation) with a validated semi-quantitative FFQ.[18] Child protein intake at the age of 1 year also was measured with a validated semi-quantitative FFQ.[19] Protein intakes were adjusted for total energy intake using the nutrient residual method.[20] Ethnicity, educational level and income were defined according to the classification of Statistics Netherlands.[21, 22] We categorized ethnicity as either European or non-European for our analysis. Education level was categorized as higher and no higher

education, and net household income was categorized as <1,400 euro and ≥1,400 euro per month. Maternal height and weight were self-reported and pre-pregnancy body mass index (BMI) was calculated (kg/m^2). Infant sex, gestational age and weight at birth were obtained from midwife and hospital registries. At the age of 6 years, child height was determined in a standing position to the nearest millimeter without shoes by a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Weight was measured using a mechanical personal scale (SECA, Almere, the Netherlands). Lean body mass was measured using whole body dual-energy x-ray absorptiometry (DXA) scans using Lunar models, (iDXA, GE-Lunar, 2008, Madison, WI, USA) and enCORE software version13.6. We calculated child body mass index (kg/m^2) and body surface area (BSA) (m^2) (using DuBois formula BSA= weight (kg)$^{0.425}$ x height (cm)$^{0.725}$ x 0.007184).[23]

Statistical analysis

We assessed differences in subject characteristics between the different breastfeeding categories using T-tests for continuous and normally distributed variables, Chi-square tests for categorical variables, and Mann-Whitney test for non-normally distributed variables. Associations of breastfeeding, breastfeeding duration and exclusivity and age at introduction of solid foods with childhood kidney outcomes at the age of 6 years were explored by using multivariable linear regression models (kidney volume, eGFR, creatinine levels and cystatin C levels) or multivariable logistic models (risk of microalbuminuria).These models were first adjusted for child's sex and age at kidney measurements (crude models) and subsequently adjusted for additional potential confounders. In the final model, we included family-based socio-demographic factors (maternal education, maternal ethnicity, and household income); maternal life style related factors (pre-pregnancy body mass index, smoking and alcohol use during pregnancy, folic acid supplement use and protein intake during pregnancy) and childhood factors (birth weight and BSA at the age of 6 years). These covariates were included in the models based on their associations with kidney outcomes in previous studies, or a change in effect estimates of >10%. Gestational age at birth did not fulfill the criteria of a 10% change in the effect estimates. In a separate model, we additionally adjusted the kidney function measures for kidney volume. Tests for trends were performed by including the exposure categories as continuous variables. To assess whether the associations were different for boys and girls, different ethnic groups, preterm and term born children, or different birthweight groups, we evaluated the statistical interaction by adding the product term of each of these covariates with breastfeeding duration to the adjusted models. To diminish potential bias associated with attrition, missing values of covariates (less than 34%), were multiple imputed by generating 5 independent datasets using the Markov Chain Monte Carlo method. The multiple imputation procedure was based on the correlation between each variable with missing values and the other subject characteristics.[24, 25] Regression coefficients were pooled by taking the average of the regression coefficients obtained in the 5 imputed datasets. The standard error of the pooled regression coefficient was obtained by calculating the within-imputation variance and the between-imputation variance. Because we found similar results in the imputed and non-imputed dataset, the final results in the present report are presented

as the pooled regression coefficients after the multiple imputation procedure. To assess whether child protein intake affects the associations between breastfeeding and kidney outcomes at age of 6 years we did a sensitivity analysis in the subgroup of children with dietary data by adjusting our models additionally for child protein intake at the age of 1 year. Because kidney size is associated with lean body mass we performed a sensitivity analysis in which we additionally adjusted the model for lean body mass. Since ethnicity and birth weight have been shown to be strongly related to kidney function,[13] we performed a sensitivity analysis in children from European mothers and in children with a normal birth weight (≥2,500 g). Statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Participant characteristics

As shown in **Table 3.2.1**, of all children with kidney volume measurements, 92% were ever breastfed of whom 27% were breastfed for 6 or more months and 22% were exclusively breastfed for at least 4 months. Children who were never breastfed had a lower weight and gestational age at birth. At the age of 6 years, mean (± SD) combined kidney volume was 120 cm^3 (±22.6), and mean eGFR was 119 ml/min/1.73m^2 (±16.4). Of all children, 7.1% had microalbuminuria. We provide descriptive characteristics of the individuals before and after imputation in the supplementary materials (**Supplementary Table 3.2.1**), and in those with eGFR and microalbuminuria measurements (**Supplementary Tables 3.2.2 & 3.2.3**). We performed a non-response analysis by comparing children with and without follow-up measurements at age 6 years (**Supplementary Table 3.2.4**). Compared with children who did not participate in the follow-up at the age of 6 years, those who did participate in the follow-up studies

Table 3.2.1. Subject characteristics according to category of breastfeeding in the subjects with kidney volume measurements (N=4,637)

	Total group N=4,637	Never breastfed N=354	Ever breastfed N=4,283	*P*-value
Maternal characteristics				
Pre-pregnancy body mass index (kg/m^2)	22.7 (18.3, 33.9)	23.4 (18.2, 37.8)	22.6 (18.3, 33.3)	**<0.01**
Ethnicity (%)				**<0.01**
- European	66	76.8	65.1	
- Non – European	34	23.2	34.9	
Education (%)				**<0.01**
- No higher education	49.2	69.4	47.5	
- Higher education	50.8	30.6	52.5	
Net household total income (%)				0.71
- <1,400 euro	24	24.1	24	
- ≥1,400 euro	76	75.9	76	
Smoking during pregnancy (%)				**<0.01**
- Never smoked	80.4	58.4	81.4	

Table 3.2.1. Subject characteristics according to category of breastfeeding in the subjects with kidney volume measurements (N=4,637) (continued)

	Total group N=4,637	Never breastfed N=354	Ever breastfed N=4,283	*P*-value
- Smoke until pregnancy was known	7.6	7.3	7.6	
- Continued smoking	12.0	24.3	11.0	
Alcohol during pregnancy (%)				**<0.01**
- Never alcohol in pregnancy	53.2	61.9	52.5	
- Alcohol until pregnancy was known	11.3	7.9	11.6	
- Continued alcohol during pregnancy	35.5	30.2	35.9	
Folic acid supplement use, (%)				0.54
- No	23.2	22.7	23.2	
- During early pregnancy	76.8	77.3	76.8	
Energy adjusted protein intake (g)	74.7 (10.1)	72.7 (9.9)	75.1 (10.1)	**<0.01**
Birth characteristics				
Girls (%)	50.1	50.3	50.0	0.80
Gestational age (weeks)	40.1 (36.0, 42.3)	39.9 (35.8, 42.4)	40.1 (36.1, 42.3)	**<0.01**
Weight (grams)	3,455 (537)	3,409 (555)	3,458 (535)	0.08
Child protein intake at 1 year (g)	25.1 (29.2)	25.9 (28.6)	25.0 (29.3)	0.37
Breastfeeding duration (%)				
- >0-1.9 months (N=925)	20	0	21.6	
- 2-3.9 months (N=775)	16.7	0	18.1	
- 4-5.9 months (N=438)	9.4	0	10.2	
- ≥6 months (N=1,143)	24.6	0	27.6	
Breastfeeding exclusivity (%)				
- Non- exclusively in the first 4 months (N=2,452)	52.9	0	57.2	
- Exclusively in the first 4 months (N=952)	20.5	0	22.2	
Introduction of solid foods (%)				**<0.01**
- <4 months (N=225)	5.7	11.3	5.3	
- 4-4.9 months (N=1,716)	40.3	43.5	40.1	
- ≥5 months (N=914)	20.4	9.3	21.3	
Child characteristics				
Age (years)	6.0 (5.6, 7.4)	6.0 (5.6, 7.3)	6.0 (5.6, 7.4)	0.94
Height (cm)	118.9 (5.6)	118.5 (5.9)	118.6 (5.6)	0.13
Weight (kg)	22.2 (17.4, 32.4)	22.2 (17.2, 36.0)	22.2 (17.4, 32.2)	0.40
Body mass index, (kg/m^2)	15.8 (13.6, 20.5)	15.9 (13.6, 22.4)	15.8 (13.6, 20.5)	0.40
Body surface area, (m^2)	0.86 (0.7, 1.1)	0.85 (0.7, 1.1)	0.86 (0.7, 1.1)	0.17
Kidney volume combined, (cm^3)	120 (22.6)	116 (23.4)	120 (22.5)	**<0.01**
eGFR, (ml/min/1.73m^2)	119 (16.4)	116 (14.2)	119 (16.5)	**<0.01**
Microalbuminuria (%)*	7.1	5.1	7.3	0.14
Creatinine (µmol/l)	37.2 (5.4)	37.9 (4.9)	37.2 (5.5)	**0.04**
Cystatin C (µg/l)	783 (81.0)	793 (85.2)	782 (81)	**0.04**

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 T-test for continuous normally distributed variables, Mann Whitney for non-normally distributed variables, and Chi-squared tests for categorical variables.

eGFR - estimated glomerular filtration rate

*Defined as levels between 2.5 and 25.0 mg/mmol (boys) and 3.5 and 25.0 mg/mmol (girls)

In **Supplementary Tables 3.2.2** & **3.2.3** we provide the descriptive characteristics in the individuals with eGFR and micro-albuminuria measurements.

Table 3.2.2. Associations of infant feeding with kidney outcomes at the age of 6 years adjusted for child age, sex, family-based socio-demographic, maternal lifestyle and childhood factors (N=5,043)

	Difference in outcome measure (95% Confidence Interval)				
	Kidney volume (cm^3) N=4,637	**eGFR ($ml/min/1.73m^2$) N=3,404**	**§eGFR ($ml/min/1.73m^2$) Adjusted for kidney volume N=3,166**	**Microalbuminuria (odds ratio) N=4,855**	**§Microalbuminuria (odds ratio) Adjusted for kidney volume N=4,457**
Breastfeeding					
Never (N=382)	**-2.69 (-4.83, -0.56)***	**-2.42 (-4.56, -0.28)***	-1.18 (-4.31, 1.94)	0.71 (0.45, 1.13)	0.61 (0.26, 1.44)
Ever (N=4,661)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Duration					
>0-1.9 months (N=993)	**-2.50 (-4.20, -0.80)***	-0.81 (-2.52, 0.91)	-1.10 (3.49, 1.29)	0.78 (0.56, 1.08)	**0.56 (0.32, 0.97)***
2-3.9 months (N=836)	-1.21 (-2.95, 0.53)	-1.17 (-2.91, 0.57)	-1.22 (-3.60, 1.16)	**0.61 (0.43, 0.87)***	**0.52 (0.29, 0.90)***
4-5.9 months (N=477)	-0.05 (-2.13, 2.03)	-0.41 (-2.45, 1.63)	-0.34 (-3.05, 2.37)	0.83 (0.55, 1.23)	0.73 (0.41, 1.31)
≥6 months (N=1,253)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	***<0.01***	*0.25*	*0.28*	***0.04***	***0.01***
Exclusive for 4 months					
No (N=2,653)	**-2.47 (-3.94, -1.01)***	**-1.55 (-3.01, -0.11)***	-1.17 (-3.17, 0.84)	0.85 (0.64, 1.12)	**0.61 (0.40, 0.93)***
Yes (N=1,036)	*Reference*	*Reference*	Reference	*Reference*	*Reference*
First solid foods					
<4 months (N=236)	0.27 (-2.35, 2.88)	-0.27 (- 2.91, 2.37)	0.21 (-3.64, 4.07)	1.09 (0.64, 1.85)	0.58 (0.19, 1.75)
4-4.9 months (N=1,864)	-0.65 (-2.13, 0.83)	-0.61 (- 2.11, 0.89)	-1.49 (-3.56, 0.59)	1.01 (0.75, 1.35)	1.15 (0.72, 1.86)
≥5 months (N=989)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.77*	*0.59*	*0.49*	*0.82*	*0.80*

Values are linear and logistic regression coefficients (95% confidence interval). Models are adjusted for child's age at visit, sex, plus family-based socio-demographic confounders (maternal education, ethnicity, income), maternal lifestyle related factors (pre-pregnancy BMI, smoking and alcohol usage during pregnancy, folic acid supplements and protein-energy intake during pregnancy), childhood factors (birth weight, BSA). 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 §eGRF, §microalbuminuria are additionally adjusted for kidney volume.

were more frequently breastfed for at least 6 months and exclusively breastfed for 4 months. **Supplementary Table 3.2.5** gives the descriptive characteristics of the study participants in the children with and without blood samples. We observed no differences in breastfeeding, breastfeeding duration, exclusivity, introduction to solid foods. However, we observed that children with blood samples available have slightly higher kidney volume and body surface area and were taller and heavier. Also, they had mothers with a higher socio-economic status compared to children without blood samples.

Infant feeding and childhood kidney outcomes

Table 3.2.2 gives the fully adjusted effect estimates for the associations between infant feeding and kidney outcomes. Compared with ever breastfed children, those who were never breastfed had smaller kidneys (-2.69 cm^3 (95% Confidence Interval (CI) -4.83, -0.56)) and a lower eGFR (-2.42 ml/min/1.73m^2 (95% CI -4.56, -0.28)). Shorter breastfeeding duration also was associated with smaller combined kidney volume and a lower risk for microalbuminuria (*p*-values <0.05) but not with eGFR. Compared with exclusively breastfed children, children who received non-exclusive breastfeeding for 4 months had a 2.47 cm^3 (95% CI -3.94, -1.01) lower kidney volume and a 1.55 ml/min/1.73m^2 lower eGFR (95% CI -3.01, -0.11). Age at introduction to solid foods was not associated with any kidney outcome. Additional adjustment for combined kidney volume, attenuated the associations of breastfeeding with eGFR into non-significant. The associations between breastfeeding and risk of microalbuminuria remained similar (**Table 3.2.2**).

Results from the analyses focused on creatinine and cystatin C levels are given in the Supplementary Materials (**Supplementary Table 3.2.6**). Non-exclusive breastfeeding was associated with higher creatinine levels. We did not observe any association of breastfeeding with cystatin C levels. Effect estimates from the crude models for the associations of infant feeding with kidney outcomes are given in **Supplementary Table 3.2.7**. When we additionally adjusted for child protein intake in the subgroup of children with protein data available, the effect estimates remained similar (**Supplementary Table 3.2.8**). Furthermore, when we additionally adjusted the kidney volume models for lean body mass, results did not change (**Supplementary Table 3.2.9**). Because the statistical interactions of breastfeeding duration with maternal ethnicity, child sex, birthweight or gestational age were not significant we did not stratify our analyses.

DISCUSSION

In this population-based prospective cohort study, we observed that breastfeeding duration and exclusivity are associated with larger combined kidney volume and higher eGFR in childhood. The associations with kidney function were explained by kidney volume. Although not clinically relevant yet, the observed subclinical changes in kidney function at a young age may predispose individuals to development of kidney disease in later life.[5]

A major strength of our study the prospective design from fetal life onward within a large population-based cohort. Of the total group of singleton live born children in our cohort, 86% of them had available information on breastfeeding. This is due to the study design in which not all parents received the infant feeding questionnaire and to non-response.[10] Of all children with information on breastfeeding, 24% did not participate in the follow-up measurements at the age of 6 years. Not all participants in the study consented to the collection of blood samples; 68% of all children provided blood samples for measurements of creatinine and cystatin C levels. This loss to follow-up would result in selection bias if the associations of infant feeding with kidney outcomes differed between those included and not included in the final analyses. Assessing breastfeeding initiation and duration by questionnaires appears to be a valid method, especially when the events being recalled are relatively recent (within 3 years), which is the case in our study. In our study, we used 3 questionnaires to assess infant feeding during the first 12 months. We performed detailed measurements of childhood kidney outcomes. Kidney size was used as a measure of kidney development. Ultrasound is a reliable method to measure kidney volume.[15] Kidney size, which is correlated with number of glomeruli, can be used in epidemiological studies as measure of kidney development.[2] However, glomerular enlargement due to hyperfiltration may also increase kidney volume.[4] We used urine albumin-creatinine ratio from a random urine sample to evaluate microalbuminuria.[26] Unfortunately, we did not have first morning void samples. Because we had random samples from throughout the day, these measurements are subject to the limitation of substantial intraindividual variation in urinary albumin excretion.[27] Finally, although we adjusted for a large number of potential maternal and childhood confounders, but residual confounding could still happen, as is the case in any observational study.

Two previous studies have investigated the relation between infant feeding and kidney outcomes. In a cohort of 631 children, Schmidt *et al.* observed that 3-months-old infants who were formula-fed had larger kidneys than infants who were exclusive breast-fed.[9] This effect in kidney size was temporary and did not persist at 18 months of age. Their findings are in line with observations from a study among 737 children participating in the EU Childhood Obesity Project.[8] In this study, the authors observed that infants aged 6 months who received high protein infant formula had significantly larger kidney volume compared with infants who received low protein formula or breastfeeding. Kidney volume in breastfed infants did not differ from those receiving low protein formula.[8] In our study, we observed that breastfed children had larger kidney volumes, which is not consistent with previous studies. The main differences between our and previous studies, are that our analysis were performed on a larger population-based study group and that kidney outcomes were measures at a later age. The long-term effects of breastfeeding on kidney outcomes might be different from the short-term effects as indicated by Schmidt *et al.*[9] Escribano *et al,*[8] observed that breastfed infants had lower renal overload, defined as urine urea levels and urine osmolarity, but higher levels of eGFR compared to formula fed infants. They suggest that this may be due to the action of other physiologic mechanisms promoted by different compounds of human milk or a higher bioavailability of human milk proteins. In accordance with this hypothesis, we observed that ever and exclusively breastfed children had a higher eGFR. A limitation of our study is that we

could not use the extended formula by Schwartz,[16] because we had no urea measurements available in our study population. Kidney size is correlated positively with eGFR.[28, 29] We observed that associations of infant feeding with kidney function were explained by increased kidney volume. In the same population as the present study, we previously observed that children with smaller kidneys had higher creatinine blood levels, leading to lower eGFR.[13] It is suggested that kidney size is related to the amount of lean body mass.[30] In our population effect estimates of breastfeeding on kidney size did not change when we additionally adjusted the model for lean body mass. Interestingly, shorter breastfeeding duration was also related to lower risk of microalbuminuria. We cannot explain this finding.

Potential mechanisms by which breastfeeding may affect kidney structure and function include the difference in proteins found in breast milk and infant formula. Breast milk has lower protein content than formula. On average, 100 ml breast milk contains 1.0 g of proteins, whereas the average protein content of formula milk is 2.0 g.[31] Also, protein types found in breast milk differ from those found in formula milk; they may be easier to digest and absorb.[32] It is suggested that higher protein intake leads to a rise in eGFR, as a result of increased glomerular plasma flow and trans glomerular pressure.[8, 28, 33] In our study we observed a higher eGFR in breastfed children. In a subgroup of the present study group (N=2,876), we had dietary data at the age of 1year.When we adjusted our models additionally for child protein intake at the age of 1 year, the regression coefficients remained similar in this subgroup, suggesting that protein intake at the age of 1 year does not affect the association between breastfeeding and kidney outcomes at age of 6 years. Next to protein intake, other nutrients and bioactive substances in breast milk also might influence kidney outcomes in childhood. Although differences between the early feeding categories are small, they may be relevant on population level. Further studies of animals and humans are needed to identify the mechanisms by which infant feeding influences kidney function.

In conclusion, in this population-based prospective cohort study, we observed that compared with children exclusively breastfed for 4 months, those who were non-exclusively breastfed or breastfed for a shorter period had smaller kidneys and lower eGFR. Although the observed differences are not clinically relevant, small differences in kidney function at a young age might relate to clinically relevant kidney outcomes at older ages. Other health consequences of breastfeeding need to be considered when developing advice on infant feeding. Further studies are needed to evaluate the long-term consequences of breastfeeding on kidney health.

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Supplementary Table 3.2.1. Maternal and child characteristics for observed and imputed data (N=5,043)

	Observed	Imputed
Maternal characteristics		
Pre-pregnancy body mass index, kg/m^2	23.5 (18.2, 34.2)	22.7 (18.2, 34.1)
Missing, %	*24.2*	
Ethnicity, %		
- European	65.4	66
- Non - European	32.9	34
Missing, %	*1.7*	
Educational level, %		
- No higher education	44.4	49
- Higher education	49.2	51
Missing, %	*6.4*	
Net household total income, %		
- <1,400 euro	18.6	23.9
- ≥1,400 euro	62.2	76.1
Missing, %	*19.2*	
Smoking during pregnancy, %		
- Never smoked	67.8	80.2
- Smoke until pregnancy was known	8.1	7.6
- Continued smoking	12.7	12.2
Missing, %	*11.4*	
Alcohol during pregnancy, %		
- Never alcohol in pregnancy	33.8	53.1
- Alcohol until pregnancy was known	11.2	11.2
- Continued alcohol	35.7	35.7
Missing, %	*19.3*	
Folic acid supplement use, %		
- No	14.8	23.2
- During early pregnancy	55.7	76.8
Missing, %	*29.5*	
Energy adjusted protein intake, g	74.9 (10.2)	74.9 (11.9)
Missing, %	*31.6*	
Birth characteristics		
Girls, %	50.2	NI
Gestational age, weeks	40.1 (36.0, 42.3)	40.1 (36.0, 42.3)
Missing, %	*0.1*	
Birth weight, g	3,452 (540)	3,452 (540)
Missing, %	*0.06*	
Breastfeeding, %		
- No	7.6	NI
- Yes	92.4	NI
Breastfeeding duration, %		
- >0-1.9 months	19.7	NI
- 2-3.9 months	16.6	NI
- 4-5.9 months	9.5	NI
- ≥6 months	24.8	NI

Supplementary Table 3.2.1. Maternal and child characteristics for observed and imputed data (N=5,043) (continued)

	Observed	Imputed
Missing, %	*29.4*	
Breastfeeding exclusivity, %		
- Non-exclusively in the first 4 months	52.6	NI
- Exclusively in the first 4 months	20.6	NI
Missing, %	*26.8*	
Introduction of solid foods, %		
- <4 months	5.5	NI
- 4-4.9 months	40.3	NI
- ≥5 months	20.3	NI
Missing, %	*33.9*	
Child characteristics		
Age, years	6.0 (5.6, 7.4)	NI
Height, cm	118.8 (5.7)	118.9 (5.7)
Missing, %	*0.02*	
Weight, kg	22.2 (17.4, 32.6)	22.2 (17.4, 32.6)
Missing, %	*0.02*	
Body mass index, kg/m^2	15.8 (13.6, 20.6)	15.8 (13.6, 20.6)
Missing, %	*0.02*	
Body surface area, m^2	0.86 (0.7, 1.1)	0.86 (0.7, 1.1)
Missing, %	*0.02*	
Kidney volume combined, cm^3	120 (23)	NI
Missing, %	*8.4*	
eGFR, ml/min/1.73m^2	119 (16)	NI
Missing, %	*32.6*	
Microalbuminuria, %	7.6	NI
Missing %	*4.1*	
Creatinine, µmol/l	37.2 (5.5)	NI
Missing, %	*32.6*	
Cystatin C, µg/l	738 (81.4)	NI
Missing, %	*32.6*	

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. eGFR-estimated glomerular filtration.
NI-not imputed

Supplementary Table 3.2.2. Subject characteristics according to category of breastfeeding in the subjects with eGFR measurements (N=3,404)

	Total group N=3,404	Never breastfed N=254	Ever breastfed N=3,150	*P*-value
Maternal characteristics				
Pre-pregnancy body mass index (kg/m^2)	22.7 (18.3, 34.0)	23.6 (18.1, 38.4)	22.6 (18.2, 33.6)	**<0.01**
Ethnicity (%)				**<0.01**
- European	66.4	76.9	65.6	
- Non - European	33.6	23.1	34.4	
Education (%)				**<0.01**
- No higher education	48.1	69.7	46.4	
- Higher education	51.9	30.3	53.6	
Net household total income (%)				0.71
- <1,400 euro	23.3	24.6	23.2	
- ≥1,400 euro	76.9	75.4	76.8	
Smoking during pregnancy (%)				**<0.01**
- Never smoked	80.6	66.1	81.8	
- Smoke until pregnancy was known	7.7	8.3	7.6	
- Continued smoking	11.7	25.6	10.6	
Alcohol during pregnancy (%)				**<0.01**
- Never alcohol in pregnancy	51.9	60.6	51.2	
- Alcohol until pregnancy was known	11.3	8.3	11.5	
- Continued alcohol during pregnancy	36.8	31.1	37.3	
Folic acid supplement use, (%)				0.54
- No	23.2	22.3	23.3	
- During early pregnancy	76.8	77.7	76.7	
Energy adjusted protein intake (g)	75 (10.1)	73.1 (9.7)	75.2 (10.1)	**<0.01**
Birth characteristics				
Girls (%)	49.1	48.0	49.1	0.80
Gestational age (weeks)	40.1 (36.0, 42.3)	39.9 (35.9, 42.1)	40.1 (36.0, 42.3)	**<0.01**
Weight (grams)	3,480 (540)	3,430 (542)	3,467 (539)	0.08
Child protein intake at 1 year (g)	26 (29,6)	27.8 (29.4)	25.8 (29.6)	0.37
Breastfeeding duration (%)				
- >0-1.9 months (N=651)	19.1	0	20.7	
- 2-3.9 months (N=565)	16.6	0	17.9	
- 4-5.9 months (N=338)	9.9	0	10.7	
- ≥6 months (N=857)	25.2	0	27.2	
Missing (N=993)	*29.2*			
Breastfeeding exclusivity (%)				
- Non-exclusively in the first 4 months (N=1,791)	52.6	0	56.9	
- Exclusively in the first 4 months (N=712)	20.9	0	22.6	
Missing (N=901)	*26.5*			
Introduction of solid foods (%)				**<0.01**
- <4 months (N=201)	5.9	10.6	5.5	
- 4-4.9 months (N=1,372)	40.3	42.5	40.1	
- ≥5 months (N=693)	20.4	7.9	21.4	
Missing (N=1,138)	*33.4*			

Supplementary Table 3.2.2. Subject characteristics according to category of breastfeeding in the subjects with eGFR measurements (N=3,404) (continued)

	Total group N=3,404	Never breastfed N=254	Ever breastfed N=3,150	*P*-value
Child characteristics				
Age (years)	6.0 (5.6, 7.4)	6.0 (5.7, 7.2)	6.0 (5.6, 7.5)	0.94
Height (cm)	119.2 (5.6)	119 (5.7)	119.2 (5.6)	0.13
Weight (kg)	22.4 (17.5, 32.6)	22.4 (17.4, 34.8)	22.4 (17.6, 32.4)	0.40
Body mass index, (kg/m^2)	15.8 (13.7, 20.5)	15.9 (13.6, 22.0)	15.8 (13.7, 20.5)	0.40
Body surface area, (m^2)	0.86 (0.7, 1.1)	0.86 (0.7, 1.1)	0.86 (0.7, 1.1)	0.17
Kidney volume combined, (cm^3)	120 (22.6)	116.8 (21.6)	120.4 (22.7)	**<0.01**
eGFR, (ml/min/1.73m^2)	119 (16.5)	116 (14.1)	119 (16.7)	**<0.01**
Microalbuminuria (%)*	7.7	5.5	7.7	0.14
Creatinine (µmol/l)	37.2 (5.5)	37.9 (4.9)	37.1 (5.5)	**0.04**
Cystatin C (µg/l)	783 (81.3)	792 (84.6)	782 (80.9)	**0.04**

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 T-test for continuous normally distributed variables, Mann Whitney for non-normally distributed variables, and Chi-squared tests for categorical variables.

eGFR - estimated glomerular filtration rate

*Defined as levels between 2.5 and 25.0 mg/mmol (boys) and 3.5 and 25.0 mg/mmol (girls)

Supplementary Table 3.2.3. Subject characteristics according to category of breastfeeding in the subjects with microalbuminuria measurements (N=4,855)

	Total group N=4,855	Never breastfed N=371	Ever breastfed N=4,484	*P*-value
Maternal characteristics				
Pre-pregnancy body mass index (kg/m^2)	22.6 (18.2, 34.1)	23.5 (18.2, 38.0)	22.6 (18.2, 33.6)	**<0.01**
Ethnicity (%)				**<0.01**
- European	65.7	76.3	64.8	
- Non - European	34.3	23.7	35.2	
Education (%)				**<0.01**
- No higher education	49.2	69.7	47.6	
- Higher education	50.8	30.3	52.4	
Net household total income (%)				0.71
- <1,400 euro	24	25.4	23.9	
- ≥1,400 euro	76	74.6	76.1	
Smoking during pregnancy (%)				**<0.01**
- Never smoked	80.1	67.7	80.0	
- Smoke until pregnancy was known	7.7	7.8	8.3	
- Continued smoking	12.2	24.5	11.7	
Alcohol during pregnancy (%)				**<0.01**
- Never alcohol in pregnancy	53.0	62.3	52.3	
- Alcohol until pregnancy was known	11.2	8.1	11.4	
- Continued alcohol during pregnancy	35.8	29.6	36.3	

Supplementary Table 3.2.3. Subject characteristics according to category of breastfeeding in the subjects with microalbuminuria measurements (N=4,855) (continued)

	Total group N=4,855	Never breastfed N=371	Ever breastfed N=4,484	*P*-value
Folic acid supplement use, (%)				0.54
- No	23.2	22.3	23.3	
- During early pregnancy	76.8	77.7	76.7	
Energy adjusted protein intake (g)	74.8 (10.2)	73.0 (10.2)	75 (10.2)	**<0.01**
Birth characteristics				
Girls (%)	49.6	50.4	49.5	0.80
Gestational age (weeks)	40.1 (36.1, 42.3)	39.9 (35.9, 42.4)	40.1 (36.1, 42.3)	**<0.01**
Weight (grams)	3,453 (538)	3,409 (556)	3,457 (536)	0.08
Child protein intake at 1 year (g)	25.4 (29.3)	28 (29)	25.2 (29.3)	0.37
Breastfeeding duration (%)				
- >0-1.9 months (N=958)	19.7	0	21.3	
- 2-3.9 months (N=815)	16.8	0	18.2	
- 4-5.9 months (N=456)	9.4	0	10.2	
- ≥6 months (N=1,203)	24.8	0	26.8	
Missing (N=1,423)	*29.3*			
Breastfeeding exclusivity (%)				
- Non-exclusively in the first 4 months (N=2,559)	52.7	0	57.1	
- Exclusively in the first 4 months (N=999)	20.6	0	22.3	
Missing (N=1,297)	*26.7*			
Introduction of solid foods (%)				**<0.01**
- <4 months (N=266)	5.5	11.3	5.1	
- 4-4.9 months (N=1,965)	40.5	43.5	40.1	
- ≥5 months (N=987)	20.3	9.3	21.3	
Missing (N=1,637)	*33.7*			
Child characteristics				
Age (years)	6.0 (5.6, 7.4)	6.0 (5.6, 7.2)	6.0 (5.6, 7.4)	0.94
Height (cm)	119 (5.6)	118.5 (5.9)	118.6 (5.6)	0.15
Weight (kg)	22.2 (17.4, 32.6)	22.2 (17.1, 35.6)	22.2 (17.4, 32.4)	0.40
Body mass index, (kg/m^2)	15.8 (13.6, 20.6)	15.8 (13.6, 22.0)	15.8 (13.6, 20.5)	0.40
Body surface area, (m^2)	0.86 (0.7, 1.1)	0.85 (0.7, 1.1)	0.86 (0.7, 1.1)	0.17
Kidney volume combined, (cm^3)	120 (22.5)	116 (23.3)	120 (22.4)	**<0.01**
eGFR, (ml/min/1.73m^2)	119 (16.5)	116.5 (14.1)	119 (16.7)	**<0.01**
Microalbuminuria (%)*	7.6	5.7	7.8	0.14
Creatinine (µmol/l)	37.2 (5.5)	37.8 (4.9)	37.1 (5.2)	**0.04**
Cystatin C (µg/l)	783 (81.2)	793 (86.5)	782 (80.7)	**0.04**

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 T-test for continuous normally distributed variables, Mann Whitney for non-normally distributed variables, and Chi-squared tests for categorical variables.

eGFR-estimated glomerular filtration rate

*Defined as levels between 2.5 and 25.0 mg/mmol (boys) and 3.5 and 25.0 mg/mmol (girls)

Supplementary Table 3.2.4. Maternal and child characteristics for population for analysis (N=5,043) and the group with information on dietary data but no follow-up at 6 years (N=1,573)

	Population for analysis (N=5,043)	No follow-up at 6 years (N=1,573)	P value
Maternal characteristics			
Pre-pregnancy body mass index, kg/m^2	22.7 (18.2, 34.1)	22.3 (17.8, 35.5)	**0.02**
Ethnicity, %			**<0.01**
- European	66	57.3	
- Non - European	34	42.7	
Educational level, %			**<0.01**
- No higher education	49	56.7	
- Higher education	51	43.3	
Net household total income, %			**<0.01**
- <1,400 euro	23.9	5.9	
- ≥1,400 euro	76.1	94.1	
Smoking during pregnancy, %			**0.02**
- Never smoked	80.2	55	
- Smoke until pregnancy was known	7.6	5	
- Continued smoking	12.2	40	
Alcohol during pregnancy, %			0.57
- Never alcohol in pregnancy	53.1	57.9	
- Alcohol until pregnancy was known	11.2	10.5	
- Continued alcohol	35.7	31.6	
Folic acid supplement use, %			0.37
- No	23.2	22.2	
- During early pregnancy	76.8	77.8	
Energy adjusted protein intake, g	74.9	73.4	0.62
Birth characteristics			
Girls, %	50.2	43.8	0.20
Gestational age, weeks	40.1 (36.0, 42.3)	40.0 (35.9, 42.3)	0.13
Birth weight, g	3,452 (540)	3,417 (564)	**0.03**
Child protein intake 1y, g	25.4 (29.4)	25.8 (31)	0.76
Breastfeeding, %			0.76
- No	7.6	9.6	
- Yes	92.4	90.4	
Breastfeeding duration, %			**<0.01**
- >0-1.9 months	19.7	39.4	
- 2-3.9 months	16.6	23.6	
- 4-5.9 months	9.5	10.5	
- ≥6 months	24.9	26.5	
Breastfeeding exclusivity, %			**<0.01**
- Non-exclusively in the first 4 months	52.8	73.8	
- Exclusively in the first 4 months	20.6	21.7	
Introduction of solid foods, %			
- <4 months	5.5	-	
- 4-4.9 months	40.3	45.5	

Supplementary Table 3.2.4. Maternal and child characteristics for population for analysis (N=5,043) and the group with information on dietary data but no follow-up at 6 years (N=1,573) (continued)

	Population for analysis (N=5,043)	No follow-up at 6 years (N=1,573)	P value
- ≥5 months	20.3	54.5	
Child characteristics			
Age, years	6.0 (5.6, 7.4)	-	
Height, cm	118.8 (5.7)	-	
Weight, kg	22.2 (17.4, 32.6)	-	
Body mass index, kg/m^2	15.8 (13.6, 20.6)	-	
Body surface area, m^2	0.86 (0.7, 1.1)	-	
Kidney volume combined, cm^3	120 (23)	-	
eGFR, ml/min/1.73m^2	119 (16)	-	
Microalbuminuria, %	7.6	-	
Creatinine, µmol/l	37.2 (5.5)	-	
Cystatin C, µg/l	738(81.4)	-	

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the non-followed up group) were evaluated using T-test for continuous normally distributed variables, Mann Whitney for non-normally distributed variables, and Chi-squared tests for categorical variables. eGFR-estimated glomerular filtration rate. Non response group, children with no kidney measurements but with available information on breastfeeding.

Supplementary Table 3.2.5. Subject characteristics for children with and without blood sample available at the age of 6 years (among population for analysis)

	With blood sample (N=3,413)	Without blood sample (N=1,630)	P value
Maternal characteristics			
Pre-pregnancy body mass index, kg/m^2	22.7 (18.2, 34.0)	22.6 (18.2, 34.2)	0.69
Ethnicity, %			0.37
- European	66.4	65	
- Non - European	33.6	35	
Educational level, %			**0.04**
- No higher education	48	50.9	
- Higher education	52	49.1	
Net household total income, %			0.09
- <1,400 euro	23.3	25.2	
- ≥1,400 euro	76.7	74.8	
Smoking during pregnancy, %			0.37
- Never smoked	79.5	78.5	
- Smoke until pregnancy was known	8.3	7.8	
- Continued smoking	12.2	13.7	
Alcohol during pregnancy, %			**<0.01**
- Never alcohol in pregnancy	51.9	55.4	
- Alcohol until pregnancy was known	11.3	11.0	

Supplementary Table 3.2.5. Subject characteristics for children with and without blood sample available at the age of 6 years (among population for analysis) (continued)

	With blood sample (N=3,413)	Without blood sample (N=1,630)	P value
- Continued alcohol	36.8	33.6	
Folic acid supplement use, %			0.91
- No	23.2	23.1	
- During early pregnancy	76.8	76.9	
Energy adjusted protein intake, g	75.0	74.6	0.18
Birth characteristics			
Girls, %	49.1	52.5	**0.03**
Gestational age, weeks	40.1 (36.0, 42.3)	40.1 (36.3, 42.3)	0.92
Birth weight, g	3,464 (539)	3,430 (538)	**0.04**
Child protein intake 1y, g	25.4 (29.4)	24.3 (29)	0.16
Breastfeeding, %			0.69
- No	7.5	7.8	
- Yes	92.5	92.2	
Breastfeeding duration, %			0.22
- >0-1.9 months	19.1	20.9	
- 2-3.9 months	16.6	16.5	
- 4-5.9 months	9.9	8.5	
- ≥6 months	25.2	24.2	
Breastfeeding exclusivity, %			0.56
- Non-exclusively in the first 4 months	52.7	52.5	
- Exclusively in the first 4 months	20.9	19.9	
Introduction of solid foods, %			0.25
- <4 months	5.9	4.7	
- 4-4.9 months	60.5	40.3	
- ≥5 months	30.6	20.1	
Child characteristics			
Age, years	6.0 (5.6, 7.2)	6.0 (5.6, 7.4)	0.26
Height, cm	118.8 (5.6)	118.3 (5.7)	**<0.01**
Weight, kg	22.4 (17.5, 32.6)	22.0 (17.2, 32.6)	**<0.01**
Body mass index, kg/m^2	15.8 (13.7, 20.5)	15.8 (13.5, 20.8)	0.50
Body surface area, m^2	0.86 (0.7, 1.1)	0.85 (0.7, 1.1)	**<0.01**
Kidney volume combined, cm^3	120 (22.6)	118.6 (22.5)	**0.04**
eGFR, ml/min/1.73m^2	119 (16)	-	
Microalbuminuria, %	7.7	7.5	0.76
Creatinine, µmol/l	37.2 (5.5)	-	
Cystatin C, µg/l	738 (81.4)	-	

Values are means (SD), percentages (%), or medians (95% range) for variables with skewed distribution. Differences in maternal, infant and childhood characteristics (compared with the non-blood sample group) were evaluated using T-test for continuous normally distributed variables, Mann Whitney for non-normally distributed variables, and Chi-squared tests for categorical variables.

eGFR-estimated glomerular filtration rate

Supplementary Table 3.2.6. Associations of infant feeding with creatinine and cystatin C at the age of 6 years adjusted for child age, sex, family-based socio-demographic, maternal lifestyle and childhood factors

	Difference in outcome measure (95% Confidence Interval)			
	Creatinine (µmol/l)	**Cystatin C (µg/L)**	**[§]Creatinine (µmol/l) Adjusted for kidney volume**	**[§]Cystatin C (µg/L) Adjusted for kidney volume**
	N=3,411	**N=3,410**	**N=3,171**	**N=3,178**
Breastfeeding				
Never (N=382)	0.44 (- 0.29, 1.16)	1.53 (- 9.75, 12.81)	0.16 (- 0.56, 0.88)	-0.50 (-11.73, 10.74)
Ever (N=4,661)	*Reference*	*Reference*	*Reference*	*Reference*
Duration				
>0-1.9 months (N=993)	0.47 (-0.10, 1.04)	-1.63 (-10.45, 7.19)	0.31 (-0.26, 0.88)	-2.69 (-11.67, 6.30)
2-3.9 months (N=836)	0.57 (-0.01, 1.14)	1.72 (-7.19, 10.64)	0.54 (-0.03, 1.12)	3.12 (-5.97, 12.21)
4-5.9 months (N=477)	0.10 (-0.59, 0.78)	-1.35 (-11.94, 9.24)	0.16 (-0.52, 0.85)	-1.43 (-12.23, 9.37)
≥6 months (N=1,253)	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	***0.05***	*0.87*	*0.15*	*0.78*
Exclusive for 4 months				
No (N=2,653)	**0.63 (0.15, 1.11)***	3.81 (-3.57, 11.19)	0.43 (-0.06, 0.92)	2.26 (-5.31, 9.84)
Yes (N=1,063)	*Reference*	*Reference*	*Reference*	*Reference*
First solid foods				
<4 months (N=263)	0.27 (-0.61, 1.15)	-2.33 (-15.72, 11.05)	0.14 (-0.74, 1.01)	-4.05 (-17.47, 9.37)
4-4.9 months (N=1,864)	**0.27 (0.23, 0.76)**	2.39 (-5.20, 9.97)	0.28 (-0.22, 0.78)	2.33 (-5.33, 9.97)
≥5 months (N=989)	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.35*	*0.94*	*0.45*	*0.91*

Values are linear regression coefficients (95% confidence intervals). Models are adjusted for child's age at visit, sex, plus family-based socio-demographic confounders (maternal education, ethnicity, income), maternal lifestyle related factors (pre-pregnancy BMI, smoking and alcohol usage during pregnancy, folic acid supplements and protein-energy intake during pregnancy), childhood factors (birthweight, gestational age, body surface area). 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 [§] creatinine and [§] cystatin C are additionally adjusted for kidney volume.

Supplementary Table 3.2.7. Associations of infant feeding with kidney outcomes at the age of 6 years adjusted for child age and sex (N=5,043)

	Difference in outcome measure (95% Confidence Interval)				
	Kidney volume (cm^3) N=4.637	**Creatinine (μmol/l) N=3,411**	**Cystatin C (μg/L) N=3,410**	**eGFR ($ml/min/1.73m^2$) N=3,411**	**Microalbuminuria (odds ratio) N=4,855**
Breastfeeding					
Never (N=382)	**-3.79 (-6.17, -1.42)***	**0.77 (0.09, 1.45)***	**10.77 (0.40, 21.14)***	**-3.16 (-5.26, -1.06)***	0.71 (0.45, 1.12)
Ever (N=4,661)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Duration					
>0-1.9 months (N=993)	**-3.44 (-5.39, -1.59)***	0.50 (- 0.04, 1.03)	1.02 (-7.14, 9.18)	-1.51 (-3.17, 0.15)	0.78 (0.57, 1.07)
2-3.9 months (N=836)	-1.90 (-3.84, 0.04)	**0.58 (0.02, 1.13)***	3.47 (-5.02, 11.95)	-1.34 (-3.06, 0.39)	**0.61 (0.43, 0.87)***
4-5.9 months (N=477)	-0.02 (-5.64, 2.33)	0.20 (-0.47, 0.86)	-1.36 (-11.43, 8.71)	-0.26 (-2.31, 1.79)	0.83 (0.56, 1.24)
≥6 months (N=1,253)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	***<0.01***	***0.03***	*0.62*	***0.04***	***0.04***
Exclusive for 4 months					
No (N=2,653)	**-1.69 (-2.53, -0.86)***	**0.65 (0.19, 1.11)***	3.75 (-3.15, 10.65)	**-1.94 (-3.37, -0.51)***	0.82 (0.63, 1.08)
Yes (N=1,036)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
First solid foods					
<4 months (N=236)	-1.71 (-4.60, 1.18)	0.23 (-0.60, 1.06)	3.29 (-3.87, 10.45)	-1.43 (-3.99, 1.13)	1.04 (0.63, 1.73)
4-4.9 months (N=1,864)	-1.36 (-3.01, 0.30)	0.35 (-0.13, 0.83)	-0.92 (-13.27, 11.43)	-0.94 (-2.43, 0.54)	0.96 (0.72, 1.28)
≥5 months (N=989)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.11*	*0.28*	*0.73*	*0.17*	*0.98*

Values are linear and logistic regression coefficients (95% confidence intervals). Models are adjusted for child's age and sex at visit. 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

Supplementary Table 3.2.8. Associations of infant feeding with kidney outcomes at the age of 6 years adjusted for child age, sex, family-based socio-demographic, maternal lifestyle and childhood factors, additionally adjusted for child protein intake at 1 y (N=2,876)

	Difference in outcome measure (95% Confidence Interval)				
	Kidney volume (cm^3) N=2,671	**Creatinine (μmol/l) N=1,940**	**Cystatin C (μg/L) N=1,941**	**eGFR (ml/min/1.73m^2) N=1,939**	**Microalbuminuria (odds ratio) N=2,773**
Breastfeeding					
Never (N=207)	**-3.45 (-6.22, -0.68)***	0.42 (0.47, 1.31)	5.25 (-8.56, 19.06)	-2.03 (-4.87, 0.80)	0.59 (0.30, 1.19)
Ever (N=2,669)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Duration					
>0-1.9 months (N=596)	**-3.35 (-5.43, -1.27)***	0.11 (-0.56, 0.79)	-2.14 (-12.48, 8.20)	-0.16 (-2.43, 2.02)	0.83 (0.54, 1.27)
2-3.9 months (N=557)	-0.67 (-2.71, 1.38)	0.13 (-0.53, 0.80)	0.18 (-10.00, 10.35)	0.29 (-1.86, 2.44)	**0.59 (0.38, 0.93)***
4-5.9 months (N=338)	-0.36 (-2.76, 2.04)	-0.26 (-1.02, 0.50)	-4.25 (-15.87, 7.38)	0.86 (-1.59, 3.31)	0.84 (0.52, 1.37)
≥6 months (N=861)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	***<0.01***	*0.63*	*0.80*	*0.91*	*0.15*
Exclusive for 4 months					
No (N=1,629)	-0.92 (-2.60, 0.76)	0.22 (-0.32, 0.76)	4.38 (-3.86, 12.62)	-0.30 (-2.03, 1.43)	0.98 (0.70, 1.39)
Yes (N=774)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
First solid foods					
<4 months (N=185)	1.50 (-1.60, 4.59)	-0.75 (-1.73, 0.23)	-6.81 (-21.89, 8.27)	2.32 (-0.84, 5.47)	1.15 (0.61, 2.21)
4-4.9 months (N=1,425)	0.17 (-1.52, 1.86)	0.03 (-0.54, 0.55)	4.05 (-4.31, 12.42)	0.21 (-1.54, 1.96)	0.57 (0.60, 1.24)
≥5 months (N=786)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
P for trend	*0.46*	*0.32*	*0.93*	*0.28*	*0.86*

Values are linear and logistic regression coefficients (95% confidence intervals). Models are adjusted for child's age at visit, sex, plus family-based socio-demographic confounders (maternal education, ethnicity, income), maternal lifestyle related factors (pre-pregnancy BMI, smoking and alcohol usage during pregnancy, folic acid supplements and protein energy intake during pregnancy), and childhood factors (birth weight, BSA, protein intake at year 1). 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. *$P < 0.05$

Supplementary Table 3.2.9. Associations of infant feeding with kidney volume at the age of 6 years adjusted for child age, sex, family-based socio-demographic, maternal lifestyle, childhood factors, and lean body mass (N=4,637)

	Kidney volume (cm^3) N=4,637
Breastfeeding	
Never (N=354)	**-2.56 (-4.69, -0.44)***
Ever (N=4,283)	*Reference*
Duration	
>0-1.9 months (N=925)	**-2.40 (-4.09, -0.70)***
2-3.9 months (N=775)	-1.13 (-2.86, 0.60)
4-5.9 months (N=438)	-0.18 (-2.26, 1.90)
≥6 months (N=1,143)	*Reference*
P for trend	***<0.01***
Exclusive for 4 months	
No (N=2,542)	**-2.34 (-3.80, -0.88)***
Yes (N=952)	*Reference*
First solid foods	
<4 months (N=225)	0.01 (-3.89, 3.90)
4-4.9 months (N=1,716)	-0.72 (-2.87, 1.43)
≥5 months (N=914)	*Reference*
P for trend	*0.93*

Values are linear regression coefficients (95% confidence intervals). Models are adjusted for child's sex, plus family-based socio-demographic confounders (maternal education, income), maternal lifestyle related factors (pre-pregnancy BMI, smoking and alcohol usage during pregnancy, folic acid supplements and protein-energy intake during pregnancy), childhood factors (birth weight, BSA and lean body mass). 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

Childhood estimates of glomerular filtration rate based on creatinine and cystatin C: Importance of body composition

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ABSTRACT

Background: Creatinine and cystatin C concentrations are commonly used to estimate glomerular filtration rate (eGFR) in clinical practice and epidemiological studies. To estimate the influence of different body composition measures on eGFR from creatinine and cystatin C blood concentrations, we compared the associations of different anthropometric and body composition measures with eGFR derived from creatinine ($eGFR_{creat}$) and cystatin C ($eGFR_{cystC}$) blood concentrations.

Methods: In a population-based cohort study among 4,305 children aged 6.0 years (95% range 5.7–8.0), we measured weight and height and calculated body mass index and body surface area, and lean and fat mass by Dual-energy X-ray Absorptiometry. At the same age, we measured creatinine and cystatin C blood concentrations and estimated the GFR.

Results: Correlation between eGFR based on creatinine and cystatin C concentrations was r = 0.40 (*p*-value <0.01). Higher body mass index was associated with lower $eGFR_{cystC}$ but not with $eGFR_{creat}$. Higher body surface area was associated with higher $eGFR_{creat}$ and lower $eGFR_{cystC}$ (*p*-values <0.05). Lean and fat mass percentages were associated with $eGFR_{creat}$ but not with $eGFR_{cystC}$.

Conclusions: Our findings suggest that both $eGFR_{creat}$ and $eGFR_{cystC}$ are influenced by body mass index and body surface area. $eGFR_{creat}$ is more strongly influenced by body composition than $eGFR_{cystC}$.

INTRODUCTION

Glomerular filtration rate (GFR) plays a key role in the management of kidney disease. Ideally, measuring GFR should be based on renal clearances of exogenous markers such as inulin, but this approach is complex, invasive and expensive.[1] Therefore, GFR is commonly estimated based on creatinine blood concentrations.[2] Using creatinine as marker of renal function has some limitations. Creatinine, is actively secreted by the proximal tubule, and is related to muscle mass, age, sex, ethnicity and dietary factors.[1, 3] Creatinine concentrations can be higher in individuals with an increased muscle mass, independent of kidney function, leading to an underestimation of eGFR.[4] Studies in adults suggest that eGFR based on creatinine concentrations can be improved if lean mass percentage could be incorporated in the formula.[4]

An alternative marker for the estimation of GFR is cystatin C, the concentrations of which are reported to be independent of muscle mass in children.[5, 6] Some authors report a superior sensitivity of cystatin C for detecting impaired GFR in pediatric patients to that of creatinine, especially in children with low muscle mass.[7] However, studies in kidney disease patients suggest that lean mass affects cystatin C concentrations.[8, 9] Many studies have explored the associations between body mass index (BMI) and eGFR, using BMI as a proxy for body composition.[10–11] To our knowledge, large population based studies in healthy children comparing the correlations and associations of detailed body composition measures, next to BMI, with the estimates of GFR are lacking.

To estimate the influence of different body composition measures on eGFR from creatinine and cystatin C blood concentrations, we compared the associations of different anthropometric and body composition measures with eGFR derived from creatinine ($eGFR_{creat}$) and cystatin C ($eGFR_{cystC}$) blood concentrations in a population-based prospective cohort study among 4,305 children who were 6 years of age.

METHODS

Design and study population

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands, which has been described in detail previously.[12] The study has been approved by Medical Ethical Committee of Erasmus MC, University Medical Center Rotterdam. All children were born between April 2002 and January 2006 and form a largely prenatally enrolled birth cohort that is currently being followed until young adulthood. Written consent was obtained for all children. The present analyses were performed among 4,305 children with body composition and kidney function measures available (**Supplementary Figure 3.3.1**).

Body composition measurements

Children's anthropometrics and body composition were measured at a median age of 6.0 years (95% range 5.7 to 8.0).[12] Height (m) was determined in standing position to the nearest millimeter without shoes using a Harpenden stadiometer (Holtain Limited, Dyfed, U.K.). Weight was measured using a mechanical personal scale (SECA, Almere, The Netherlands). We calculated BMI (kg/m^2) and body surface area (BSA) (m^2). For BSA, we used the DuBois formula: BSA = weight (kg)$^{0.425}$ x height (cm)$^{0.725}$ x 0.007184. Whole body dual-energy X-ray absorptiometry (DXA) scans (iDXA, GE-Lunar, 2008, Madison, WI, USA) were performed to estimate fat and lean mass. We calculated lean mass percentage as (lean mass (kg) /weight (kg)) and fat mass percentage as (fat mass (kg) /weight (kg)).

Kidney function measurements

Non-fasting blood samples were drawn by antecubital venipuncture and centrifuged for 10 minutes and stored at -80 °C at one location in the STAR-MDC laboratory. As previously described, creatinine concentrations were measured with enzymatic methods and cystatin C concentrations with a particle enhanced immunoturbidimetric assay (using Cobas 8000 analyzers, Roche, Almere, the Netherlands). Quality control samples demonstrated intra-assay and inter-assay coefficients of variation of 0.51% for creatinine and 1.65% for cystatin C, and 1.37% for creatinine and 1.13% for cystatin C, respectively.[13] We calculated the eGFR based on creatinine concentrations according to the revised Schwartz 2009 formula: $eGFR_{creat}$ = 36.5 * (height (cm)/ serum creatinine (µmol/l)),[14] and eGFR based on cystatin C concentrations using Zappitelli's formula: $eGFR_{cystC}$ = 75.94/[CysC (mg/L)$^{1.17}$].[15]

Statistical analysis

We performed a non-response analysis by comparing subject characteristics between children with and without kidney function measurements using T-tests, Chi-square tests and Mann-Whitney tests. We created standard deviations scores (SDS) for all body composition measures to enable comparison between effect estimates. Next, we examined the Pearson rank correlation coefficients between childhood anthropometrics, body composition and eGFR measures. Third, we used multiple linear regression analyses to examine the associations of anthropometric and body composition measures with creatinine, cystatin C, $eGFR_{creat}$ and $eGFR_{cystC}$. The linear regression models were adjusted for child sex, age at measurements, and ethnicity. Additionally, we explored the associations of childhood BMI clinical cut-offs with creatinine, cystatin C and eGFRs. Because of the already reported associations of ethnicity with kidney function markers, we performed a sensitivity analysis in children of European ethnicity, the largest ethnic subgroup.[16] Based on previous literature we assessed whether the explored association differed by sex, which was not the case in this study.[17, 18] All analyses were performed using the Statistical Package for the Social Sciences version 21.0 for Windows (SPSS IBM, Chicago, IL, USA).

RESULTS

Participant characteristics

Supplementary Figure 3.3.1 describes the selection of the study population. At the median age of 6 years (95% range 5.7, 8.0) a total of 8,305 children participated in the study follow-up measurements. Of these children, 6,509 (78%) visited the research center for body composition measurements. In this study, we excluded children with congenital kidney abnormalities (N=12). A total of 6,497 children were available for kidney function measurements. Of these children, N=2,192 did not have kidney measurements. The present analyses were performed among 4,305 children with body composition and kidney function measures available.

Table 3.3.1 shows the characteristics of the participants. In the full group the mean (SD) $eGFR_{creat}$ and $eGFR_{cystC}$ were 118.9 ml/min/1.73m^2 (15.9) and 101.6 ml/min/1.73m^2 (11.2), respectively. The histograms of creatinine, cystatin C, $eGFR_{creat}$ and $eGFR_{cystC}$ are provided in **Supplementary Figure 3.3.2**. Results from the non-response analyses are given in the **Supplementary Table 3.3.1**. Children with kidney function measurements had higher lean mass percentage and lower fat mass percentage compared to children who did not have kidney function measurements.

Table 3.3.1. Subjects characteristics (N=4,305)

Subjects characteristics	
Age at measurements (y)	6.0 (5.7, 8.0)
Sex, Girls (%)	48.3
Ethnicity (%)	
- Dutch or European	65.1
- Non-European	34.9
Height (cm)	119.7 (6.0)
*SD-score (mean, sd)	-0.2 (1.0)
Weight (kg)	23.4 (4.2)
*SD-score (mean, sd)	0.1 (1.0)
Body mass index (kg/m^2)	16.2 (1.8)
*SD-score (mean, sd)	0.3 (0.9)
Body surface area (m^2)	0.88 (0.09)
Lean mass percentage (%)	71.7 (5.4)
Fat mass percentage (%)	24.7 (5.6)
Creatinine (µmol/l)	37.4 (5.3)
Cystatin C (µg/l)	787.3 (74.4)
$eGFR_{creat}$ (ml/min/1.73m^2)	118.9 (15.9)
$eGFR_{cystC}$ (ml/min/1.73m^2)	101.6 (11.2)

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. *The standard deviation scores were obtained using Dutch reference growth curves (Growth Analyzer 3.0, Dutch Growth Research Foundation, Rotterdam, the Netherlands). Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood concentrations; $eGFR_{creat}$ = 36.5 * (height (cm)/ serum creatinine (µmol/l)); $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood concentrations $eGFR_{cystC}$ = 75.94/[CysC(mg/L)$^{1.17}$].

Correlations of childhood anthropometrics and body composition measures with eGFR

The Pearson rank correlation coefficient between $eGFR_{creat}$ and $eGFR_{cystC}$ was r = 0.40 (*p*-value <0.01) (**Table 3.3.2**). Childhood height, weight, BMI and BSA were positively correlated with creatinine and cystatin C concentrations, with stronger coefficients for creatinine concentrations (*p*-values <0.01). Lean mass percentage was positively correlated with creatinine concentrations and negatively correlated with $eGFR_{creat}$ (r = 0.13, *p*-value <0.01). Similar results, but in opposite directions were observed for fat mass percentage. Lean mass percentage and fat mass percentage were not correlated with cystatin C concentrations or $eGFR_{cystC}$.

Table 3.3.2. Correlation coefficients of the investigated variables

	Height	Weight	BMI	BSA	FMP	LMP	Creat	Cyst C	$eGFR_{creat}$	$eGFR_{cystC}$
Height	1.00									
Weight	0.77**	1.00								
BMI	0.31**	0.83**	1.00							
BSA	0.90**	0.97**	0.69**	1,00						
FMP	0.18**	0.57**	0.69**	0.46**	1,00					
LMP	-0.19**	-0.56**	-0.68**	-0.45**	-0.999**	1,00				
Creat	0.30**	0.28**	0.16**	0.30**	-0.05**	0.05**	1,00			
Cyst C	0.05**	0.06**	0.05**	0.06**	-0.001	0.003	0.40**	1,00		
$eGFR_{creat}$	0.06**	0.01	-0.04**	0.03	0.12**	-0.13**	-0.92**	-0.40**	1,00	
$eGFR_{cystC}$	-0.05**	-0.06**	-0.05**	-0.06**	0.01	-0.01	-0.39**	-0.99**	0.40**	1,00

** Correlation is significant at the 0.01 level (2-tailed).

Abbreviations: BMI- body mass index, BSA- body surface area, FMP- fat mass percentage, LMP- lean mass percentage, Creat- creatinine, Cyst C- cystatin C, $eGFR_{creat}$ estimated glomerular filtration creatinine-based, $eGFR_{cystC}$ estimated glomerular filtration cystatin C-based.

Associations of childhood body composition measures with eGFR

Table 3.3.3 shows the results from the linear regression models. Childhood height was associated with creatinine concentrations (*p*-value <0.05), but not with cystatin C concentrations. Higher childhood weight was associated with both higher creatinine and cystatin C concentrations (*p*-value <0.01). Higher childhood height was associated with higher $eGFR_{creat}$ (*p*-value <0.01), but not with $eGFR_{cystC}$ whereas higher childhood weight was associated with higher $eGFR_{creat}$ and lower $eGFR_{cystC}$ (*p*-value <0.01).

Each 1-SD increase in BSA was associated with 1.81 ml/min/1.73m^2 (95% confidence interval (CI) 1.24, 2.37) higher $eGFR_{creat}$ and 0.57 ml/min/1.73m^2 (95% CI -0.98, -0.17) lower $eGFR_{cystC}$. BMI was negatively associated with $eGFR_{cystC}$ (*p*-value <0.05) but not with $eGFR_{creat}$. We observed tendencies for similar effect estimates when we restricted the analyses to Europeans only (N=2,727) (**Supplementary Table 3.3.2**). The associations of BMI clinical cut-offs with creatinine, cystatin C and the eGFR are given in **Supplementary Table 3.3.3**.

Higher lean mass percentage was associated with higher creatinine concentrations and with lower cystatin C concentrations (**Table 3.3.3**) (*p*-values <0.05). A 1-SD increase in lean

Table 3.3.3. Associations of anthropometric and body composition measures with creatinine, cystatin C and eGFR (N=4,305)

	Difference (95% Confidence Interval)			
	Creatinine (µmol/l)	**Cystatin C (µg/l)**	**$eGFR_{creat}$ (ml/min/1.73m²)**	**$eGFR_{cystC}$ (ml/min/1.73m²)**
Anthropometrics and body composition (SDS)				
Height	0.97 (0.79, 1.15)***	1.99 (-0.70, 4.70)	2.78 (2.22, 3.35)***	-0.29 (-0.70, 0.12)
Weight	0.91 (0.73, 1.08)***	4.49 (1.91, 7.07)***	1.16 (0.61, 1.71)***	-0.66 (-1.05, -0.27)**
Body mass index	0.52 (0.36, 0.68)***	4.15 (1.80, 6.51)***	-0.37 (-0.87, 0.13)	-0.61 (-0.96, -0.26)**
Body surface area	1.02 (0.84, 1.20)***	3.93 (1.27, 6.59)**	1.81 (1.24, 2.37)***	-0.57 (-0.98, -0.17)**
Lean mass percentage	0.30 (0.07, 0.54)*	-2.73 (-5.28, -0.18)*	-2.74 (-3.27, -2.20)***	0.36 (-0.03, 0.74)
Fat mass percentage	-0.48 (-0.65, -0.31)***	2.86 (0.32, 5.40)*	2.68 (2.14, 3.21)***	-0.38 (-0.76, 0.01)

Values are beta coefficients and 95% confidence intervals, from linear regression models adjusted for child age, sex and ethnicity. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood concentrations; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood concentrations
P value for the associations *<0.05 **<0.01 ***<0.001.

mass percentage was associated with 2.74 ml/min/1.73m² (95% CI -3.27, -2.20) lower childhood $eGFR_{creat}$. No association was observed of lean mass percentage with $eGFR_{cystC}$.

Similar results but in an opposite direction were observed for fat mass percentage (**Table 3.3.3**). We observed similar effect estimates when we restricted our analyses to European subjects only, although not all associations were significant (**Supplementary Table 3.3.2**).

DISCUSSION

Results of this cross-sectional study in healthy 6-year-old children suggest that BMI and BSA are associated with creatinine-based eGFR and cystatin C-based eGFR. Lean mass percentage and fat mass percentage are associated with creatinine-based eGFR, but not with cystatin C-based eGFR.

Interpretation of main findings

To our knowledge, this is the first large population-based study in healthy school-age children comparing the association of detailed measures of body composition using DXA scans with estimates of GFR based on creatinine and cystatin C concentrations. Both creatinine and cystatin C concentrations can be influenced by different factors. Creatinine is produced in active muscle and is reported to be determined by muscle mass and dietary intake, which may account for the variations in the concentrations of serum creatinine observed among different age and ethnic groups.[1, 3] However, we have previously reported that childhood protein intake does not influence the eGFR.[19] Cystatin C is another marker to evaluate renal function, although is not used as commonly as creatinine.[6, 20] Cystatin C is produced by all nucleated cells and is reported to be less strongly related to body weight and height in chil-

dren compared to creatinine.[5, 7] Besides, adult studies suggest that cystatin C concentrations are related to age, sex, height and weight and influenced by corticosteroid use.[17, 21]

In children, the Schwartz formula is widely used to estimate GFR from creatinine concentrations.[14] The Schwartz formula is known to overestimate eGFR compared to inulin clearance GFR.[6, 22, 23] Schwartz formula estimates GFR using creatinine concentrations and child height.[14] Therefore, the observed effect estimates in the current study of height on $eGFR_{creat}$ might be clinically not relevant. Next to Schwartz's formula we estimated GFR using the Zappitelli's formula.[15] This formula is not dependent of any anthropometric measures. It estimates GFR by using only the cystatin C concentrations.[15] In a study among 42 healthy adults, eGFR using creatinine and cystatin C concentrations was compared with measured GFR. This study suggested that eGFR based on cystatin C concentrations was a better marker then eGFR based on creatinine concentrations for estimating kidney function.[24]

It has previously been reported that lean body mass, indicating muscular mass, is an important determinant of the GFR.[25] So far results from studies comparing the effects of body composition measures on creatinine and cystatin C concentrations and their derived eGFR are contradictory.[8, 26] A number of studies have explored the associations of BMI with eGFR, using BMI as a proxy of body composition.[10–11] The associations of BMI with creatinine and cystatin C differed between populations studied. We observed that higher BMI was associated with higher creatinine and cystatin C concentrations and with lower $eGFR_{cystC}$, but not with $eGFR_{creat}$. In the subgroup of Europeans, we observed that BMI was associated with creatinine, cystatin C concentrations, and their derived eGFRs. In line with our findings in the European subgroup, in the general Japanese population, BMI was associated with lower $eGFR_{creat}$.[11] Similar to what we observe, studies among both healthy and kidney diseased adults suggested that eGFR based on cystatin C concentrations is not independent of BMI.[8, 27] These findings appear to be different among children with various kidney diseases, where BMI does not have a clinically relevant effect on $eGFR_{cystC}$.[10] Next to BMI, we observed that higher BSA was associated with higher creatinine, cystatin C concentrations, $eGFR_{creat}$ and lower $eGFR_{cystC}$. The effect estimates for the associations of BMI and BSA with eGFRs are relatively small. Studying detailed measures of body composition will therefore likely add to the understanding of the associations of body composition and kidney function measures.

Studies among healthy adults have shown lean mass to be associated with serum creatinine but not with cystatin C concentrations.[4, 26] A study among 67 healthy individuals of ages between 18 and 52 years has shown that creatinine concentrations were highly affected by muscle mass, whereas cystatin C concentrations were affected by fat mass.[28] The associations between lean mass and cystatin C are reported to be different among kidney disease patients.[8] Among 77 chronic kidney disease patients lean mass affected cystatin C concentrations and GFR estimation based on cystatin C concentrations improved when lean mass was included in the formula, especially in patients with extreme body composition.[8] In severely obese children lean mass percentage has been reported to correlate with both creatinine and cystatin C concentrations.[29] In the current study, we observed that lean and fat mass percentage correlate with creatinine concentrations and $eGFR_{creat}$. Higher lean mass percentage and lower fat mass percentage were associated with higher creatinine concentrations and lower

$eGFR_{creat}$. We did not observe a significant correlation of lean mass percentage or fat mass percentage with cystatin C concentrations. Our study shows that eGFR based on cystatin C concentrations is independent of lean mass percentage and fat mass percentage.

Our findings suggest that $eGFR_{creat}$ is more strongly influenced by body composition than $eGFR_{cystC}$. However, their impact on clinical care may be limited. As the revised Schwartz formula ($eGFR_{creat}$) is the most widely used formula both in epidemiological studies and clinical practice, our findings suggest that body composition measures should be considered when GFR is estimated based on creatinine concentrations. Ideally, body composition measures would have been incorporated in the $eGFR_{creat}$ equations and compared with GFR based on renal clearances of exogenous markers, but unfortunately this is not possible in our study. Considering the feasibility and costs of performing DXA scans in school-aged children, whether and to what extent detailed body composition measures should be used in the clinical practice when eGFR can be argued. Other studies are needed to assess whether using $eGFR_{cystC}$ instead of $eGFR_{creat}$ leads to better care for pediatric kidney patients.

Strengths and limitations

To the best of our knowledge, this is the first and largest cross-sectional multiethnic study in a healthy pediatric population-based cohort examining the associations of body composition with estimates of GFR. GFR was estimated based on creatinine and cystatin C concentrations. Except height and weight to calculate BMI and BSA, we also measured fat mass and lean mass with DXA. Of all children 61% provided blood samples for measuring creatinine and cystatin C concentrations. Children without data on kidney function measures were shorter, had a higher fat mass percentage and lower lean mass percentage. The difference in fat and lean mass percentage may be explained by the higher percentage of girls in the group without available kidney measures. There was no difference in BMI between children with and without available kidney function measures. We observed tendencies for similar effect estimates among Europeans only, although not all associations were significant in this subgroup. This might be due to the smaller study group, but may also reflect an effect of ethnicity. Ideally, we would have been able to compare the explored associations with the measured GFR and validate our findings. Unfortunately, we do not have the urinary or plasma clearance of an ideal filtration marker, such as inulin, iothalamate or iohexol, as the gold standard for the measurement of GFR.[30] Our findings are based on a healthy pediatric population of a narrow age category and may not be generalizable to older, younger, or diseased populations.

CONCLUSION

Our results suggest that eGFR based on both creatinine and cystatin C concentrations are influenced by BMI and BSA, whereas only eGFR based on creatinine concentrations is influenced by lean mass percentage and fat mass percentage. Beside anthropometric measurements, body composition measures should be considered when estimating GFR in children. Further studies to compare these results with measured GFR are needed.

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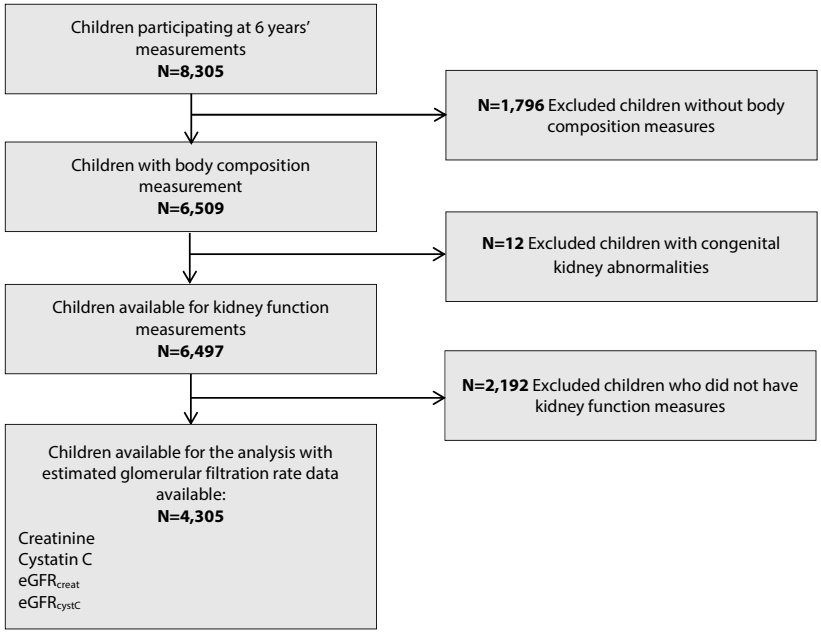
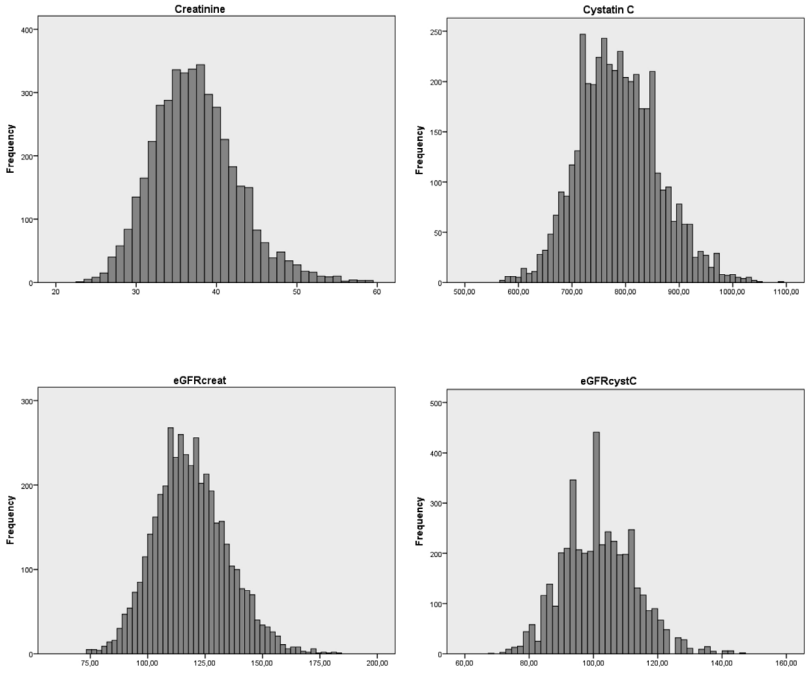
Supplementary Figure 3.3.1. Flowchart of the study participants



Supplementary Figure 3.3.2. Histograms of kidney function measures



Supplementary Table 3.3.1. Subject characteristics between children with and without kidney function measurements (N=6,509)

Subjects characteristics	With eGFR data (N=4,305)	Without eGFR data (N=2,204)	*P*-value
Age at measurements (y)	6.0 (5.7, 8.0)	6.0 (5.7, 7.6)	0.20
Sex, Girls (%)	48.3	53.4	0.07
Ethnicity (%)			0.26
- Dutch or European	65.1	63.8	
- Non-European	34.9	36.2	
Height (m)	119.7 (6.0)	119.0 (6.0)	**<0.01**
*SD-score (mean, sd)	-0.2 (1.0)	-0.3 (1.1)	**<0.01**
Weight (kg)	23.4 (4.2)	23.1 (4.3)	**0.01**
*SD-score (mean, sd)	0.1 (1.0)	0 (1.1)	0.07
Body mass index (kg/m^2)	16.2 (1.8)	16.2 (1.9)	0.74
*SD-score (mean, sd)	0.3 (0.9)	0.3 (1.0)	0.91
Body surface area (m^2)	0.88 (0.09)	0.87 (0.09)	**<0.01**
Fat mass percentage (%)	24.7 (5.6)	25.3 (5.9)	**<0.01**
Lean mass percentage (%)	71.7 (5.4)	71.1 (5.7)	**<0.01**

Values are percentages for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. *The standard deviation scores were obtained using Dutch reference growth curves (Growth Analyzer 3.0, Dutch Growth Research Foundation, Rotterdam, the Netherlands).

Supplementary Table 3.3.2. Associations of anthropometric and body composition measures with creatinine, cystatin C and eGFR in European children (N=2,727)

	Difference (95% Confidence Interval)			
	Creatinine (µmol/l)	Cystatin C (µg/l)	$eGFR_{creat}$ (ml/min/1.73m^2)	$eGFR_{cystC}$ (ml/min/1.73m^2)
Anthropometrics and body composition (SDS)				
Height	0.83 (0.60, 1.05)***	2.33 (-1.15, 5.80)	3.13 (2.42, 3.84)***	-0.35 (-0.87, 0.17)
Weight	1.16 (0.92, 1.39)***	5.84 (2.16, 9.51)**	0.75 (-0.01, 1.52)	-0.85 (-1.40, -0.30)**
Body mass index	0.86 (0.64, 1.08)***	5.33 (1.94, 8.72)**	-1.53 (-2.23, -0.83)***	-0.78 (-1.29, -0.27)**
Body surface area	1.13 (0.90, 1.36)***	4.80 (1.18, 8.43)**	1.72 (0.97, 2.47)***	-0.71 (-1.25, -0.17)*
Lean mass percentage	0.30 (0.07, 0.54)*	-2.60 (-6.17, 0.98)	-2.11 (-2.85, -1.37)***	0.29 (-0.25, 0.82)
Fat mass percentage	-0.28 (-0.52, -0.05)*	2.83 (-0.74, 6.40)	2.02 (1.28, 2.75)***	-0.32 (-0.85, 0.21)

Values are beta coefficients and 95% confidence intervals, from linear regression models adjusted for child age and sex. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood concentrations; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood concentrations
P value for the associations *<0.05 **<0.01 ***<0.001.

Supplementary Table 3.3.3. Associations of BMI clinical cut-offs with creatinine, cystatin C and eGFR (N=4,305)

	Difference (95% Confidence Interval)			
	Creatinine (µmol/l)	**Cystatin C (µg/l)**	**$eGFR_{creat}$ (ml/min/1.73m^2)**	**$eGFR_{cystC}$ (ml/min/1.73m^2)**
Body mass index (kg/m^2)				
Underweight (n=201)	-0.87 (-1.62, -0.12)*	5.14 (-5.45, 15.73)	1.90 (-0.36, 4.17)	-0.60 (-2.20, 1.00)
Normal weight (n=3,383)	*Reference*	*Reference*	*Reference*	*Reference*
Overweight (n=547)	0.98 (0.50, 1.46)***	5.54 (-1.28, 12.36)	-0.38 (-1.83, 1.08)	-0.77 (-1.80, 0.26)
Obese (n=166)	1.61 (0.77, 2.45)***	12.36 (0.45, 24.27)*	1.23 (-1.31, 3.78)	-1.78 (-3.58, 0.02)

Values are beta coefficients and 95% confidence intervals, from linear regression models adjusted for child age, sex and ethnicity. Abbreviations: $eGFR_{creat}$ estimated glomerular filtration rate calculated based on creatinine blood levels; $eGFR_{cystC}$ estimated glomerular filtration rate calculated based on cystatin C blood levels. P value for the associations *<0.05 ** <0.01 ***<0.001.

Chapter 4

General discussion

INTRODUCTION

Chronic kidney disease prevalence is increasing.[1] Epidemiological evidence suggests that the susceptibility for chronic kidney disease is established in early life.[2] Studies relate fetal growth restriction and low birth weight with increased risks of chronic kidney disease in later life.[3,4] In addition, historical studies associate maternal undernutrition with an increased risk of cardiovascular and renal disease in the adult offspring.[5,6] All these observations relate with the "Developmental Origins of Health and Disease" Hypothesis, which proposes that an organism may develop in different ways, depending on the environment it is exposed to.[7,8] Adverse exposures, such as maternal malnutrition, during fetal life may lead to impaired fetal growth, low birth weight and smaller kidneys with a reduced number of nephrons.[3,9] Post mortem studies in humans have shown that lower nephron number is associated with low birth weight.[10–12] The "Glomerular Hyperfiltration Theory" suggests that a reduced nephron number in smaller kidneys in early life may lead to glomerular hyperfiltration and hypertrophy, which in turn lead to glomerulosclerosis and chronic kidney disease and hypertension in later life.[13–15] An impaired renal function in childhood, possibly as a consequence of smaller kidney with a lower number of nephrons, could be an early sign of developing renal disease.

The aim of this thesis was to identify specific nutritional factors during fetal life, and genetic and childhood factors related with early growth and measures of kidney volume and function in school-aged children. In this chapter, we provide a brief summary of the main findings of the studies in this thesis, a general discussion of methodological issues, and possible clinical implications of these studies. Furthermore, this chapter provides suggestions for future research.

MAIN FINDINGS

Fetal factors

Various adverse maternal nutritional and lifestyle-related characteristics have been associated with the risk of adverse birth outcomes, and child cardiovascular and renal health.[5,6,16–20] Evidence from the Dutch Hunger studies suggest that the fetal nutrition supply line may be directly affected by the dietary intake of the mother during pregnancy.[6,21] Thus far, not much is known about common, contemporary nutritional exposures during pregnancy that affect fetal growth and offspring kidney health. As fetal life is a critical period for growth and nephron formation, identification of specific nutrients and life style determinants may be important for early prevention of adverse birth outcomes and of renal and cardiovascular disease in adulthood.

First, we analysed whether protein intake in early pregnancy was associated with childhood kidney function. The results of our study suggested that higher maternal total protein intake in early pregnancy was associated with higher estimated glomerular filtration rate (eGFR) in children. These association were indipendent of many maternal lifestyle and sociodemografic factors and child characteristics. Further analyses showed that the observed

associations were mainly driven by maternal vegetable protein intake, rather than animal protein intake. Additionally the associations of maternal protein intake with childhood eGFR were not explained by later diet of the child. In the literature, contraversary findings are reported on the effect of protein intake in renal health.[22, 23] However, our findings are in line with animals studies which suggest that specifically maternal dietary protein intake during pregnancy influences offspring's kidney function.[20, 24] Our results shade light on the potential porgraming role of maternal protein consumption during pregnancy on childhood kidney development. Maternal diet may program the embryonic kidney by altering cell turnover and gene expression at a time when nephrons and glomeruli have yet to form.[25] Other underlying pathways that may drive these associations can be marked changes in the renal expression of the glucocorticoid receptor and components of the renin-angiotensin system due to a low protein diet. Also, protein restriction during pregnancy could affect the growth hormone-insulin-like growth factor and the prostaglandins axis in the offspring.[26]

Strong relations have been reported between various micronutrients and risk factors for cardiovascular and renal disease in the offspring.[16, 27] During periods of rapid cell growth and division maternal folate need increases. A deficient folate supply may result in impaired deoxyribonucleic acid (DNA) synthesis and inability to methylate cellular proteins, lipids, and DNA. In this scenario complications such as neural tube defects may occur.[28] In healthy infants, a relationship between maternal folate concentrations during pregnancy and vascular endothelial function has been demonstrated.[29] Whereas, adults studies have shown associations of elevated homocysteine concentrations with an accelerated decline in kidney function.[30] However, no previous study has explored the associations of first trimester maternal and cord blood folate, vitamin B_{12} and homocysteine concentrations with kidney outcomes in children. The results of our study suggest that higher maternal folate concentrations were associated with larger childhood combined kidney volume, whereas higher maternal vitamin B_{12} concentrations were associated with higher childhood (estimated glomerular filtration rate based on cystatin C concentrations) $eGFR_{cystC}$. These associations were independent of homocysteine concentrations. Higher maternal homocysteine concentrations were associated with smaller combined kidney volume and lower childhood $eGFR_{cystC}$. Further analysis showed that the association of maternal homocysteine concentrations with childhood $eGFR_{cystC}$ was largely explained by combined kidney volume. Higher cord blood homocysteine concentrations were associated with larger combined kidney volume and lower $eGFR_{cystC}$. Low folate and high homocysteine concentrations are previously shown to be associated with endothelial dysfunction and altered vascular development.[29, 30] The potential underlying pathway can be that high homocysteine concentrations may impair endothelial vasodilatation by inhibiting the generation of endothelial mediators and promoting adhesion between neutrophil and endothelial cells.[31] Suboptimal vascular development and endothelial dysfunction could lead to hypertension in childhood which could be a predictor to chronic kidney diseases in later life.[13, 32]

Vitamin D is widely recognised for its beneficial effects, especially on bone health.[33] Next to the skeletal health, associatons of vitamin D and extraskeletal outcomes are reported.[17, 34–36] Vitamin D deficiency, defined as 25-hydroxyvitamin D concentrations (25(OH)D) <50nmol/L,

is becoming a public health concern, especially among pregnant women.[37, 38] Many authors debate if vitamin D deficiency is really pandemic.[39] It is important to acknowledge the controversy behind the assesment of adequate vitamin D status and recommended dietary allowance of vitamin D intake.[39] We measured 25(OH)D concentrations in women during mid-pregnancy and at birth in cord blood. Based on the Institute of Medicine (IOM), the Endocrine Society recommendations and based on previous cohort studies, we created four vitamin D groups, including severely deficient (<25.0 nmol/L), deficient (25.0 to 49.9 nmol/L), sufficient (50.0 to 74.9 nmol/L) and optimal (≥75.0 nmol/L).[18, 40–42] IOM defines vitamin D as deficient (<50nmol/L), and sufficient (≥50nmol/L), which would lead to categorizing our severely deficient and deficient groups as deficient, and categorizing the sufficient and optimal groups as sufficient or adequate. We consider an advantage of our categories as compared to IOM categories that we have more groups and can compare our results with previous pregnancy studies. In our cohort (N=7,176), 53% of the pregnant mothers were 25(OH)D deficient (<50 nmol/L). We analyzed vitamin D continuously, using quartiles and using cut-offs based on current recommendation.

We first explored the associations of mid-pregnancy maternal vitamin D and fetal growth patterns and birth outcomes. So far, most published studies focused on the associations of maternal vitamin D status during pregnancy with fetal development were mainly based on birth weight and showed inconsistent results.[43–46] Nonetheless, birth weight is just a proxy for fetal growth and development. Different fetal growth patterns and body proportions may lead to the same birth weight. We observed that mothers with mid-pregnancy 25(OH)D concentrations in the lower quartiles had offspring with third trimester fetal growth restriction leading to a smaller birth head circumference, shorter birth length and lower weight at birth as compared to mothers with 25(OH)D concentrations in the highest quartile. Mothers who had 25(OH)D concentrations in the lowest quartile had increased risks of preterm delivery and small-size for gestational age children. Most importantly we observed that the associations were not based on extreme 25(OH)D deficiency, but present within the common ranges. A recent Mendelian Randomization study provided some evidence to support a possible causal association with birthweight, still further exploration in larger numbers of pregnancies are required.[47]

Second, we explored the associations of mid-pregnancy maternal and cord blood 25(OH)D concentrations with childhood body composition and cardiovascular outcomes. We observed that as compared to children from mothers with optimal 25(OH)D concentrations, those of severely deficient vitamin D mothers had higher fat mass percentage and lower lean mass percentage. Additional analyses suggested that these associations were independent of child current vitamin D status. Potential pathways of vitamin D on body composition have been shown.[48] Vitamin D may increase adipose tissue lipolysis and decrease adipogenesis, suggesting a programing effect on childhood body composition.[48] In our study, maternal and cord blood 25(OH)D concentrations were not associated with cardiovascular risk factors in children.

Third, we explored the associations of mid-pregnancy and cord blood 25(OH)D concentrations with kidney structure and function in children. We observed that higher maternal

25(OH)D concentrations were associated with lower childhood eGFR. These associations were partly explained by childhood vitamin D status. Maternal 25(OH)D concentrations were not consistently associated with childhood combined kidney volume whereas, cord blood 25(OH)D concentrations were not associated with childhood kidney outcomes. More research is needed in replicating these findings, establishing the causality of the associations, the underlying mechanisms, and the long term-clinical consequences.

One of the most important determinants of the fetal supply line is placental function, reflected by placental weight and hemodynamic function. Gestational hypertensive disorders are considered as the most extreme form of hemodynamic placental dysfunction. According to previous studies, gestatonal hypertensive disorders have been associated with elevated blood pressure in offspring during childhood and adolescence.[49, 50] However, gestational hypertensive disorders do not represent the full spectrum of blood pressure development during pregnancy. We examined the associations of maternal blood pressure throughout pregnancy and hypertensive disorders in pregnancy with childhood blood pressure. Specific focus was on the comparison with paternal blood pressure effects, the identification of critical periods and the role of birth outcomes and childhood body mass index in the observed associations. Both maternal and paternal blood pressure were positively associated with childhood blood pressure. Since both maternal and paternal blood pressure were associated with childhood blood pressure, we must be careful to conclude direct intra-uterine effects. Early, mid- and late pregnancy maternal blood pressure were all independent and positively associated with childhood blood pressure, with the strongest effect estimates for early pregnancy. As compared to children from mothers without hypertensive disorders in pregnancy, those from mothers with hypertensive disorders in pregnancy had higher diastolic blood pressure. Further analyses suggested that the observed associations were not materially affected by birth outcomes and childhood body mass index.

Some of these factors are potentially modifiable and might provide targets for intervention to improve fetal growth, renal and cardiovascular health on a population level. Altogether, the findings of these studies indicate the importance of a balanced diet during fetal life, rich in protein and vitamins may support healthy growth and childhood kidney development.

Genetic and childhood factors

Large genome wide association studies (GWAS) in adults have identified common genetic variants associated with impaired kidney function.[51–53] An impaired renal function in childhood, possibly as a consequence of smaller kidney with a lower number of nephrons, could be an early sign of developing renal disease. The associations of kidney measures in early life with kidney disease in later life can be partly explained by common genetic variants that lead to both smaller kidneys with lower kidney function in early childhood and kidney disease in adulthood. We examined the associations of a genetic risk score combining previously identified common genetic variants related to adult (estimated glomerular filtration rate based on creatinine concentrations) $eGFR_{creat}$ with kidney outcomes in children aged 6.0 years. The genetic risk score based on variants related to impaired kidney function in adults was associated with lower kidney volume and lower eGFR in children. These findings suggest

that subjects who develop kidney disease in later life do already have smaller kidneys with lower eGFR in early life.

Infant diet is a key component of in infant growth. Infant diet habits are related with the risk of overweight and cardiovascular health in early childhood.[54, 55, 56] However these results are not consistent.[56] Nevertheless, the results of a meta analyses acknowledge the long-term protective effects of breastfeeding on blood pressure, obesity and diabetes.[57] Whether breastfeeding also influences childhood kidney development is unknown. Only one small study has examined the associations of breastfeeding versus formula feeding and kidney outcomes and observed that infants who were exclusively formula fed had increased kidney growth during the first 3 months, but this difference was no longer apparent at the age of 18 months.[58] We examined the associations of breastfeeding duration and exclusivity, and age at introduction of solid foods with kidney outcomes at school-aged children. The results of our study showed that never breastfed children had a smaller kidney volume compared to ever breastfed children. Among breastfed children, shorter duration of breastfeeding was associated with a smaller kidney volume and with a lower risk of microalbuminuria. Compared to exclusive breastfeeding for 4 months, non-exclusive breastfeeding in the first 4 months was associated with a smaller kidney volume and a lower eGFR. Further analysis showed that the associations with eGFR were largely explained by kidney volume. Age at introduction of solid foods was not associated with any of the kidney outcomes. It is imperative to explore whether early life nutrition also affects the risk of kidney disease in adulthood and what are the potential mechanisms that drive these associations.

Throughout this thesis we have used eGFR when we express kidney function. It is widely recognised that the best measure to asses kidney function is GFR.[59] Due to the difficulties and cost in measuring GFR, kidney function is estimated using creatinine blood concentrations.[60] Next to creatinine, cystatin C blood concentrations can be used to estimate GFR.[61, 62] In children estimation of GFR is challenging. Creatinine, is actively secreted by the proximal tubule, and is related to muscle mass, age, sex, ethnicity and dietary factors.[59, 63] Some authors report a superior sensitivity of cystatin C for detecting impaired GFR in pediatric patients compared to that of creatinine, especially in children with low muscle mass.[64] An advantage of cystatin C in children is that it is considered less related to body weight and height.[64, 65] Still, it is reported that lean mass affects cystatin C concentrations in kidney disease patients.[66, 67] We explored the influence of different body composition measures on eGFR from creatinine and cystatin C blood concentrations. Additionally we compared the associations of different anthropometric and body composition measures with eGFR derived from creatinine ($eGFR_{creat}$) and cystatin C ($eGFR_{cystC}$) blood concentrations. We observed that higher body mass index was associated with lower $eGFR_{cystC}$ but not with $eGFR_{creat}$. Our results suggest that eGFR based on both creatinine and cystatin C concentrations are influenced by BMI and BSA, whereas only eGFR based on creatinine concentrations is influenced by lean mass percentage and fat mass percentage. $eGFR_{creat}$ is more strongly influenced by body composition than $eGFR_{cystC}$. Although our study lacked the actual measurements of GFR, it suggests that beside anthropometric measurements, body composition measures should be considered when estimating GFR in children. Further studies to compare these results with measured GFR are needed.

Main findings

- Higher total and vegetable, but not animal, maternal protein intake during early pregnancy is associated with a higher eGFR in childhood.
- Folate, vitamin B_{12} and homocysteine concentrations during fetal life are associated with offspring kidney development.
- Low maternal 25(OH)D concentrations are associated with proportional fetal growth restriction and with increased risks of preterm birth and small-size for gestational age at birth.
- Severe maternal 25(OH)D deficiency during pregnancy is associated with an adverse childhood body fat profile, but not with childhood cardiovascular risk factors.
- Maternal 25(OH)D levels during pregnancy may influence childhood kidney outcomes. These results should be considered hypothesis-generating.
- Both maternal and paternal blood pressure affect childhood blood pressure, independent of fetal and childhood growth measures, with the strongest effect for maternal blood pressure in early pregnancy.
- Common genetic variants related to impaired kidney function in adults already lead to subclinical changes in childhood kidney outcomes.
- Breastfeeding is associated with subclinical changes in kidney outcomes in childhood.
- Both $eGFR_{creat}$ and $eGFR_{cystC}$ are influenced by body mass index and body surface area. $eGFR_{creat}$ is more strongly influenced by body composition than $eGFR_{cystC}$.

METHODOLOGICAL CONSIDERATIONS

Specific strengths and limitations for the studies presented in this thesis are described in **Chapter 2** and **Chapter 3** of this thesis. In the following paragraphs, we discuss general methodological considerations regarding selection bias, information bias and confounding bias.

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 did not participate in the study, but were eligible for the study. Of all children eligible at birth, the overall response to participate in the Generation R Study was 61%. The participants in the study were more of a Dutch ethnicity and belonged to a higher socio-economic class as compared to non-participants. Furthermore, the percentages of participating women with gestational hypertensive disorders or preterm born children were lower than expected from the population figures in Rotterdam. Altogether, these suggest a selection towards a relatively more affluent and healthy population.[68] This selection towards a more affluent and healthy population at baseline may have led to reduced statistical power, due to lower prevalence rates, and subsequently affecting the generalizability of our findings to other populations. However, we consider it less likely because in large cohort studies biased estimates mainly arise from loss to follow-up rather than from non-response at baseline.[69] Selective loss to follow-up may result in selection bias when the association between the determinant and the outcome of

interest is different between those who continued participation in the study and those who were lost to follow-up. At the age of 6 years children were invited to participate in body composition, cardiovascular and kidney follow-up measurements. The response rate for the studies presented in this thesis was around 70%. Overall, mothers of children who were lost to follow-up had more often a lower socio-economic status and unhealthy life style habits. This selection might have biased the effect estimates presented in this thesis.

Information bias

Information bias is a bias that arises in a study because of misclassification of determinant or outcome measurements.[70] Misclassification of either determinant or outcome can be classified as non-differential or differential. Non-differential misclassification involves misclassification where the determinant status is not related to the outcome status, and vice versa. Non-differential misclassification generally leads to an underestimation or dilution of the effect estimates. Differential misclassification involves misclassification of determinant status related to the outcome status, and vice versa. Differential misclassification may lead to biased effect estimates. Most of the determinant data used in this thesis were assesed before the outcome, therefore the differential missclasification of the determinant is unlikely. Whereas, non-differential misclassification might have occured.

In some of the stuides presented in this thesis we have used questionnaires to asses information on the determinant. Dietary assessment in epidemiological studies is challenging. Questions on the frequency and amount of consumption of regularly eaten foods by a food frequency questionnaire (FFQ) is a commonly used method. However, a disadvantage of this method is its dependence on recall. Validation studies have shown that reported values from FFQs are subject to substantial error, in cohort studies in principle non-differential.[71] It may be that especially overweight women tend to underestimate their intake. To deal with this issue, the dietary analyses were corrected for pre-pregnancy BMI. Furthermore, energy adjustment may also help to resolve the issue of under- or over-reporting.[72] Also, information on infant feeding was obtained by questionnaires, which has been proven to be difficult to acquire reliable measurement of adverse lifestyle-related factors by self-reported questionnaires. Studying biomarkers that describe nutritional status may help to reduce the issue of misclassification. In the studies presented in this thesis we had biomarkers of folate,vitamin B_{12}, homocysteine and vitamin D. In our studies, the outcome was assessed using medical records, or standardized hands-on assessments of body composition, cardiovascular and kidney development. Furthermore, the observers were blinded to the determinant status, which makes differential misclassification of the outcomes less likely.

Confounding

A confounder is an extraneous variable that is associated with both the determinant and the outcome of interest and is not an intermediate step in the causal pathway between the determinant and outcome.[70] We adjusted our analyses for many potential confounders. The confounders were selected based on previous literature or a change of more than 10% in the effect estimates. In some of the studies, adjustment for potential confounders attenuated

significant associations into non-significant, indicating that the unadjusted association is confounded. In other studies, the association did not attenuate or only slightly, indicating the association is possibly a true association between the determinant and the outcome. Although in the Generation R Study, there are many potential confounders available, it still may be that we did not measure all potential confounders. Also, measurement error of the confounding variables can occur. Therefore, residual confounding might still be an issue. Where possible, we assessed the associations of both maternal and paternal determinants during pregnancy. This in order to understand, if the explored associations were through direct intra-uterine mechanisms.[73]

CAUSALITY

Due to the observational design of our study, we were unable to establish causality in the observed associations. Bradford Hill's Criteria on Causation attempt to establish scientifically valid causal connections between potential exposures and outcomes.[74] They outline the minimal conditions needed to establish a causal relationship between the two items.[74] In our observational study, Hill's criteria on causation were applicable as detailed below.

Hill's criteria on causation

1. *Strength*
   In the studies presented in this thesis we observe associations of early life determinants with fetal growth and kidney function in children. The effect estimates on eGFR are relatively small.
2. *Consistency*
   The results of studies presented in this thesis are consistent with previous studies showing that early life exposures are associated with kidney function in later life.
3. *Specificity*
   We observed that different specific early life factors were associated with kidney structure and function measures in children. Furthermore, maternal vitamin D concentrations were associated with fetal growth patterns, body composition, and kidney function in children.
4. *Temporality*
   In our studies, the exposures were collected before the outcomes.
5. *Biological gradient*
   Dose response effects were observed in most of the studies. Changes in the outcomes rates followed the corresponding changes in exposure.
6. *Plausibility*
   For the associations observed plausible underlying mechanisms are suggested.
7. *Coherence*
   The observed associations are compatible with existing knowledge. There is coherence from animal studies showing that adverse factors during early life can affect nephrogenesis and subsequently predispose the individual to chronic kidney disease in later life.
8. *Experiment*
   The experimental criteria could not be fulfilled.
9. *Analogy*
   The analogous criteria could not be fulfilled.

To establish causation and possibly identifying mechanisms that underlie the associations, randomized controlled trials are preferred. Still, this design is not possible when exploring exposures such as gestational hypertensive disorders. Studies on causality are not easy to perform when the exposure of interest is difficult to modify. For other exposures, like maternal nutrition or breastfeeding, this might be feasible and randomized controlled trials could overcome the residual confounding issue. Observational studies that can provide sophisticated tools to obtain further insight into causality are important. Mendelian randomization studies use genetic variants, which are associated with the exposure of interest and not affected by confounding, as an instrumental variable for a specific exposure, to examine whether an exposure is causally related to the outcome.[75, 76] For this type of study large sample sizes are necessary in order to obtain sufficient statistical power. Another method to compare intrauterine mechanisms from associations explained by shared family-based lifestyle-related characteristics is by comparing effect size of maternal-offspring and paternal-offspring associations.[77] Therefore it is important that fathers are included in future studies, and have information of the same exposures as mothers to allow these types of comparisons.

CLINICAL IMPLICATIONS

In the studies presented in this thesis, fetal life, genetic and childhood determinants were associated with fetal growth and kidney function in children. Although the results presented in this thesis alone do not provide a basis to make strong statements and further research is certainly warranted, they provide suggestions for future prevention strategies. Implications for prevention are:

- Supporting population-strategies to improve vitamin D concentrations and to increase folate and vitamin B_{12} concentrations in pregnant women.
- Improving maternal diet during pregnancy. A diet rich in vegetable protein may support offspring kidney development.
- Considering parental factors in screening guidelines for childhood hypertension.
- Supporting the World Health Organization recommendations on infant breastfeeding.

Developing preventive strategies focused on consuming a healthy diet, sun exposure, and vitamin supplements for pregnant women may help to improve fetal growth, offspring kidney function, and prevent future kidney disease. This thesis highlights that both fetal life and early childhood provide a possible window of opportunity to improve renal health.

FUTURE RESEARCH

The results of our studies together with previous published evidence suggest that both fetal life and early childhood are important periods for growth and kidney health.[78] Future studies should be focused on the causality, underlying mechanisms, and potential population effects for the observed associations.

Detailed and better markers on kidney development and kidney function are required to provide further insights in the studied associations. So far, the best surrogate marker for assessing nephron number in epidemiological studies is kidney weight or kidney size measured by ultrasound.[79] In our studies we used ultrasound to estimate kidney volume, as a measure of nephron number, which seems to be a reliable surrogate for nephron endowment.[14] More reliable ways should be used to determine nephron number in the general population. Animal studies have suggested that magnetic resonance imaging techniques can provide individual counts of glomerular number, which is equivalent to the nephron number.[80] If these techniques can be implemented in humans we can assess how early life determinants affect nephron number and thereafter the development of chronic kidney disease and hypertension.

GFR is the most widely used marker to asses kidney function.[61] The estimation of GFR in children remains challenging in daily practice: even though the Lund strategy appears to give reliable results in adults, to date in children there is no real consensus.[81] With the design of our study it was not possible to compare and validate the explored associations of body composition with the estimates of GRF based on creatinine and cystatin C concentrations with the measured GFR. Thus, effort should be made on evaluating creatinine- and cystatin-derived formulas, to help physicians in daily practice and also epidemiological studies to assess renal function in the general pediatric population.

A potential mechanism on renal development is alterations in nephron endowment due to adverse fetal exposures.[82] The number of nephrons, which is determined during fetal development, is an important determinant of chronic kidney disease and hypertension during adult life. Autopsy studies in humans have shown that a lower nephron number is associated with hypertension.[10, 12] Therefore, biopsy studies can help to assess nephron number, which can help in distinguishing the mechanisms of the observed associations. However, this approach can only be used in clinical settings and may be difficult and risky to perform due to its invasive procedure, potential complications and costs.[83]

In addition, epigenetic changes that persist throughout life may lead to the development of risk factors for chronic kidney disease and hypertension in adult offspring. Epigenetic modifications such as DNA methylation may occur in response to fetal nutrition, regulating gene activity.[84] These epigenetic modifications may have phenotypic consequences throughout the life course, subsequently affecting the risk of kidney disease in later life.[84] The largest variation of methylation is expected periconceptionally, therefore it is important that future population-based cohort studies start early in preconceptional or fetal life.[85] Future studies are needed to obtain insight in the role of epigenetics in the associations between adverse fetal nutritional exposures and chronic kidney disease in adult life.[86]

The observed effect estimates for the associations of fetal, genetic and childhood determinants with fetal growth and childhood kidney outcomes were small. However, they provide insights from a developmental perspective and may be important in a population level. It is previously shown that childhood cardiovascular and kidney measures track from childhood into adulthood.[87, 88] Therefore, the observed subclinical differences in kidney function in childhood may be related to the development of chronic disease in later life. Health care

approaches should start early in life and take a cross-generational perspective.[89] We should be aware of the importance of nutrition during pregnancy and its long-lasting effects in offspring health. Research and clinical practice should focus on identifying high risk individuals and developing preventive strategies or interventions from early pregnancy onwards.

CONCLUSION

The findings from this thesis suggest that early life nutrition is associated with fetal growth patterns and childhood kidney function. Future studies should focus on elucidating the underlying mechanisms and confirming the long-term consequences of these findings.

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Chapter 5

Summary & Samenvatting

SUMMARY

In this thesis, we examined the fetal, genetic and childhood determinants of growth and childhood kidney function. Adverse environmental exposures during early life may lead to fetal growth adaptations and influence on the development of chronic kidney disease in later life, as indicated by many lines of evidence. From both an etiological and a prevention perspective, it is important to identify specific factors, especially modifiable factors that lead to an impaired kidney development. The studies presented in this thesis were mainly focused on the identification of specific nutritional factors during fetal life and early childhood factors.

Chapter 1 gives the background of the studies presented in this thesis. It describes the aim of the performed studies and describes the outline of the thesis. The studies included in this thesis were conducted within the Generation R Study, a population-based prospective cohort study from fetal life onwards.

Chapter 2 describes the associations of fetal life factors with early growth, cardiovascular and kidney outcomes in childhood. In **Chapter 2.1** we explored the associations of early pregnancy maternal protein intake with childhood kidney volume and measures of kidney function. We observed that higher total and vegetable, but not animal, maternal protein intake during early pregnancy was associated with a higher estimated glomerular filtration rate (eGFR) in childhood. These associations were not explained by protein intake in early childhood. In **Chapter 2.2** we explored the associations of folate, vitamin B_{12} and homocysteine concentrations measured in early pregnancy and at birth with childhood kidney outcomes. We concluded that folate, vitamin B_{12} and homocysteine concentrations during fetal life were associated with offspring kidney development. These associations were independent of maternal, birth and childhood potential confounders. In **Chapter 2.3** we investigated whether maternal vitamin D concentrations in mid-pregnancy were associated with fetal growth patterns and subsequently adverse birth outcomes. We showed that low maternal vitamin D concentrations were associated with proportional fetal growth restriction and with increased risks of preterm birth and small-size for gestational age at birth. These associations were not restricted to the extremes, but tended to be present across the full spectrum of maternal vitamin D concentrations. In **Chapter 2.4** we examined the associations of mid- pregnancy maternal and cord blood vitamin D concentrations with childhood body composition, blood pressure, lipids and insulin concentrations. We observed that severe maternal vitamin D deficiency during pregnancy was associated with an adverse childhood body fat profile, but not with childhood cardiovascular risk factors. Cord blood vitamin D concentrations were not associated with childhood body composition or cardiovascular risk factors. In addition, in **Chapter 2.5** we explored whether maternal vitamin D concentrations were associated with childhood kidney health. Our findings suggest an association of maternal vitamin D concentrations during mid- pregnancy with childhood eGFR. The associations of maternal vitamin D concentrations with childhood eGFR were partly explained by childhood vitamin D status. In **Chapter 2.6** we examined the associations of maternal blood pressure throughout pregnancy and hypertensive disorders in pregnancy with childhood blood pressure. Our specific focus was on the comparison with paternal blood pressure effects, the identification

of critical periods and the role of birth outcomes and childhood body mass index in these associations. We observed that both maternal and paternal blood pressure were associated with childhood blood pressure with similar effect estimates. Early, mid- and late pregnancy maternal blood pressure were all independent and positively associated with childhood blood pressure, with the strongest effect estimates for early pregnancy. All the observed associations were independent of fetal and childhood growth measures.

Chapter 3 describes the associations of genetic and childhood exposures with kidney outcomes. In **Chapter 3.1** we observed that common genetic variants related to impaired kidney function in adults were associated with kidney volume and eGFR in school-aged children. This chapter suggests that the well-known associations of kidney measures in early life with kidney disease in later life may at least be partly explained by common genetic variants. **Chapter 3.2** explores the associations of breastfeeding duration, exclusivity, and age at introduction of solid foods with kidney outcomes at school-aged children. This chapter shows that breastfeeding was associated with subclinical changes in kidney outcomes in childhood. Children who were shorter or non-exclusively breastfed had smaller kidney and a lower eGFR. In **Chapter 3.3** we compared the associations of different anthropometric and body composition measures with eGFR derived from creatinine and cystatin C blood concentrations. This chapter suggests that eGFR based on both creatinine and cystatin C concentrations were influenced by body mass index and body surface area, whereas only eGFR based on creatinine concentrations was influenced by lean mass percentage and fat mass percentage. However, this chapter concludes that further studies are needed to assess whether using $eGFR_{cystC}$ instead of $eGFR_{creat}$ leads to better care for pediatric kidney patients. Finally, in **Chapter 4** the main findings of this thesis are evaluated. It discusses the methodological issues of the included studies and concludes with suggestions for future research and clinical application.

In conclusion, the findings of this thesis suggest that early life nutrition is associated with fetal growth patterns and childhood kidney function. Further studies are needed to unravel these mechanisms and to identify whether these subclinical changes in kidney development have consequences for the development of chronic kidney disease in later life.

SAMENVATTING

In dit proefschrift hebben we verschillende determinanten van vroege groei en nierfunctie op de leeftijd van 6 jaar onderzocht. We hebben gekeken naar genetische determinanten en naar determinanten tijdens de foetale tijd en de kindertijd. Zoals eerder aangetoond kan blootstelling aan nadelige omgevingsfactoren tijdens het vroege leven leiden tot aanpassingen in de foetale groei en mogelijk invloed hebben op de ontwikkeling van chronische nierziekten op latere leeftijd. Vanuit etiologisch perspectief en voor de ontwikkeling van preventiestrategieën is het belangrijk om factoren te identificeren die kunnen leiden tot een verminderde ontwikkeling van de nierfunctie, in het bijzonder beïnvloedbare factoren. De studies die beschreven worden in dit proefschrift zijn met name gericht op de identificatie van voedingsgerelateerde factoren tijdens de foetale tijd en de vroege kindertijd.

In **Hoofdstuk 1** beschrijven we de achtergrond van de studies die zijn opgenomen in dit proefschrift. Tevens wordt het doel van elke studie en de opbouw van het proefschrift beschreven. De studies die in dit proefschrift zijn opgenomen, zijn uitgevoerd binnen het Generation R onderzoek, een populatie-gebaseerd prospectief cohort onderzoek vanaf het foetale leven tot in de jongvolwassenheid.

In **Hoofdstuk 2** beschrijven we de associaties tussen foetale factoren met vroege groei, cardiovasculaire uitkomsten en de ontwikkeling van de nieren in de kindertijd. In **Hoofdstuk 2.1** hebben we de associaties onderzocht tussen eiwitinname door de moeder tijdens de vroege zwangerschap en niervolume en nierfunctie van het kind op 6-jarige leeftijd. We zagen dat een hogere totale eiwitinname en een hogere inname van plantaardige eiwitten, maar niet van dierlijke eiwitten, tijdens de vroege zwangerschap was geassocieerd met een hogere geschatte glomerulaire filtratiesnelheid (estimated glomerular filtration rate: eGFR) van het kind op de leeftijd van 6 jaar. Deze associaties werden niet verklaard door eiwitinname van het kind. In **Hoofdstuk 2.2** hebben we de relatie onderzocht tussen foliumzuur-, vitamine B_{12}- en homocysteïne-concentraties, gemeten in de vroege zwangerschap en bij de geboorte, en nierfunctie van het kind op 6-jarige leeftijd. We hebben geconcludeerd dat foliumzuur, vitamine B_{12} en homocysteïne concentraties tijdens het foetale leven zijn geassocieerd met de ontwikkeling van de nierfunctie van het kind. Deze associaties waren onafhankelijk van mogelijke confounders met betrekking tot moeder, geboorte en kind. In **Hoofdstuk 2.3** hebben we onderzocht of vitamine D concentraties bij de moeder tijdens de zwangerschap geassocieerd waren met foetale groeipatronen en complicaties bij de geboorte. We hebben aangetoond dat lage maternale vitamine D concentraties mogelijk leiden tot proportionele foetale groeirestrictie en tot een verhoogd risico op vroeggeboorte en een laag geboortegewicht in verhouding tot de zwangerschapsduur. Deze associaties werden gezien in het gehele spectrum van maternale vitamine D concentraties en waren dus niet alleen zichtbaar bij extreme vitamine D waarden. In **Hoofdstuk 2.4** hebben we de associaties onderzocht tussen vitamine D concentraties tijdens het tweede trimester van de zwangerschap, gemeten in maternaal bloed en navelstrengbloed, met lichaamssamenstelling, bloeddruk, lipiden en insulineconcentraties bij het kind. We zagen dat ernstige maternale vitamine D-deficiëntie tijdens de zwangerschap kan leiden tot een schadelijk lichaamsvetprofiel bij het

kind, maar geen invloed heeft op cardiovasculaire risicofactoren. Vitamine D concentraties in navelstrengbloed waren niet geassocieerd met lichaamssamenstelling of cardiovasculaire risicofactoren bij het kind. Daarnaast hebben we in **Hoofdstuk 2.5** onderzocht of maternale vitamine D concentraties geassocieerd waren met de nierfunctie van het kind. Onze bevindingen suggereren een verband tussen maternale vitamine D concentraties tijdens het tweede trimester en eGFR in de kindertijd. Deze associaties werden gedeeltelijk verklaard door vitamine D status in de kindertijd. In **Hoofdstuk 2.6** hebben we het verband onderzocht tussen maternale bloeddruk door de zwangerschap heen en hypertensieve aandoeningen tijdens de zwangerschap met de bloeddruk van het kind. In deze associaties lag onze focus op de vergelijking met het effect van paternale bloeddruk, op de identificatie van kritieke perioden en op de rol van geboorte uitkomsten en body mass index van het kind. We zagen dat zowel maternale als paternale bloeddruk geassocieerd waren met de bloeddruk van het kind. Beiden hadden een vergelijkbaar effect. Gedurende de gehele zwangerschap was de maternale bloeddruk onafhankelijk en positief geassocieerd met de bloeddruk van het kind. Het sterkste verband werd gevonden tijdens de vroege zwangerschap. Alle associaties waren onafhankelijk van foetale groei en groei tijdens de kindertijd.

In **Hoofdstuk 3** beschrijven we de relatie tussen genetische determinanten en determinanten in de kindertijd met nierfuncties op de leeftijd van 6 jaar. In **Hoofdstuk 3.1** zagen we dat veel voorkomende genetische varianten, geassocieerd met verminderde nierfunctie in volwassenen, waren geassocieerd met niervolume en eGFR in basisschoolkinderen. Dit hoofdstuk suggereert dat eerder onderzochte verbanden tussen nierfunctie in het vroege leven en nierziekten in het latere leven mogelijk deels worden verklaard door veel voorkomende genetische varianten. In **Hoofdstuk 3.2** onderzochten we de associaties tussen de duur van borstvoeding, het geven van uitsluitend borstvoeding en de leeftijd waarop vaste voeding wordt geïntroduceerd met nierfunctie in de kindertijd. Dit hoofdstuk toont aan dat borstvoeding is geassocieerd met subklinische veranderingen in de nierfunctie op 6-jarige leeftijd. Kinderen die korter of niet uitsluitend borstvoeding kregen hadden kleinere nieren en een lagere eGFR. In **Hoofdstuk 3.3** hebben we de associaties vergeleken van verschillende maten van antropometrie en lichaamssamenstelling met eGFR, afgeleid van creatinine en cystatine C concentraties. Dit hoofdstuk suggereert dat eGFR gebaseerd op zowel creatinine en cystatine C concentraties werd beïnvloed door body mass index en lichaamsoppervlakte, terwijl eGFR gebaseerd op enkel creatinine concentraties werd beïnvloed door vetvrije massa en vetpercentage. In dit hoofdstuk werd geconcludeerd dat verder onderzoek nodig is om te beoordelen of het gebruik van $eGFR_{cyst}$ in plaats van $eGFR_{creat}$ leidt tot betere zorg voor jonge nierpatiënten. Tot slot zijn in **Hoofdstuk 4** de belangrijkste bevindingen van dit proefschrift weergegeven. Ook worden hier de methodologische aspecten van de geïncludeerde studies besproken en worden de conclusies weergegeven, waarbij tevens ingegaan wordt op suggesties voor vervolgonderzoek en toepassing in de klinische setting.

Concluderend suggereren de bevindingen van dit proefschrift dat voeding tijdens het vroege leven is geassocieerd met foetale groeipatronen en nierfunctie in de kindertijd. Verder onderzoek is nodig om de onderliggende mechanismen te achterhalen en om te identi-

ficeren of deze subklinische veranderingen in de ontwikkeling van de nieren consequenties hebben voor de ontwikkeling van chronische nierziekten op latere leeftijd.

Appendices

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Kozeta Miliku was born on the 11th of July 1988 in Korçë, Albania. She grew up in Korçë where she attended "Preca College" high school. In 2013, she was accepted in the Medical University of Tirana, Albania to follow her medical studies. During her studies, she also worked as a medical assistant. Kozeta obtained her medical degree in 2013. In the last year of her medical school, she received a scholarship to participate in a Master of Science program in Health Sciences at the Netherlands Institute of Health Sciences (NIHES). For her Master of Science thesis, she joined the Generation R Study group. In 2014, she obtained her Master of Science degree. In the same year, she received another grant to expand her research project into the PhD-project presented in this dissertation under the supervision of dr. J.F. Felix (Departments of Epidemiology and Pediatrics) and prof. dr. V.W.V. Jaddoe (Departments of Epidemiology and Pediatrics) at the Erasmus University Medical Center in Rotterdam.

Kozeta is very much looking forward to start working as a postdoctoral researcher in the CHILD Study, in Canada as of August 2017. She would like to homologate her medical degree in Canada.

PUBLICATION LIST AND MANUSCRIPTS

Miliku K, Voortman T, Bakker H, Hofman A, Franco OH, Jaddoe VW. Infant breastfeeding and kidney function in school-aged children. *Am J Kidney Dis. 2015; 66(3):421–8*.

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Miliku K, Felix JF, Voortman T, Tiemeier H, Eyles DW, Burne TH, McGrath JJ, Jaddoe VW. Associations of maternal and fetal vitamin D status with childhood body composition and cardiovascular outcomes. *Submitted.*

Bakker H, **Miliku K**, Franco OH, Dorresteijn EM, Cransberg K, Steegers EA, Jaddoe VW. Early longitudinal kidney growth patterns and glomerular filtration rate at school-age. *Submitted.*

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Master of Science in Health Sciences, NIHES, Rotterdam, the Netherlands	2013-2014	
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General courses		
Principles of Research in Medicine and Epidemiology	2013	0.7
Clinical Decision Analysis	2013	0.7
Methods of Public Health Research	2013	0.7
Health Economics	2013	0.7
Markers and Prognostic Research	2013	0.7
The Practice of Epidemiologic Analysis	2013	0.7
Study Design	2013	4.3
Biostatistical Methods I: Basic Principles	2013	5.7
Clinical Epidemiology	2013	5.7
Methodologic Topics in Epidemiologic Research	2013	1.4
Biostatistical Methods II: Classical Regression Models	2013	4.3
Causal Inference	2014	0.7
History of Epidemiologic Ideas	2014	0.7
Advances in Epidemiologic Analysis	2014	0.4
Conceptual Foundation of Epidemiologic Study Design	2015	0.7
Causal Mediation Analysis	2015	0.7
Advanced courses		
Repeated Measurements in Clinical Studies	2014	1.4
Women's Health	2014	0.9
Planning and Evaluation of Screening	2014	1.4
Quality of Life Measurement	2014	0.9
From Problem to Solution in Public Health	2014	1.1
Public Health in Low and Middle Income Countries	2014	3.0
Bayesian Statistics	2015	1.4
Missing Values in Clinical Research	2015	0.7
Principles of Epidemiologic Data-analysis	2015	0.7
Maternal and Child Health	2015	0.9
Psychology in Medicine	2015	1.4

	Year	Workload (ECTS)
Skills courses		
English Language	2013	1.4
Courses for the Quantitative Researcher	2013	1.4
Introduction to Medical Writing	2014	1.1
General academic courses		
Endnote, Medical Library, Erasmus MC	2014	0.3
Systematic Literature Search, Medical Library, Erasmus MC	2016	0.6
Research Integrity	2016	2.0
Seminars and workshops		
Seminars, Epidemiology	2013-2016	1.0
Generation R Research Meetings	2013-2016	1.0
Research Meetings Nutritional Epidemiology (SIGN-E)	2013-2014	0.5
Generation R maternal and child health meetings	2013-2016	1.0
Sophia Research Day, Erasmus MC	2015-2016	1.2
PhD Day, Erasmus MC	2016	0.6
International Conferences		
International Congress of Biomedical Sciences, Tirane - *Poster presentation*	2014	1.0
DOHaD Developmental Origins of Health and Disease, Cape Town, - *Oral presentations*	2015	1.4
The Power of Programming, Early Nutrition, Munich - *Poster presentations*	2016	1.4
Scholarships and Grants		
ERAWEB Master Student Grant	2013-2014	
ERAWEB PhD Student Grant	2014-2017	
Vereniging Trustfonds Erasmus Universiteit Rotterdam grants	2015-2016	
Trainee Travel Fund of the ISRHML-Family Larsson Rosenquist Foundation	2017	
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Tamara Marinkovic, MSc thesis Clinical Epidemiology, NIHES	2015	2.0
Florianne Vehmeijer, MSc thesis Clinical Epidemiology, NIHES	2016	2.0
Teaching assistant in the SPSS practicals, Biostatistics I NIHES	2016	0.5
3. Other Activities		
Peer review of articles for scientific journals: Schizophrenia Bulletin	2016	

WORD OF GRATITUDE

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